UNIVERSITY OF SURREY

THE ENZYME IMMUNOASSAY OF PROGESTERONE IN BOVINE MILK

BY

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TO

MUM AND DAD
The reproductive endocrinology of the cow during the oestrous cycle and early pregnancy has been reviewed, with emphasis on progesterone and how its measurement in milk may be used to improve reproductive performance. Competitive colorimetric enzyme immunoassays (EIAs) performed in microtitre plate wells were developed, enabling progesterone concentrations in unextracted milk samples to be determined.

Dicyclohexylcarbodiimide/N-hydroxysuccinimide or disuccinimidyl carbonate was used to synthesize N-hydroxysuccinimide esters of progesterone derivatives: these enabled formation of enzyme-labelled steroids in a more efficient manner than conventional procedures.

Poor sensitivity was observed with seven anti-progesterone 11α-hemisuccininate antisera when 11α-hemisuccinate, 11α-hemimaleate or 3-carboxymethylloxime bridges were used to link progesterone with the enzyme label: this problem of bridge recognition was greatly reduced when the 11α-glucuronide bridge was employed. Penicillinase as label provided more sensitive EIAs than alkaline phosphatase, peroxidase or β-galactosidase but alkaline phosphatase offered practical advantages.

The heterologous EIA finally adopted employed progesterone 11α-glucuronide-alkaline phosphatase as label, with progesterone 11α-hemisuccinate antibody. Assay sensitivity was similar to RIA, with a limit of detection of 5pg and 50% label displacement with 24pg progesterone. Analytical recovery, linearity of response and precision of the assay also compared favourably with RIA. Results of EIA of milk samples showed good correlation with determinations made after isolation of progesterone by HPLC ($r = 0.910$) and with determinations by RIA ($r = 0.933$).
The assay enabled accurate monitoring of corpus luteum function in dairy cattle. This allowed insemination to be performed without the need for observation of behavioural oestrus or reduction of the pregnancy rate. When used for pregnancy testing, good correspondence was found with results derived from RIA performed in a commercial laboratory. Positive pregnancy tests by EIA proved 94% accurate when compared with diagnosis by palpation per rectum and in a separate study 93.5% were confirmed as correct according to calving dates.
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GLOSSARY

AI  Artificial insemination
BSA  Bovine serum albumin
$B_0$  The quantity of labelled analyte bound to antibody in the absence of competing analyte.
$P_{50}/B_0$  The quantity of labelled analyte bound to antibody in the presence of 50pg of competing analyte relative to $B_0$.
CV  Coefficient of variation
$ED_{50}$  The dose of analyte effective in reducing $B_0$ by 50%
HPLC  High pressure liquid chromatography
OD  Optical density
ODS  Octadecyl silica
ovX milk  Milk taken from an ovariectomized cow
s.d.  Standard deviation
uv  Ultraviolet
CBC  Ministry of Agriculture Fisheries and Food, Cattle Breeding Centre, Shinfield, Reading
IRAD  Institute for Research on Animal Diseases, Compton
NIRD  National Institute for Research in Dairying, Shinfield, Reading
GUILDHAY  Guildhay Antisera, University of Surrey, Guildford
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CHAPTER 1:

GENERAL INTRODUCTION
1.1. THE IMPORTANCE OF MILK PROGESTERONE ASSAY IN THE MANAGEMENT OF REPRODUCTION IN DAIRY CATTLE.

The development of radioimmunoassay (RIA, Berson & Yalow, 1959; Ekins 1960) heralded a revolution in the field of endocrinology by introducing a simple and accurate technique for determination of protein and polypeptide hormone concentrations in large numbers of blood samples. This in turn enabled detailed studies of hormone secretion patterns to be made.

Although the potential of RIA in the steroid hormone field was not realised until several years later (Murphy, 1967; Abraham, 1969) it became and is still the most widely used technique for their detection (van der Molen, 1979) and has proved invaluable in elucidating the mechanisms involved in regulating the reproductive process of many species. It has thus become possible to place our basic understanding of the secretion patterns and concentrations of the ovarian steroid hormones in blood in the context of ovarian status and the control of the oviducal and uterine environment. This has allowed various aspects of reproductive performance of individual animals to be assessed, provided a basis for the identification of major causes of sub-fertility and enabled the diagnosis and assignment of treatment for specific reproductive problems.

The cyclic rise and fall of progesterone in the blood of cows over the 21 day period of the oestrous cycle directly relates to the functional status of the corpus luteum, the cycle ceasing and levels remaining high in the event of pregnancy. The quantitation of progesterone in the milk of dairy cows (Laing & Heap, 1971) by RIA has enabled a particularly valuable extension of such observation to be made (see Pope & Swinburne, 1980) since its concentration closely reflects that in plasma (Heap, Gwyn, Laing & Walters, 1973; Hoffman & Hamburger, 1973) and samples may be simply and routinely taken by stockmen during milking.

In practice, however, there are severe limitations on the use of RIA
procedures for the rapid and routine monitoring of ovarian function in
cattle and other domestic species in order to improve the management of
reproduction. The performance of RIA requires technical expertise and the
use of expensive equipment and reagents and radioisotopic health hazards
restrict its use to licensed specialised laboratories. Thus although
determination of progesterone concentrations in milk by RIA has provided
a practical research tool in the investigation of ovarian function in
herds and in individual cows (Hoffmann, Gunzler, Hamburger & Schmidt, 1976;
Lamming & Bulman, 1976; Foote, Oltenacu, Kummerfeld, Smith, Riek & Braun,
1979; Ball & Jackson, 1979) its full potential remains untapped. The
potential for management of reproduction can only be realised through a
test capable of being performed locally, either in a veterinary laboratory
or on-farm. Such a test must be safe, simple and rapid, with results
available within a few hours as well as being cheap, since the resulting
economic benefit, though considerable, is finite.

RIA by its very nature cannot fulfill these criteria and as a result
commercial application of progesterone determination in milk has remained
restricted to the provision of a postal pregnancy testing service. This
application is of limited value since it does not enable animals which
have not conceived to artificial insemination to be identified before the
next oestrus and thereby precludes repeat insemination at that oestrus.

The economic value to the dairy farmer of a test fulfilling the above
criteria may be summarised by its potential to reduce the calving index
(the mean period between calvings of all the cows in a herd) and tighten
calving patterns (defining and shortening to a few months the period over
which all cows in a herd calve). It is well established (Esslemont, 1982)
in the UK that effecting a calving index of 365 days enables substantial
economic benefit to be achieved from increased milk and calf production:
tight control of the calving period further ensures that maximum milk
production coincides with the highest price for that commodity. The value
of shortening the calving index is clear given that the mean calving index
in the UK is between 381-385 days (Warren, 1984): the financial consequences of failure to achieve the optimum index have recently been described by Baillie (1982).

The optimum index may be attained by reducing the number of unobserved and incorrectly identified oestrus events, by early identification of inseminated but non-pregnant cows and identification and treatment of subfertile individuals. Although all these measures are possible through the use of a programme of progesterone determinations in milk such applications have not hitherto been widely practised.

Additional applications of a suitably simple progesterone assay exist in research projects where large numbers of samples are required to be assayed. These may include investigation of the occurrence of early embryo death and the efficacy of hormone treatment in improving fertility and oestrous synchronisation regimes. Embryo transfer programmes would benefit since it is important to establish the stage of the oestrous cycle of both the donor and the recipient.

The elements of a cheap and easily performed test exist in enzyme-immuno-assay (EIA). Published EIA techniques are, however, far from ideal since they offer no particular advantages of speed or convenience over RIA and have only been applied to the assay of extracted serum samples. In its potential, EIA possesses all the advantages of RIA in terms of sensitivity and specificity without the hazards of radioactive isotopes and the necessity for expensive counting equipment. The possibility of achieving a colorimetric end-point sets EIA aside in its advantage over many other immunoassay procedures since this could enable the visual assessment of analyte concentrations, an ideal for a simple on-farm test.

1.2. THE DETECTION OR SYNCHRONISATION OF OESTRUS AND THEIR IMPORTANCE IN THE EFFICIENT MANAGEMENT OF HERDS USING ARTIFICIAL INSEMINATION (AI).

In the United Kingdom 78% of cows are bred by artificial insemination (Anon, 1982) and since pregnancy rates (calving rates) are generally in
the range 50-60% (Laing, 1970; Anon 1977) many cows have to be inseminated a second time at an interval of 21 days (the length of the oestrous cycle). Since AI eliminates nature's means of ensuring that the timing of introduction of spermatozoon to ovum is optimum for fertilization, its successful use is almost totally dependent upon good oestrous detection by the stockman. The economic importance of this in seasonally calving dairy herds in particular is considerable (Esslemont, 1982): when oestrous detection is poor calving to conception intervals are extended, conception rates lowered, calving patterns scattered and a high culling rate for infertility often results. The mean duration of pregnancy in cows is 282 days (Hansel & McEntee, 1977) and if a calving interval of around 365 days is not maintained (i.e. conception not achieved within 85 days postpartum) losses may be made by the farmer in terms of decreased milk production: in the UK for 1982 this was estimated to be of the order of £2.00 per cow for each day the cow is not pregnant beyond this target (Baillie, 1982).

1.2.1. The Timing of Insemination

In the cow and heifer, there is considerable variation in the timing and duration of oestrus and of the intensity of oestrous behaviour (Asdell, 1964). In heifers, 85% of cycles are between 18 and 22 days in length (mean 20 days), while in cows, 84% fall between 18 and 24 days (mean 21 days). The mean duration of overt oestrus as reported by several authors varies between 9.3 ± 2.3 and 19.3 ± 2.3 hours (see Esslemont & Bryant, 1974), the variation reported probably reflecting the relative frequency (12) or infrequency (2) of observations made per day and which signs were considered as indicative of heat (O'Farrel, 1982). The study of Esslemont, Glencross, Bryant & Pope, (1980) involving 24 heifers in which observations were made day and night for 24 days indicated a duration of 10 hours. O'Farrel (1984) subsequently reported that duration was not affected by herd size (range 46 - 120 cows) and that 23.3% of oestrous periods were of four hours duration or less.
Ovulation occurs about 14 hours after the end of the oestrus period and again there is much individual variation: in relation to the LH peak, however, its timing is not so variable and occurs about 25 hours after peak concentrations have been attained (mean ± sd = 25.7 ± 6.9hrs; Schams et al, 1977). Maximum fertility is achieved when AI is performed between the middle and the end of the oestrous period (Trimberger, 1948; Roberts, 1971). Based on the findings of Trimberger (1948) the recommended practice for AI in Britain is for insemination on the same day if oestrus is observed during the morning or early the following morning if observed during the afternoon. Although precise data regarding the viability in vivo of spermatozoa and ova are difficult to obtain, it has become axiomatic that both are incapable of retaining full viability and fertility in the female tract for much longer than 24 hours (Austin, 1969). Inaccurate diagnosis of oestrus can lead to AI at a time too early for survival until ovulation occurs and conversely, if too late, at a time when the ovum is no longer viable.

1.2.2. Oestrous Behaviour and the Detection of Oestrus

Oestrus is in theory well defined and characterized in terms of intensified behavioural traits occurring over a short period before ovulation, the components of which include aggression (butting), investigation (sniffing, rubbing, licking, chin-resting and orientation), disordered mounting (mounting without standing and disorientated mounting) and mounting with standing (Esslemont, Glencross, Bryant & Pope, 1980). In practice, however, it is hindered by a number of factors. From the herdsman's point of view, the best single criterion for oestrus is to see a cow stand and allow mounting by other animals (Esslemont, 1974a; Foote, 1975; Kiddy, 1979). Since not all animals show this clear cut "behavioural oestrus" and a proportion may not be observed (for instance at night) clearly its detection does not enable all cows to be artificially inseminated at the earliest appropriate time.
Williamson, Morris, Blood, Cannon & Wright (1972a) using continuous observation found that this "standing" behaviour was only 79% accurate when compared with palpation of follicles per rectum: other outward signs of oestrus such as mounting of other cows, ruffling of rump hair and abrasion of rump skin, vulval relaxation, moistness and erythema, oestral mucus from the vulva and sensitivity to palpation of the rump were much less reliable as sole indicators of oestrus.

In temperate regions there is a tendency for oestrus to occur during the evenings and at night rather than in the day (Esslemont & Bryant, 1976; O'Farrel, 1982) making oestrous observation during normal working hours difficult. Observations may be further hampered in autumn calving herds because the conception period should by definition be during the autumn and winter months when the influence of colder weather, shorter days and a fall in nutritional status result in attenuated oestrous behaviour which is of shorter duration (see De Kruif, 1978).

The oestrus detection rate may be particularly low during the early postpartum period since there is an associated high incidence of "silent oestrus" related particularly to the first postpartum ovulation (Morrow, Roberts & McEntee, 1969a; King, Hurnik & Robertson, 1976; Lamming & Bullman, 1976; Greve & Lehn-Jensen, 1980; Karg, Claus, Günzler, Rattenberger & Pirchner, 1980; Ball & Lamming, 1983) and as a result there is considerable variation in the interval between calving and the first observed oestrous period (Morrow, Roberts & McEntee, 1969b). Hartigan, Langley, Nunn & Griffin (1974) found a mean interval of 48 ± 22 (mean ± sd) days and observed that ovulation (deduced following palpation per rectum of a corpus luteum) occurred in 58% of animals prior to the first oestrus.

Although there is little doubt that silent oestrus occurs, in general terms its incidence is considered by many to be over-estimated and to be more a reflection of the accuracy of heat detection and the infrequency of observations (Zemjanis, Fahning & Shultz, 1969; Williamson, Morris, Blood, Cannon & Wright, 1972b; Esslemont, 1973; McCaughey & Cooper, 1980;
It has now been indicated that a substantial proportion of animals which fail to conceive to AI may do so through incorrect diagnosis of oestrus (Williamson et al., 1972b): estimates were between 12 to 36% as determined by independent observation and 17% as indicated by determination of progesterone concentrations in milk (section 1.2.3., p 1.11) at AI (Appleyard & Cook, 1976; McCaughey & Cooper, 1980).

In 1981/82, 78.2% of UK dairy cattle were artificially inseminated (Anon, 1982) for which accurate detection is necessary if conception is to result. It has been demonstrated by numerous authors, however, that on many dairy farms about 50% of oestrous periods are not detected (Bozworth, Ward, Call & Bonewitz, 1972; Esslemont, 1974a; Barr, 1975; King, Hurnik & Robertson, 1976).

Taken together, these findings indicate that even under ideal conditions behavioural signs alone are inadequate for the detection of all oestrous periods. In order to maintain a tight calving pattern and a mean calving interval within 365 days it is most important to serve a high proportion of cows from the earliest possible date postpartum (MacMillan & Watson, 1973; O'Farrell, 1975; Esslemont, 1982): given the elapse of 35-40 day period to achieve uterine involution and resumption of normal fertility (Lamming & Bulman, 1976) this allows for three inseminations to be performed, if necessary, within the 85 day period. In order to take maximum advantage of these opportunities it is apparent that means of oestrus detection other than or additional to behavioural observations are required.

1.2.3. Oestrus Detection Aids

Numerous aids to oestrus detection have been devised, used and assessed in field trials although the majority have proved of equivocal value in practice. They appear to offer, at most, equal detection rates to behavioural observations: their failings appear to be due either to
technical problems or variability or insensitivity of response. Such methods include the use of chalk or crayon marks, tail paint or pressure sensitive devices to determine whether the cow has been mounted (Foote, 1975; Kiddy, 1979; Ducker, Haggett, Fisher, Bloomfield & Morant, 1983; Kerr & McCaughey, 1984). The fitting of chin devices to hormonally treated cows or steers or bulls with surgically diverted penises has provided a means of marking the oestrus cow when mounted by the treated animal (Hurnik, King & Robertson, 1975; McDonald, Foote, Drost, Lu, Potrascu & Hall, 1976; Britt, 1980).

Videotapes and pedometers have been used to show the increase in physical activity (Kiddy, 1977; Holdsworth & Markillie, 1982; Lewis & Newman, 1984) and probes and transducers to indicate changes in electrical resistance associated with the changing biochemical composition of vaginal mucus (Leidl & Stolla, 1976; Foote, Oltenacu, Mellinger, Bean, Scott & Marshall, 1979; Foulkes, Hartley & Stewart, 1981) or changes in body or milk temperature (Ball, Morant & Cant, 1978; Kiddy, 1979; Schlüs sen, Schön, Artman, Paul & Speckman, 1981; Lewis & Newman, 1984) around oestrus. Dogs trained to respond to specific odours of cows or their milk indicative of the oestrous period have also been used as a means of oestrus detection (Kiddy, Mitchell, Bolt & Hawk, 1978; Hawk, Conley & Kiddy, 1984).

Demise of the corpus luteum at the end of the oestrous cycle results in a dramatic fall in progesterone concentration in blood and milk over a short period approximately 3 days before ovulation and is a pre-requisite for oestrus to occur. Its measurement thus provides a near ideal method of predicting oestrus and the optimum time for AI and additionally it provides an aid for the diagnosis of ovarian dysfunction. Although progesterone determination by RIA has been used in a number of studies as a definitive and objective means of retrospectively assessing the accuracy of oestrus detection (Bulman & Lamming, 1978; Appleyard & Cook, 1976; Hoffmann, Günzler, Hamberger & Schmidt, 1976; McCaughey & Cooper, 1980) only in one study (Ball & Jackson, 1979) was it used in this predictive sense. The latter study indicated that although a highly effective means of determining the
timing of AI without recourse to behavioural observations a more rapid and simple procedure would be required for it to be of value in farm practice. The value and potential of this approach will be discussed and illustrated further in section 1.6.

1.2.4. Oestrus Synchronisation

Alternatives to the determination of the oestrous period or the prediction of its occurrence to enable AI to be correctly timed have been developed. These involve hormonal treatments aimed at regulating the timing of oestrous cycle events in such a way that ovulation will occur at a predictable time following treatment or withdrawal of treatment: large numbers of animals can thus be synchronised and AI performed at a predetermined time without the necessity for oestrous observation.

Two methods (of which there are numerous variants) have been developed enabling cycle synchronisation and fixed time AI. The first is to treat all cattle with a progestational compound to inhibit oestrus and ovulation for sufficient time to allow regression of the corpora lutea of all animals (Hansel & Malven, 1960; Hansel & Beal, 1979). In theory, following withdrawal of the compound all animals will come into oestrus and ovulate at a similar time. The second method involves induction of luteolysis in cows in the luteal phase by injection of prostaglandin F$_2$α (or an analogue) followed by insemination at oestrus or 3 and 4 days later (see Roche, 1979).

Although promising for simplifying dairy herd breeding management and circumventing the need for oestrous detection both treatments have given rise to problems in practice.

In earlier studies, progesterone or synthetic prostagen were administered by a variety of methods including orally in water or food, silastic implant or vaginal pessaries. Treatments were for periods of 18-20 days and although providing a good degree of oestrous synchronisation resulted in lower conception rates than untreated controls (Hansel, 1965;
Roche, 1974a). This may be due to formation of abnormal ova (van Niekerk & Belonje, 1970) or interference with the early cleavage stages of embryonic development and may also be associated with asynchrony between the conceptus and the uterine and oviductal environment (Wishart & Young, 1974; Hansel & Beal, 1979). Both proestrous plasma progesterone and oestrogen concentrations are altered from the normal by the treatments (Britt & Ulberg, 1972; Henricks, Hill & Dickey, 1973). Reduction of the period of treatment to 9 or 12 days improved conception rates to a level comparable with the controls but gave rise to less effective synchronization and a poor oestrus response (Roche, 1974b). Combination of the short treatment with the use of a luteolytic agent such as oestradiol valerate (Wiltbank, Sturges, Wideman, Le Fever & Faulkner, 1971) or prostaglandin $F_2\alpha$ (Thimonier, Chupin & Pelot, 1975; Chupin, Pelot & Mauleon, 1977) appeared to resolve this problem since it gave rise to a high incidence of observed oestrus and conception rates comparable with untreated controls. The degree of synchronization achieved by the latter protocol shows promise for effective fixed time AI since pregnancy rates (by rectal palpation) at 45-60 days were higher (66%) than following two prostaglandin injections (52%) and compared with a pregnancy rate of 73% for control animals (Smith, Pomerantz, Beal, McCann, Pilbeam & Hansel, 1984).

Synchronization methods using prostaglandin treatment alone need to take account of the inability of this treatment to cause luteolysis while the corpus luteum is developing, i.e. during the first five days of the oestrous cycle (Rowson, Tervit & Brand, 1972). To ensure that all animals are synchronized by this treatment numerous treatment protocols have been devised (see Roche, 1979).

The most common protocols involve single or double prostaglandin treatments. Two prostaglandin $F_2\alpha$ (or analogue) injections at 11 or 12 day intervals may be used followed by insemination at ensuing oestrus or 72 and 96 h after the second injection. Alternatively, close
observation for oestrus and insemination over a five day period enables treatment of all remaining animals with a single injection of prostaglandin $F_{2\alpha}$ on day 5: insemination may then be performed following observation of ensuing oestrus or 72 and 96 h after treatment (Hansel & Beal, 1979). Although in particular circumstances good synchronization may be achieved and normal fertility rates obtained using a single insemination 72 h after treatment (Lamming, Hafs & Manns, 1975) a reduced fertility rate has been noted by others (Cooper, Hammond, Harker & Jackson, 1976; Roche, 1979). In maiden heifers it appears that an insemination at 72 and 96 h gives normal fertility rates in most cases (see Roche, 1979).

In dairy cows, however, synchronization using prostaglandin treatment has proven far more problematic and the efficacy of the 11 day double injection regime has been held in question (de Kruif, Zikken, Kommerij & de Bois, 1976; Macmillan, Curnow & Morris, 1977) since lower conception rates result. This may possibly be due to greater variation in the timing of onset of oestrus and ovulation due to incomplete luteolysis or, once luteolysis has occurred, to prolongation of the follicular phase (Roche, 1979; Baishya, Ball, Leaver & Pope, 1980). This indicates that a longer interval between injections (13 days) may be beneficial (Chupin, Pelot & Mauleon, 1978). Certainly a critical factor is ensuring that only cyclic animals are treated: this applies particularly to cows since this can not be ensured for a period of at least 42 days post-partum (Macmillan et al, 1977; Roche & Prendiville, 1979; Ball & Lamming, 1983).

As with oestrous cycle control methods using progesterone treatment, highest pregnancy rates are achieved with dairy cows when insemination is performed at observed oestrus rather than at a fixed time (Schultz, 1978; Hyttel & Greve, 1983; Ball & Jackson, 1984) and thus these procedures have not yet obviated the necessity for oestrus observation.
1.3. HORMONAL EVENTS ASSOCIATED WITH THE BOVINE OESTROUS CYCLE AND EARLY PREGNANCY.

1.3.1. The Bovine Oestrous Cycle

Events associated with the onset of breeding activity and the oestrous cycle in cows have been the subject of numerous reviews (Cupps, Anderson & Cole, 1969; Robinson, 1977; Hansel & Convey, 1983).

The onset of puberty in heifers occurs between five months and two years after birth (Hammond, 1927; Asdell, 1964). Its timing appears to be more closely related to achievement of a particular body size than to the absolute age of the animal: under good nutritional conditions a heifer will attain puberty at approximately two-thirds adult size (Robinson, 1977).

With the onset of puberty, heifers exhibit oestrous behaviour (heat) for the first time (i.e. standing to be mounted or mounting other cattle) the behaviour being of variable duration. The subsequent recurrence of oestrus at mean intervals of 20 days (21 days in cows) is called the oestrous cycle: in temperate climates cycles occur continuously (polyoestrus) independent of season and normally cease only in the event of pregnancy (Asdell, 1964). The period of oestrous behaviour (day 0 of the oestrous cycle) corresponds with peak secretion of oestradiol-17β by the developing follicle prior to ovulation (Glencross et al., 1973). The release of LH by the pituitary results in ovulation, typically 12-14 h after the onset of oestrus. Since the release of LH occurs in the absence of external stimuli, ovulation is referred to as spontaneous. Only one follicle normally develops to the stage of ovulation (mono-ovulatory) and following ovulation luteinization and proliferation of the granulosa and thecal cells of the ruptured follicle give rise to the corpus luteum and thus to the secretion of progesterone: in the event of pregnancy the corpus luteum and associated progesterone secretion are maintained to term. If the animal does not become pregnant regression of the corpus luteum (luteolysis) occurs between days 17-19 of the cycle, a new follicle
Fig. 1.1. Schematic representation of concentrations of a) progesterone, oestradiol-17β and LH in plasma during the oestrous cycle and b) progesterone in whole milk after parturition and during the early stages of pregnancy of the cow.
develops to ovulation and the cycle continues (Fig. 1). The measurement of progesterone concentrations in plasma (Robertson & Sarda, 1971) or milk (Laing & Heap, 1971) at a fixed time following insemination has thus provided a means of testing for pregnancy in cattle.

1.3.2. Hormonal Control of the Oestrous Cycle

Ovarian events during the oestrous cycle may be conveniently divided into three periods in relation to the nature of hormonal control, follicular development and ovulation, corpus luteum formation and luteolysis.

a) Follicular development and ovulation

During the period of follicular development, ovarian oestradiol-17β secretion increases (Glencross, Munro, Senior & Pope, 1973; Hansel, Concannon & Lucaszewska, 1973), reaching a peak at oestrus: the rising levels of oestradiol-17β are thought to provide the stimulus which triggers the ensuing surge in concentrations of the gonadotrophins, luteinizing hormone (LH; Beck & Convey, 1977) and follicle stimulating hormone (FSH; Martin, Henricks, Hill & Rawlings, 1978) which have been shown to occur around the onset of oestrus (Akbar, Reichert, Dunn, Kaltenback & Niswender, 1974). Progesterone would appear to have a negative feedback effect on gonadotrophin release, however, and thus surges of FSH and LH can only occur following a substantial reduction of circulating progesterone concentration such as normally occurs with luteolysis (Chenault, Thatcher, Kalra, Abrams & Wilcox, 1975; Kesner, Convey & Anderson, 1981). During this period, LH, characterized by the pulsatile nature of its release, is secreted with increased frequency resulting in overall increased circulating concentrations (Rahe, Owens Fleeger, Newton & Harms, 1980) and may be responsible for stimulation of oestradiol-17β secretion from the pre-ovulatory follicle (Hansel & Convey, 1983). A mechanism whereby LH and FSH may work in concert to induce oestrogen synthesis in these follicles has been proposed in which LH stimulation of androgen synthesis by the theca interna occurs: the thecal cells are incapable of androgen aromatization and transference
of androgen to the granulosa cells occurs where oestrogen is synthesized under stimulation from FSH (Hansel & Fortune, 1978; Hansel & Convey, 1983).

As the follicle grows into a vesicular pre-ovulatory follicle the increasing LH influence may result in stimulation and luteinization of the follicle. This may be inhibited by the presence, within the follicle, of a "luteinization inhibitor" (Bernard, 1975). The LH surge is probably responsible for the removal of this inhibitory effect and results in oocyte maturation, ovulation following about 24 h after the LH peak (Thibier, 1976; Schams, Schallenburger, Hoffman & Karg, 1977).

The release of LH from the anterior pituitary is induced by the pulsatile release of the decapeptide gonadotrophin releasing hormone (GnRH) from the hypothalamus. In vitro the pituitary shows a marked increase in sensitivity to GnRH during the pre-ovulatory period due to high oestradiol-17β levels (Padmanabhan, Leung & Convey, 1982). It has been shown that both an increase in GnRH secretion and in pituitary responsiveness to it are necessary for the pre-ovulatory LH and FSH surges (Kesner & Convey, 1982). The termination of the surges appears to result from the refractoriness of the pituitary to GnRH (Kesner & Convey, 1982) rather than the depletion of gonadotrophin content (Convey, Kesner, Padmanabhan, Caruthers & Beck, 1981). Following the LH surge, LH, FSH oestradiol-17β and progesterone concentrations in blood are basal (see Hansel & Convey, 1983). Approximately 24 h following the pre-ovulatory gonadotrophin surge a significant rise in FSH concentration occurs (Dobson, 1978; Ireland & Roche, 1983) which may play a role in recruitment of pre-antral follicles for the next cycle.

b) Corpus luteum formation

The luteinization and growth of granulosa and thecal cells following ovulation results in formation of the corpus luteum (Hansel, Concannon & Lukaszawska, 1973) which reaches its mature size by day 7 of the cycle (Hammond, 1927). A large follicle develops concurrently and with this (Matton, Adelakoun, Coutre & Dufour, 1981; Ireland & Roche, 1983) the
Oestrogen concentration in blood rises to a peak at day 6 (Glencross et al., 1973; Glencross & Pope, 1981). The post-ovulatory increase in FSH may play a role in this development along with a secondary but slightly later rise in LH concentrations (Dobson, 1978). Neither FSH, LH or oestradiol rise to concentrations associated with the pre-ovulatory period and the follicle, failing to develop to ovulation, becomes atretic. It is thought that further increases in gonadotrophin secretion and follicular development are inhibited by negative feedback of progesterone produced by the developing corpus luteum (Glencross, 1982).

LH is believed to be the main luteotrophic hormone in the cow, prolactin having no direct luteotrophic effect (Hansel, 1967; Hansel et al., 1973; Hoffman, Schams, Bopp, Ender, Giminez & Karg, 1974). It acts after binding to receptors in the corpus luteum cell membrane through intra-cellular stimulation of the "second messenger", cyclic AMP (Sutherland, Butcher, Robinson & Hardman, 1967). The latter activates intra-cellular protein kinase which in turn stimulates the steroidogenic enzymes (Marsh, 1976; Ling & Marsh, 1977).

The progesterone and 20β-dihydroprogesterone content of the corpus luteum (Hafs & Armstrong, 1968) and progesterone concentrations in peripheral blood (Glencross et al., 1973; Hansel et al., 1973) continue to rise during the luteal phase, reaching a maximum 10-11 days after oestrus. Degenerative changes become apparent by day 17-19 (Mares et al., 1962) if the cow is not pregnant, the subsequent drop in peripheral progesterone concentrations permitting the development of a new follicle to ovulation. The source and nature of the signal initiating the onset of luteolysis is yet to be fully established.

c) Luteolysis

It has been demonstrated that the uterus controls the lifespan of the bovine corpus luteum and is responsible for the induction of luteal regression during the cycle since hysterectomy leads to prolongation of the luteal phase (Wiltbank & Cassida, 1956; Malven & Hansel, 1964). Indeed the induction of luteolysis is specifically related to the uterine
horn ipsilateral to the corpus luteum since unilateral hysterectomy results in maintenance of a corpus luteum of an ipsilateral but not a contralateral ovary (Mapletoft, Del Campo & Ginther, 1976).

One view of the physiological mechanism of luteolysis revolves around the possibility of transfer of a luteolysin via the uterine vein to the ovarian artery by a counter-current transfer process as originally demonstrated for sheep (McCracken, Carlson, Glew, Goding, Baird, Green & Samuelsson, 1972). Such a mechanism is made possible because the ovarian artery convolutes around and closely adheres to the walls of the utero-ovarian vein and at the area of contact the walls of the vein and the artery are very thin: various surgical re-arrangements of the vein and artery have provided evidence for such a mechanism (Ginther, 1974, 1981; Hixon & Hansel, 1974; Mapletoft et al, 1976). More recent evidence, again obtained from sheep, suggests that counter-current transfer is not the only mechanism involved and that the lymphatic drainage system may play a role in transport of luteolysin to the ovary (Abdel Rahim, Bland & Poyser, 1984; Heap, Fleet & Hamon, 1985).

A substantial body of information suggests that the luteolysin produced by and transferred from the uterus is prostaglandin F\textsubscript{2}\alpha (PGF\textsubscript{2}\alpha, Hansel et al, 1973; Thibier, 1976; Thatcher & Chenault, 1976) or its precursor, arachidonic acid (Hansel, 1975). Certainly, administration of PGF\textsubscript{2}\alpha or its synthetic analogues mimics normal functional and morphological luteolysis in hysterectomized cows (La Voie, Poncelet, Han, Soliday, Lambert & Moody, 1975) and causes premature luteolysis followed by resumption of normal cyclicity in intact cows (Hansel et al, 1973). It has further been demonstrated that passive immunization against PGF\textsubscript{2}\alpha can halt luteolysis and thus prolong the oestrous cycle (Fairclough, Smith & McGowan, 1981). It has not, however, been adequately demonstrated that simultaneous or sequential changes in PGF\textsubscript{2}\alpha concentrations occur in both uterine venous and ovarian arterial blood prior to a decline in ovarian progesterone output during the oestrous cycle of normal animals.
The luteolytic effect of PGF$_2\alpha$ is thought to be achieved through antagonism of the luteotrophic action of LH, possible at the receptor level, through inhibition of the adenylate cyclase-cyclic AMP system (Henderson & McNatty, 1975).

It has been suggested (Thatcher, Wolfsen, Curl, Rice, Knickerbocker, Bazer & Drost, 1984) that oestrogens may regulate the onset of luteolysis through stimulation of uterine PGF$_2\alpha$ release since administration of free or conjugated oestrogens during the luteal phase shortens the lifespan of the corpus luteum but is not effective in hysterectomized heifers and elevated levels of uterine PGF$_2\alpha$ coincide with the start of the pre-ovulatory rise in follicular oestradiol.

With the recent finding that oxytocin may be synthesized in the bovine corpus luteum (Wathes & Swann, 1982) renewed attention has been paid to its possible role in regulating corpus luteum function. Measurements of arterial venous differences in oxytocin concentration across the ovine ovary have shown that the corpus luteum can secrete oxytocin (Flint & Sheldrick, 1983) and immunizing against oxytocin caused prolongation of the oestrous cycle of sheep (Sheldrick, Mitchell & Flint, 1980; Schams, Prokopp & Barth, 1983). Experiments, also in sheep, have indicated that uterine arterial administration of oxytocin gives rise to increased concentrations of PGF$_2\alpha$ in uterine venous blood (Roberts & McCracken, 1976) and that this arises from endometrial tissues (Roberts, McCracken, Gavagen & Soloff, 1976). Current evidence thus suggests that in sheep and possibly in cattle, oxytocin may work through positive feedback on PGF$_2\alpha$ release from the endometrium to induce a more rapid completion of luteolysis (Flint & Sheldrick, 1983; Wathes, 1984; Wathes, Swann & Pickering, 1984).

It has recently been proposed (McCracken, Schramm & Okulicz, 1984) that
the timing of this process is strictly regulated by the presence of oxytocin receptors in the endometrium, the synthesis of which is stimulated by oestradiol-17β and blocked by progesterone during the luteal phase. The block ceases after a physiologically defined period (for reasons not established) enabling the low levels of oestradiol-17β present in plasma during the luteal phase to exert their stimulatory effect on oxytocin receptor synthesis.

1.3.3. The Maternal Recognition of Pregnancy

Progesterone is essential for the maintenance of all stages of pregnancy in the bovine and is provided by the corpus luteum through at least the first half of pregnancy. The continued presence of circulating progesterone is achieved by prolongation of the lifespan of the corpus luteum formed in the cycle of conception. The means by which the conceptus prevents luteolysis has yet to be fully established and is considered in detail in Appendix 1. Evidence that the conceptus may exert a direct luteotrophic effect on the corpus luteum is equivocal and it may additionally or alternatively exert its influence indirectly by reducing uterine prostaglandin $\text{F}_{2\alpha}$ secretion or inducing release of an antagonist to its action. It is clear, however, that the mechanism must be operative prior to day 17 or the pregnancy will fail.
1.4. THE SECRETION, TRANSPORT AND TISSUE DISTRIBUTION OF PROGESTERONE

1.4.1. The Discovery of Characterization of Progesterone

It became apparent from the beginning of the century that corpora lutea of the ovaries of several mammals produced a substance which was responsible for the secretory activity of the endometrium and necessary for the implantation of the conceptus (Fieser & Fieser, 1959). Indeed, extracts of sow corpora lutea had been shown to maintain pregnancy in ovariectomized rabbits (Allen & Corner, 1929; 1930). By 1957 the substance involved had also been identified in adrenal (Beall, 1938) and placental (Salhanik, Neall, Zarrow & Samuels, 1952) tissue and in blood (Short, 1957).

The active principle of the corpus luteum was initially termed "progestin" (Allen, 1930) because of its action but subsequent to the isolation and structural determination of the biologically active pure crystalline compound (see Allen, 1974; Butenand & Westphal, 1974) the name "progesterone" was adopted (League of Nations, 1935) and still is the accepted trivial name. 4-Pregnene-3, 20-dione is the currently accepted systematic name (IUPAC-IUB, 1967) and its structural formula is shown in Fig. 1:2.

Fig. 1.2. The structural formula of progesterone.
1.4.2. Hormone Function and Control

Hormones provide a biochemical means by which a variety of physiological functions are regulated. By definition, they are secreted directly into the blood and transported to specific target organs where they exert their specific influence remote from their site of production. They may, for instance, induce tissue growth, differentiation of cells or alterations in their metabolic activity. The target organ itself may be an endocrine gland which may in turn release hormones acting on other target organs or exert a feedback effect on the gland which stimulated its activity: this feedback interaction provides the means by which the endocrine system is regulated.

Ovarian steroid hormone production is controlled by a system which in its simplest form involves the hypothalmic-pituitary-ovarian axis. Ovarian steroid synthesis is stimulated by FSH and LH which originate in the anterior pituitary. Their release is in turn stimulated by the release of GnRH and may be modulated by feedback from the ovarian steroids. GnRH is produced in the hypothalamus and secreted directly into the anterior pituitary via the pituitary portal vessel. The control mechanisms involved have been reviewed by Hansel and Convey (1983) and are considered further in section 1.3.2.
1.4.3. Secretion of Steroid Hormones by the Bovine Ovary

Although a multiplicity of steroids are synthesized by the ovary, some only appear to serve as intermediaries in oestrogen biosynthesis. The presence of steroids in ovarian vein blood at concentrations higher than those in ovarian arterial blood or the systemic circulation has been taken to be indicative of ovarian origin. Such steroids include progesterone (Gomes & Erb, 1965; Dobrowolski, Stupnicki & Domanski, 1968) and oestradiol-17β (Nancarrow et al., 1973). Changes in concentrations of plasma progesterone and oestradiol-17β during the bovine oestrous cycle are shown in Fig. 1.1.

The main source of oestrogen during the oestrous cycle is the ovarian follicle (Saumande & Testart, 1974; Saumande & Pelletier, 1975) while progesterone produced during the oestrous cycle and pregnancy derives largely from the corpus luteum (Pope, Gupta & Munro, 1969; Henricks, Dickey & Niswender, 1970). The levels of these hormones in peripheral plasma closely reflects the stages of development and regression of these structures (Pope et al., 1969; Henricks et al., 1970; Saumande & Testart, 1974; Saumande & Pelletier, 1975).

The bovine adrenal is capable of synthesizing and secreting progesterone into the peripheral circulation but its contribution is small compared with that of the corpus luteum (Wagner, Strohbehn & Harris, 1972; Dorfman, 1973; Watson & Munro, 1984). Although the foeto-placental unit is capable of synthesizing progesterone (Dorfman, 1973) the corpus luteum is considered to be the main source of progesterone throughout pregnancy (Edqvist, Ekman, Gustafsson & Lindell, 1973) and removal of the latter prior to day 200-230 of pregnancy may result in abortion (McDonald, McNutt & Nichols, 1953; Estergreen, Frost, Gomes, Erb & Bullard, 1967; Wendorf & First, 1972).

1.4.4. Transport of Ovarian Steroids

During transport to their respective target organs, only a small proportion of the steroid hormones are in free solution. As much as 98% may be bound to serum proteins, the most significant of which are albumin (Ka = 1 x 10^5 M^-1) and in primates, the steroid binding globulins (Ka = 1 x 10^8 M^-1). The high
binding affinities of the latter ensure preferential binding and are thought to prevent indiscriminate exposure of body tissue to high concentrations of sex steroids: only cells with high affinity receptors would therefore be capable of effective retention (King & Mainwaring, 1974; Westphal, Stroupe & Cheng, 1977). The free and bound hormones exist in equilibrium and only the free steroid in blood is thought to exert biological activity or to be metabolized by the liver. A likely function of the binding proteins, therefore, would be to provide an efficient transport mechanism which effectively buffers circulating concentrations by reducing metabolic clearance (Clark & Peck, 1979).

1.4.5. Tissue distribution of Progesterone and its Mode of Action in the Uterus.

Early attempts to demonstrate preferential retention of steroids by target organ tissues using radiolabelled tracers were confounded because their relatively low specific activity necessitated the use of pharmacological rather than physiological doses of hormone. Following the synthesis of \((^3\text{H})\text{hexoestrol}, (^3\text{H})\text{oestradiol-17\beta}\) and later \((^3\text{H})\text{progesterone}\) of high specific activity, however, it was shown that the uterus, vagina and pituitary retained significant quantities of \text{oestradiol-17\beta}\) and progesterone against a marked concentration gradient (Glascock & Hoekstra, 1959; Jenson & Jacobson, 1962; Laumas & Farooq, 1966). This finding provided an illustration that the proposals of Langley (1905) and Ehrlich (1913) that "drugs do not act unless they bind" also applied to hormones. Although much of the evidence is indirect or circumstantial it is now widely accepted that binding of steroid to specific cytoplasmic receptors followed by translocation of the steroid-receptor complex to the cell nucleus is a pre-requisite for the biological response (see Jensen, Mohla, Gorella, Tanaka & DeSombre, 1972; O'Malley & Means, 1974). The role of receptors in steroid hormone action has been reviewed recently by Clark & Peck (1979), Stormshak (1979) and Catelli & Mester (1983).
Current descriptions of the action of both progesterone and oestradiol-17β involve a mechanism in which steroid enters the target cell and is specifically bound to a high affinity (\(K_a \times 10^9 - 10^{10} \text{M}^{-1}\)) cytosol receptor. Translocation to the cell nucleus is preceded by a change in size of the complex ("transformation"): once inside the nucleus binding of the steroid-receptor complex to "acceptor sites" in the chromatin initiates genome transcription allowing expression of the particular characteristics of the target cell. This may result, for instance, in cell division, the synthesis of specific proteins and the secretion of specific products in particular circumstances (King & Mainwaring, 1974; Baulieu, Atger, Best-Belpomme et al., 1975).

The process by which the steroid enters the cell may simplistically be described by diffusion down a concentration gradient (maintained by removal of the free steroid on binding to the receptor). Other processes by which this might be more efficiently achieved remain a matter of speculation but include (i) the concept of specific accumulation and retention of albumin bound steroid by target tissues such as the uterus (Peterson & Spatziani, 1971), (ii) initial binding within the target cell to a receptor of lower affinity but higher capacity than the classical cytosol receptor (Clark & Peck, 1979) or (iii) a mechanism analogous with the low density lipoprotein system effective in cholesterol uptake, storage and metabolism, involving endocytosis after binding to cell surface receptors (Goldstein & Brown, 1977; Clark & Peck, 1979).

During the oestrous cycle of mammals, progesterone secretion occurs after a period of oestrogen dominance so that it normally acts on an oestrogen-primed uterus. Thus the actions of progesterone on the reproductive tract in animals during the oestrous cycle cannot be considered in isolation from those of oestradiol-17β. Morphologically, during the follicular phase when the uterus is oestrogen dominated, growth and proliferation of the cells of the endometrium are evident (endometrial proliferative phase). Without this phase the endometrium is unable to respond to the progesterone secretion which follows ovulation and corpus luteum formation. Classically, the oestrogen-primed glandular
epithelium responds to the influence of progesterone by way of secretion of histotrophe (endometrial secretive phase) in preparation for the arrival of the conceptus. The control over the reproductive tract exerted by oestradiol-17β and progesterone has been observed through all stages of the reproductive process. This includes the control of transport of the fertilized egg to and the timing of its arrival in the uterus (control of contractility and ciliary activity of the oviduct; see Anderson, 1977), the production of the oviductal and uterine histotrophe (provision of nutrients for the developing conceptus: Finn & Porter, 1975), the development of a uterine environment receptive to attachment or implantation of the conceptus (Cook & Hunter, 1978; Sauer, 1979) and the maintenance of myometrial quiescence until parturition (Catchpole, 1977).

1.5. BIOSYNTHESIS AND METABOLISM OF PROGESTERONE

1.5.1. Ovarian Synthesis and Metabolism of Progesterone

Pathways leading to the production of progesterone from cholesterol in the ovary have been established from in vitro studies in which sliced or minced ovarian tissue or isolated ovarian compartments have been incubated with radiolabelled precursors and the labelled products identified. Four types of enzyme are responsible for the synthesis and catabolism of progesterone: the dehydrogenases add or remove hydrogen from carbon atoms of the steroid nucleus, the hydroxylases add hydroxyl groups to the steroid nucleus, the isomerases transfer a double bond between carbon atoms after a dehydrogenase has acted and the lyases cause splitting of a side chain or loss of methyl groups (Feder, 1981).

The similarity of the enzyme systems involved, their sub-cellular sites and their control by the trophic hormones (see section 1.3.2) indicate that the same synthetic pathways are operable in the majority of steroid producing tissues (Henricks & Mayer, 1977) and are outlined in Fig. 1.3 Cholesterol for ovarian steroid synthesis is mainly derived from plasma.
Fig. 1.3 The biosynthesis of progesterone from cholesterol.
Fig. 1.4 Progesterone metabolism in the bovine ovary.

\[ \Delta^4\text{-PATHWAY} \]

PROGESTERONE → 17-HYDROXYPROGESTERONE → ANDROSTENEDIONE → OESTRONE

\[ \Delta^5\text{-PATHWAY} \]

PROGESTERONE → 17-HYDROXYPREGNENOLONE → DEHYDROEPIANDROSTERONE → ANDROSTENEDIOL

OESTRADIOL-17β

Fig. 1.5 Reductive metabolism of progesterone in bovine liver and kidney

KIDNEY

20β-hydroxy-4-pregnen-3-one → PROGESTERONE

5α-pregnan-3,20-dione

3β-hydroxy-5α-pregnan-20-one

3α-hydroxy-5α-pregnan-20-one

5α-pregnan-3β,20β-diol

LIVER

5β-pregnan-3α,20β-diol

3α-hydroxy-5β-pregnan-20-one

5β-pregnan-3α,20β-diol

5β-pregnan-3,20-dione
even though the corpus luteum possesses all the enzymes required for de novo synthesis from acetate (Hellig & Savard, 1965) and does produce some cholesterol. Its conversion to pregnenolone is brought about in the mitochondria by a multicomponent enzyme system which hydroxylates and cleaves the side chain between C<sub>20</sub> and C<sub>22</sub> (Fig. 1.3). The side chain cleavage system is membrane bound and thus pregnenolone, when formed, is free to traverse the mitochondrial membrane to be utilized for the synthesis of hormones by the microsomal enzyme systems.

Pregnenolone is the main precursor for the steroid hormones produced by the ovary, adrenal gland and placenta (Henricks & Mayer, 1977). The involvement of progesterone as an intermediary in the synthesis of the androgens and oestrogens, however, will depend on whether the Δ<sup>4</sup> 3-keto (4-en-3-one) configuration of progesterone or the Δ<sup>5</sup> 3β-hydroxy (3β-hydroxy-5-en) configuration of pregnenolone is maintained (Fig. 1.4) in the so-called Δ<sup>4</sup> and Δ<sup>5</sup> pathways respectively (van der Molen, 1979). In both cases the enzymes associated with transformations to the androgens and oestrogens are found in the endoplasmic reticulum. It has been speculated that the enzymes are associated in an organized group such that the substrate is exposed to each enzyme within the complex in turn and "released" only when the end of the pathway is reached (Savard, 1973; Tamaoki, 1973).

1.5.2. Metabolism of Progesterone in the Bovine Ovary

The Δ<sup>5</sup> pathway would appear to provide the predominant anabolic route in bovine follicular tissue (Short, 1962; Lacroix, Eechaute & Leusen, 1974; Mori, 1975). The formation of oestrogens is thought to result from the action of the synthetic enzymes of the granulosa or thecal follicular tissue. The likelihood is that both cell types work in concert (Short, 1964; Lacroix et al., 1974) since the thecal cells possess the capability of androgen synthesis but not aromatization and the granulosa cells are capable of oestrogen synthesis only when provided with aromatizable substrate or co-cultured with thecal tissue (Hansel & Fortune, 1978). The corpus luteum,
however, is incapable of production of oestrogens, either from acetate or from testosterone since it lacks the 19-hydroxylase-aromatase enzyme complex for the conversion of androgens to oestrogens (Savard & Telegdy, 1965). The bovine corpus luteum thus produces a more limited range of metabolites than any other species studied (Savard, 1973), consisting of progesterone (as the main product) 20β-hydroxy-4-pregn-3-one, pregnenolone (Hayano, Lindberg, Wiener, Rosencrontz & Dorfman, 1954; Mason, Marsh & Savard, 1962; Axelson, Schumacher, Sjovall, Gustafsson & Lindell, 1975) and 17-hydroxyprogesterone (Gomes & Erb, 1965; Thibier, Castanier, Tea & Scholler, 1973). Pregnenolone and allopregnanolone (3β-hydroxy-5α-pregn-20-one) (Axelson et al., 1975) and their fatty acid esters (Albert, Porticovo & Lieberman, 1980) have also been reported to be present in bovine corpora lutea, although the significance of the latter remains a matter of speculation.

1.5.3. Catabolism of Progesterone in the Bovine

The levels of progesterone in blood are determined by its rate of synthesis and entry into the circulation compared with its rate of excretion and metabolism. Production rates can be calculated by determination of the metabolic clearance rate (MCR) since the MCR may remain relatively constant regardless of physiological state and hormone concentration (Heap et al., 1975). Changes of hormone levels in blood accurately reflect production rates and secretory activity of the organ and the availability of the steroid to the target organ.

Since the hormones constitute a control mechanism and the intensity of hormone activity is proportional to the hormone concentration in blood it is important that blood hormone levels reflect secretion rate. The circulating steroid hormones have relatively short half lives, typified by that of progesterone in the bovine peripheral circulation (36.3 mins; Miller, Williams, Pipes & Turner, 1963). This enables a rapid response to pituitary control to be made (Samuels & Eik-Nes, 1968). Target-cell intracellular half lives are longer however since the avidity of binding to receptors is several orders of magnitude higher than to plasma proteins and they remain bound to the
receptor during receptor transformation and translocation (King & Mainwaring, 1974).

Physiologically potent steroids are largely deactivated in the liver. Circulating steroids are, for the most, bound to plasma proteins and the liver can only act on that proportion of steroid which is free.

In the case of progesterone, metabolism occurs by reduction at C-3, C-20 and at the C-4, C-5 double bond in ring A (Fig. 1.5) and the configuration of the metabolites formed is very much tissue-dependent. In the bovine liver, *in vivo* and *in vitro* studies have indicated essentially complete reduction of progesterone. Products of reduction at the C-4, C-5 double bond are of 5β configuration and hydroxylation at C-3 results in products of α-configuration (Purdy, Durocher, Moore & Rao, 1980; Clemens & Estergreen, 1982). Hydroxylation at C-20 gave rise to 20β-hydroxy metabolites *in vivo* (Purdy *et al*, 1980) whereas *in vitro* both 20α-hydroxy (the majority) and 20β-hydroxy metabolites were found (Clemens & Estergreen, 1982). In both studies, greater than 40% of hydroxylated products were found to be in the form of glucurononides, and that these were of the 3α type (Purdy *et al*, 1980).

The pattern of *in vivo* metabolism by kidney indicated that two thirds of the progesterone content is in a reduced form. In contrast with liver reduction at C-4, C-5 gave rise to 5α products. The main metabolites were 20β-hydroxy-4-pregnen-3-one and the 3α-hydroxy and 3β-hydroxy-5α-pregnan-20-one and the 5α-pregnane-3β, 20β-diol (Purdy *et al*, 1980).

Adipose tissue is known to provide a reservoir for endogenous progesterone in dairy cows (McCracken, 1964) and also possesses the ability to metabolize it into the 3α-hydroxy-5β-pregnan-20-one and to form the 20β and 20α-4-pregnen-3-one *in vitro* (Clemens & Estergreen, 1982). These metabolites have also been isolated *in vivo* together with 3α-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5β-pregnan-20-one and 20α-hydroxy-5α-pregnan-3-one (Lin, Estergreen, Moss, Willet & Shimoda, 1978).

*In vivo* metabolism of progesterone by bovine muscle indicated 46% conversion,
principally to the products 5α-pregnane-3, 20-dione, 20β-hydroxy-4-pregnen-3-one and 3α-hydroxy-5β-pregnan-20-one (Lin et al, 1978).

Metabolism of progesterone to products other than hormone precursors has also been noted in the corpus luteum of the cow. Its ability to produce substantial quantities of 20β-hydroxy-4-pregnen-3-one has long been known (Hayano et al, 1954) although a function for this metabolite has yet to be established. While these reduction reactions generally lead to the production of more polar steroids, the products are still essentially hydrophobic and would not, therefore, be displaced readily from plasma proteins to enable filtration and excretion via the kidneys. The hydroxylated metabolites are made hydrophilic through glycoside formation or esterification in the liver or intestinal wall, most commonly with glucuronic acid derived from uridine diphosphogluconic acid (Isselbacker, 1956) or sulphate from adenosine 3′-phosphate-5′-phosphosulphate (Robbins & Lipman, 1956). The enzymes involved are the glucuronyl transferases and the sulphokinases and these form the glucosiduronates (glucuronides) and sulphates respectively. The glucuronides are readily filtered by the kidney and excreted in urine without reabsorption at a rate similar to creatinine: sulphates are eliminated more slowly.

1.5.4. Excretion of Progesterone and its Metabolites

In an experiment involving administration of (14C)-progesterone to a pregnant lactating dairy cow, 45%, 1.7% and 0.03% of the radioactive material was recovered in faeces, urine and milk respectively (Williams, 1962). Similar observations were made in a study of six lactating, non-pregnant cows and four steers where 50% and 12% were found in faeces and 2.0% and 1.2% in urine for the cows and steers respectively: an additional 0.25% was removed with the milk (Estergreen, Lin, Martin et al, 1977). The main excretion route of progesterone appears to be through the liver and bile to the faeces since far larger quantities of radioactivity were found in bile and faeces than urine and in liver compared with kidney (Estergreen et al, 1977).
A range of hormones have been found in the milk of the dairy cow (see Foote, 1979; Richards, 1979; Pope & Swinburne, 1980; Sack, 1980; Strbak, 1985) and these include the major ovarian hormones progesterone (McCracken, 1963) and oestradiol (Turner, 1958). It has been widely reported that during the oestrous cycle milk concentrations of both progesterone (Fig. 1.1b; Heap, Gwyn, Laing & Walters, 1973; Hoffmann & Hamburger, 1973; Schiavo, Matuszczak, Oltenacu & Foote, 1975; Lamming & Bulman, 1976; Pope, Majzlik, Ball & Leaver, 1976) and oestradiol-17β (Monk, Erb & Mollet, 1975; Erb, Chew & Keller, 1977; Gyawu & Pope, 1983; Abeywardene, Hathorn & Glencross, 1984) correlate well with those in peripheral plasma and thus with the functional status of the ovary. Thus, although the most common means of evaluating the functional status of the ovary of farm animals has been through the determination of steroid hormone levels in blood, an alternative non-invasive procedure is possible for lactating animals. The practical significance of this lies in the ease with which large numbers of milk samples can be collected by dairymen during the normal milking period without causing discomfort to the cow: this contrast with blood sampling, for which veterinary or technical input would inevitably be required.

Although the assay of oestradiol-17β in milk has provided a useful research tool in the investigation of events associated with follicular development (Erb et al., 1977; MacDonald, Sauer & Watson, 1982; Abeywardene et al., 1984), in contrast with progesterone determination, its application to the monitoring of oestrous cycle events has not become common practice, in part because its low concentrations in milk (0-10pg/ml range: Glencross, Abeywardene, Corney & Morris, 1981; MacDonald, Sauer, Watson & Foulkes, 1982) militate against the use of simple assay protocols.

1.6.1. The Occurrence of Progesterone and its Metabolites in Milk

Progesterone in cows blood is largely derived from the corpus luteum.
Studies involving the intravenous injection of \((^{14}\text{C})\text{progesterone}\) into lactating cows indicated that concentrations of radiolabel in milk accounted for only 0.03 - 0.25% of that administered (Miller et al., 1963; Estergreen et al., 1977) indicating that milk forms a very minor excretory route for progesterone and its metabolites.

Steady state intravenous infusion studies have indicated that mammary extraction of labelled progesterone could vary from nil to 24.4% in lactation but could be as high as 84.3% in non-lactating animals when blood flow was low (Heap et al., 1975). Comparison of the concentration of labelled compounds in mammary arterial and venous blood indicated that in particular circumstances mammary metabolism could be substantial: total ether extractable radioactivity represented by progesterone was reduced from 79% to 64% respectively in one cow and from 72% to 11% respectively in another (in which mammary blood flow was low). The same study indicated that in milk 90% of the ether extractable radioactivity was associated with authentic progesterone and about 10% with a component less polar than progesterone: the latter component was thought to correspond with 5α-pregnanedione, a compound identified in milk as representing the major unconjugated progesterone metabolite by others using in vitro tracer studies (Purdy et al., 1980) or gas chromatography (Darling, Laing & Harkness, 1974). The predominance of 5α reduced metabolites in milk (Darling et al., 1974; Purdy et al., 1980) indicates that these are derived, at least in part, from mammary metabolism since the liver, the major site of metabolism, produces exclusively metabolites of 5β configuration (Purdy et al., 1980; Clemens & Estergreen, 1982).

Reports of the proportion of progesterone metabolites represented as conjugated steroids (non ether-extractable) vary from 45% (Heap et al., 1975) and 15% (Purdy et al., 1980) to as low as 1% (Estergreen et al., 1977). The reasons for these large differences remain unexplained but are unlikely to be due to differences in extraction procedure: differences in stage of oestrous cycle or pregnancy are not stated in the latter two studies and could partly
account for the disparities. Although the conjugated metabolites may well represent a substantial proportion of the progestin content of the milk, their nature has not been reported.

1.6.2. The Determination of Progesterone in Milk by Saturation Analysis

Progesterone was first reported to occur in milk by McCracken (1963) who used ultra-violet absorption methods for its estimation. Gupta (1967) confirmed its presence using gas liquid chromatography (GLC) and Darling, Kelly, Laing & Harkness (1972) made a definite identification using gas chromatography and mass spectrometry. Subsequent to these studies concentrations of progesterone in milk have, for routine purposes, been determined exclusively by saturation analysis (see section 1.8): initially competitive protein binding assays were used (Laing & Heap, 1971; Schiavo et al., 1975) and later RIA (Heap et al., 1973; Hoffmann & Hamburger, 1973; Lamming & Bulman, 1976; Pope et al., 1976).

To ensure maximum cost-effectiveness of milk progesterone determinations in fertility management, assay methodology has become increasingly simplified. Although some laboratories continue to apply extraction and chromatographic procedures to milk samples prior to assay (Hoffmann et al., 1979), many laboratories perform RIA determinations by direct addition procedures: whole milk (Heap et al., 1976; Holdsworth, Chaplin & Booth, 1979; Bulman & Lamming, 1979) or defatted milk (Pope et al., 1976; McCaughey & Gordon, 1979) is added to the assay system without prior extraction or purification. As a result procedures of this type clearly rely upon the specificity of the antiserum and constancy of non-specific binding effects to ensure accuracy. A number of laboratories have established the concentrations of progesterone which occur in the different types (fore, composite or strippings; section 1.6.4) and fractions of milk (Pope et al., 1976) and from cows of varied reproductive status using extraction and chromatographic purification prior to assay by gas chromatography and/or RIA (Darling, Laing & Harkness, 1974; Ginther et al., 1974; Nuti et al., 1975). There have been no reports, however, in which authors using direct addition
procedures have evaluated their assays by comparison with extraction/purification methods performed on the same samples. Despite this, there is a broad level of agreement between independent reports of concentrations by these different methods.

The majority of early studies of progesterone concentrations in milk involved extraction or extraction and chromatographic purification of progesterone prior to assay (Heap et al, 1973; Hoffmann & Hamburger, 1973; Darling et al, 1974; Ginther, Nuti, Wentworth & Tyler, 1974; Nuti et al, 1975; Schiavo et al, 1975). These reports were largely concerned with determinations in whole milk samples from pregnant animals since pregnancy testing was a major application of the assay: concentrations between day 30-120 of pregnancy were reported to range between 5-15ng/ml (quantitation by gas chromatography, values uncorrected for losses, Darling et al, 1974), 20-50ng/ml (quantitation by gas chromatography and RIA, Nuti et al, 1975), 23-25ng/ml (quantitation by RIA, Ginther et al, 1974) and 20-44ng/ml (quantitation by competitive binding assay, Heap et al, 1973) when extraction and chromatographic purification were employed. When extraction alone was used prior to quantitation by RIA or competitive protein binding assay, concentrations ranged from between 7-22ng/ml (Heap et al, 1973) to in excess of 30ng/ml (Hoffmann & Hamburger, 1973). The limited studies carried out at that time on extracted composite whole milk samples collected throughout the oestrous cycle indicated that follicular phase concentrations were about 1ng/ml or less (Heap et al, 1973; Hoffmann & Hamburger, 1973; Schiavo et al, 1975) rising to luteal phase concentrations varying between an average of 9ng/ml (Schiavo et al, 1975) to about 30ng/ml (Hoffman & Hamburger, 1973). Following extraction and isolation by chromatography, concentrations were found to be around 0.8ng/ml during the follicular phase, rising to between 7 and 12ng/ml in the luteal phase (Nuti et al, 1975): no significant difference was found between estimation using two different RIA systems or GLC for quantitation.

Following this period it was found that determinations could be greatly simplified by direct addition of the whole milk sample to the RIA system thereby circumventing the extraction and purification steps (Heap et al, 1973; Heap
et al., 1976; Lamming & Bulman, 1976). A major oversight during this switch in procedures was that none of these authors had directly compared estimates on the same samples using "direct addition RIA" with those found following purification. This was probably overlooked partly because concentrations were in general agreement with those found previously: Bulman (1979) has compiled and compared values obtained by several laboratories when using direct addition procedures. In these studies, values for the majority of composite whole milk samples fell between 0-3ng/ml during the oestrous period, 10-30ng/ml during the luteal phase and 12-40ng/ml during early pregnancy (Holdsworth, Chaplin & Booth, 1979; Booth, Davies & Holdsworth, 1979; Bulman & Lamming, 1979).

1.6.3. Potential Sources of Interference with Progesterone Determination by Immunoassay

Of the steroids which have been identified in milk 5α-pregnanedione and to a lesser extent 5β-pregnanedione (Darling et al., 1974; Purdy et al., 1980) commonly show appreciable cross-reactivity against readily available anti-progesterone sera (see section 1.8). In vivo metabolic studies using (14C)progesterone (Estergreen et al., 1977) have allowed isolation of steroid fractions from milk with chromatographic properties like pregnanediones and mono-, di- and trihydroxylated progesterone metabolites: these represented 11.5%, 27.9% and 4.5% respectively of total radioactivity in milk compared with progesterone and could again constitute potential significant cross-reactants.

Of the steroids which have been specifically assayed in milk, oestradiol (MacDonald, Sauer, Watson & Foulkes, 1982; Glencross & Abeywardene, 1983), oestrone sulphate (Heap & Hamon, 1979; Holdsworth, Heap, Booth & Hamon, 1982), androstenedione (Gaiani, Chiesa, Mattioli, Nanetti & Galleali, 1984), 5α-androstáne-3, 17-dione (Darling et al., 1974), testosterone (Gaiani et al., 1984) and cortisol (Schwalm & Tucker, 1978), would be expected to show low cross-reactivity with the specific antisera in use in the majority of laboratories and due to their low concentrations in milk are unlikely to influence determinations of progesterone by RIA.
It has been demonstrated that aqueous milk constituents have a considerable capacity to bind progesterone non-specifically and assumed that casein, by far the most abundant protein in milk (80%, see Renner, 1983) may be responsible for this effect. Tracer studies involving ultracentrifugation of milk have indicated that 80% of radio-labelled progesterone was contained in the lipid fraction, 19% in the pellet and less than 1% in the aqueous phase (Heap, Henville & Linzell, 1975). Thus, when milk samples are estimated by direct addition RIA this phenomenon presents a potential source of inaccuracy since radio-labelled progesterone will bind specifically to antibody and non-specifically to casein. In practice this problem may be overcome by addition of milk with negligible progesterone content (milk from an ovariectomised cow or a cow at oestrus) to the assay standards thereby producing equivalent non-specific binding in standards and test samples (Heap et al., 1973; Gadsby, Heap, Henville & Laing, 1974).

1.6.4. The Assay of Progesterone in Milk: Practical Considerations of Sampling

 Since milk is not a homogenous body fluid a number of parameters have been investigated to evaluate and minimize possible sources of error in sampling and handling. These include the type of sample taken at milking, the fraction of milk assayed and, where appropriate, methods of sample storage.

 Three alternative types of milk sample have been considered as practical, the first or fore-milk, composite milk (a representative sample taken from the whole milking) or the strippings (taken from the teat by hand following milking). Most research has been carried out using the composite sample since this is most representative (Schiavo et al., 1975; Heap et al., 1976; Lamming & Bulman, 1976; Pope et al., 1976) although fore-milk (Thibier, Fourbert & Farez, 1976) and strippings (Hoffmann et al., 1976; van de Wiel et al., 1978) have been used by some groups in part because they better suit the particular management and milking systems in operation.

 Between 80 and 87% of the progesterone content of milk is associated with the lipid fraction (Heap, Henville & Linzell, 1975; Pope et al., 1976) and in
keeping with the fat content of milk types (stippings > composite > fore; Pope et al, 1976; Smith, 1959), the progesterone content is highest in the stippings, intermediate in composite milk and lowest in fore-milk (Schiavo et al, 1975; Pope et al, 1976). Although the fat content of milk may differ according to the udder quarter from which it is drawn and according to the time of day and the physiological status of the cow (Copeland, 1929; King, 1977; Dodd & Griffin, 1979), work by Schiavo et al (1975) and McCaughey & Gordon (1979) indicated that progesterone concentration did not differ significantly in milk taken from the four udder quarters irrespective of the stage of cycle. It has been found however that progesterone concentrations are significantly higher in milk taken at the evening milking than that taken in the morning (Heap et al, 1976; Batra, Pahwa, Suri & Pandey, 1980): it is not clear whether this was related to an increased secretion rate or to a higher fat content of the milk during the evening (Heap et al, 1976).

There is a close positive correlation between the fat content of milk and its progesterone concentration (Hoffmann & Hamburger, 1973; Ginther, Nuti, Garcia, Wentworth & Tyler, 1976; Pope et al, 1976): this is perhaps to be expected since progesterone is thought to occur in milk as a result of diffusion from blood down a concentration gradient and that its higher concentration in milk than plasma is probably related to its high solubility in and effective sequestration by milk lipids (Heap et al, 1975). Clearly this indicates that different concentration limits will need to be set when assaying luteal function in breeds producing milk of higher fat content (e.g. Guernsey and Jersey cattle; Pennington, Spahr & Lodge, 1981) or when fore-milk or stippings are assayed rather than composite milk.

The errors in interpretation of ovarian status as a result of large fluctuations in fat content of particular samples (e.g. due to decreased fat yield at oestrus or faulty sampling) may be avoided by the assay of progesterone in milk fat (Hoffmann et al, 1976; Claus & Rattenburger, 1979) or defatted milk (Pope et al, 1976; McCaughey & Gordon, 1979). The benefits accrued from these procedures must, however, be balanced against the extra time and effort.
involved in preparing milk fat or defatted milk. The work of Lamming & Bulman (1976) indicates that although errors associated with fluctuations in milk fat content may be reduced by correcting for fat content only a small percentage of animals (3.2%) were displaced in category of ovarian status by not doing so: this percentage would probably be reduced if successive samples were being studied as an aid to the diagnosis of sub-fertility. Pennington et al (1976) have stressed that errors associated with fat content of whole milk will be less pronounced with a composite milk sample rather than fore-milk (see also Heap et al, 1976) or strippings and that although milk fat content normally varies by less than two fold (2.5 - 5%) a greater than ten fold change in progesterone concentrations occurs between the follicular and luteal phase of the cycle (<2ng/ml to >20ng/ml respectively).

1.6.5. Preservation of Milk Samples

Milk samples may require to be stored before assay and storage frozen or at 4°C after the addition of preservative have been considered.

Storage at -10°C or -20°C has proved convenient (Ball & Pope, 1976) although after 3-6 months this may result in precipitation of casein micelles following thawing (Pope et al. 1976): the addition of EDTA solution to the sample may overcome this problem, however (Pope & Swinburne, 1980). Nuti, Wentworth, Karavolas, Tyler & Ginther (1975) and Oltenacu & Foote (1976) found small but non-significant changes in progesterone concentration during storage at -10°C for 10-12 months.

The use of various preservatives including potassium dichromate/mercuric chloride tablets (Lactabs Mk II: Heap et al, 1976; Lamming & Bulman, 1976; Foote et al, 1979; McCaughey & Gordon, 1979), sodium azide (Pope et al, 1976), formalin (Bishop, Bond & Roberts, 1976), formaldehyde (Batra, Arora, Bachlaus & Pandey, 1979) and chloramphenicol (Hoffmann et al, 1974) have been reported and compared (Durdevic, Maksimovic, Stojic & Kercov, 1979) and found to enable storage of milk samples at 4°C for many months without significant alterations in progesterone content as determined by immunoassay. Of these, Lactab
treatment (1 tablet/20 ml milk) is perhaps the most convenient and commonly employed: potassium dichromate tablets (Lactab Mk III) have recently been found to be equally effective (R.J. Holdsworth, personal communication).

1.6.6. The Diagnostic Value of Determinations of Progesterone in Milk

The relative ease of progesterone determination offered by RIA rapidly lead to the appreciation that its measurement in the milk of dairy cows provided an accurate and valuable tool in the assessment of the functional status of the corpus luteum and thus of reproductive events associated with and controlled by its secretions. Such determinations have enabled testing for pregnancy at 21-24 days post AI (Laing & Heap, 1971; Heap et al, 1973; Heap, Holdsworth, Gadsby, Laing & Walters, 1976; Pennington, Spahr & Lodge, 1976; Pope et al, 1976) and have highlighted problems associated with acyclicity and the timing of the postpartum return to ovarian cyclicity (Bulman & Lamming, 1978; Foote, Oltenacu, Kummerfeld, Smith, Riek & Braun, 1979; Günzler, Rattenburger, Görlach, Hahn, Höcke, Claus & Karg, 1979; van de Wiel, Kalis & Shah, 1979), the incidence and occurrence of silent or missed oestrous periods (Lamming & Bulman, 1976; Karg et al, 1980; Greve & Lehn-Jensen, 1980), the accuracy of behavioural oestrous detection (Hoffmann, Günzler, Hamburger & Schmidt, 1976; Bulman & Lamming, 1979; Günzler et al, 1979; McCaughey & Cooper, 1980; Oltner & Edqvist, 1981; Henriksen et al, 1982; Laitinen, Remes, Tenhunen, Hänninen & Alanko, 1985) and the occurrence of early embryo mortality (Ball, 1978; Bulman & Lamming, 1978; Humblot, 1982). The diagnosis of the anovulatory condition using this method and its ability to distinguish between follicular and luteal cysts enable the appropriate choice of hormone therapy to be made (Hoffmann et al, 1976; Lamming & Bulman, 1976; Dobson, Rankin & Ward, 1977; Günzler et al, 1979; van de Wiel et al, 1979).

In addition the test may be used to confirm the accuracy of an oestrous observation if a milk sample is taken on the day of AI (Günzler et al, 1979). Ball & Jackson (1979) took this a step further and used an appropriate milk sampling and testing regime to enable prediction of the timing of oestrus and thus of AI: this demonstrated that cows are capable of conceiving at the time of ovulation even if this is not preceded by observed oestrus. Günzler et al (1979) have
indicated the possibility of applying progesterone assays to embryo transfer programmes since synchronisation of cycle stage and conception age is critical.

It is becoming clear that access to a simple and rapid progesterone test may help resolve some of the problems which arise in fertility management and which have emerged from the studies cited above. With regard to early post-partum studies on ovarian activity, general agreement was found with earlier investigations involving palpation or determination of plasma progesterone concentrations (Labhsetwar, Tyler & Casida, 1963; Morrow, Roberts, McEntee & Gray, 1966; Pope, Gupta & Munro, 1969; Robertson, 1972; Glencross & Munro, 1974): the first ovulation normally took place around the 24th day postpartum and was often not preceded by oestrous behaviour (Bulman & Lamming, 1978; Foote et al, 1979; Günzler et al, 1979; van de Wiel et al, 1979). Although it may not be desirable to inseminate at the first ovulation postpartum due to the likelihood of low fertility at this time, it is important to inseminate at the following and subsequent oestrous period, where appropriate, in order to achieve a mean calving interval of one year and to achieve and maintain a tight calving pattern. This requires that animals not pregnant to AI must be identified, ideally within 21 days to enable re-insemination at the next oestrous period. Similarly, sub-fertile animals must be correctly identified at an early stage postpartum so that appropriate action can be taken.

The incidence of early embryonic mortality, often considered to be of the order of 15-20% at around day 24 (see Gwazdaukas, Lineweaver & Vinson, 1981), may have been over-estimated due to problems associated with poor oestrous detection at AI or subsequent silent heat 21 days post AI: a more realistic figure may be of the order to 5-10% (Ball, 1978; Kummerfeld, Oltenacu & Foote, 1978; Humblot, 1982).

Unfortunately, progesterone does not provide a specific positive test for pregnancy: although it is possible to determine non-pregnancy, by assay of a single sample taken at day 21-24, with near 100% accuracy, pregnancy estimations are only about 80% accurate (Hoffmann, Hamburger, Günzler, Korndörfer & Lohoff, 1974; Heap et al, 1976; Hoffmann et al, 1976; Pennington et al, 1976; Pope et al,
False positive results may be due to an incidence of incorrect oestrous detection (luteal phase insemination), irregular cycle length, faulty milk sampling or embryonic death. There is currently no alternative early test for pregnancy in dairy cattle but the use of real time ultrasonic scanning devices presents a possibility for the future (Reeves, Rantanen & Hauser, 1984; von Kähn, 1985). The occurrence of oestrone sulphate in milk provides a specific test for pregnancy being a product of the conceptus but can only be performed with confidence after 105 days of gestation (Heap & Hamon, 1979; Holdsworth, Heap, Booth & Hamon, 1982).

In the United Kingdom a commercial pregnancy testing service is offered by the Milk Marketing Board at a single central laboratory and is based on milk progesterone determination by RIA. Although this provides a valuable service the test is statistically most reliable if conducted between 22-26 days post AI (Holdsworth et al., 1979). This and the inevitable delays in communicating the results means that the non-pregnancy data is available too late for insemination 21 days after the first AI.

It is clear that the determination of progesterone concentrations in milk offers what is perhaps the only single aid by which the majority of fertility management problems can be assessed and possibly corrected. RIA is, however, fatally flawed in that it is neither cheap nor fast enough or sufficiently simple to perform to be of value for the majority of its potential applications. The development of alternative non-isotopic immunoassays, based on principles similar to RIA, are a means by which these objections may be circumvented and may provide a means by which the test can be performed locally at a veterinary laboratory or, eventually, on the farm by the dairyman. Such methods will be discussed in a later section (1.9).

1.7. METHODS OF PROGESTERONE DETERMINATION

Prior to the late 1960's, methods for the detection and estimation of progesterone in biological fluids and tissue were based upon its physico-
chemical properties and biological effects. These methods have been reviewed by van der Molen & Aakvaag (1967) and van der Molen (1979) and include determination of ultra-violet or (following derivatization) visible light absorption (Perlman & Cerceo, 1953; Hinsberg, Pelzer & Seuken, 1956; Short, 1958), fluorescence (Touchstone & Murawec, 1960; Heap, 1964), isotope labelling with isotope dilution (van der Molen, Rünnenbaum, Nishizawa, Kristen, Kirschbaum, Wiest & Eik-Nes, 1965; Riondel, Tait, Gut & Little, 1965), and detection after gas-liquid chromatography (flame ionization, or, following derivatization, electron capture or nitrogen detection: Lurie, Villee & Reid, 1966; van der Molen & Groen, 1967; Stabenfeld, Ewing, Patton & McDonald, 1969; Frith & Phillipou, 1981) and bioassay (effects on endometrium, ovary or the capacity to maintain pregnancy - McGinty, Anderson & McCullough, 1939; Hooker & Forbes, 1949; Miyake & Pincus, 1958; Miyake, 1962). Their use has, however, been limited to quantitative or semi-quantitative determinations or to quantitation of relatively small numbers of samples. This is due to the relatively low concentrations of progesterone in biological fluids combined with the lack of specificity and sensitivity of the methods or to the time and expertise required. These methods are based on "reagent excess" principles (Ekins, 1976) since they rely on the use of an excess of specific reagent (chemical, physical or biological) which is converted by interaction with the hormone to give a detectable change directly proportional to the amount of hormone present. Assays in most common use today, however, are based on a totally different principle, that of "saturation analysis" (Ekins, 1976).

1.8. THE PRINCIPLE OF SATURATION ANALYSIS AND ITS APPLICATION TO THE IMMUNOASSAY OF PROGESTERONE.

Assays based on the principle of saturation analysis utilize a specific reagent, present in a limited and defined quantity (usually less than the amount of analyte in the system) and observations are made of the proportion of analyte which, as a result of its interaction with the reagent attains altered characteristics (Ekins, 1976). This principle was first exploited for the determination of insulin by
Berson and Yalow (1959) and of thyroxine by Ekins (1960) and was later adopted for the measurement of steroid hormones by methods more specifically termed competitive protein binding assay (CPBA: Murphy, 1967; Neill, Johansson & Knobil, 1967; Yoshimi & Lipsett, 1968) and radioimmunoassay (RIA: Abraham, 1969). The specific binding reagents in RIA are antibodies and in CPBA endogenous binding proteins such as corticosteroid-binding globulin. Although both procedures provided assays several orders of magnitude more sensitive than those using reagent excess, RIA soon proved to be the method of choice for the determination of steroids in biological fluids since it provided a higher degree of accuracy, precision, specificity and sensitivity than CPBA (van der Molen, 1979; Fotherby, 1979). This enabled reliable measurements of some hormones in body fluids and tissues to be made for the first time and is still the most widely used method for progesterone determination.

Immunoassays based on the saturation analysis principle require the provision of three components, a) a limited and constant quantity of antibody specific for the analyte, b) a limited and constant quantity of labelled analyte and c) standard quantities of analyte for calibration purposes (or unknown quantities of analyte in the test sample). When the three components are mixed, labelled and unlabelled analyte compete for the limited (saturable) number of antibody binding sites: since each test contains the same amount of antibody and labelled analyte, the greater the quantity of unlabelled analyte present the smaller will be the quantity of labelled analyte binding to the antibody. A fourth requirement for the assay is d) the availability of a means of distinguishing between and quantitating free labelled analyte and that bound to antibody: standard amounts of analyte thus enable calibration curves to be constructed from these data and quantitation of analyte in the unknown sample by interpolation. The components a), b) and d) are worthy of further consideration and are discussed below in the context of progesterone determination.
1.8.1. The Production and Characteristics of Progesterone Antibodies

The determination of progesterone by immunoassay requires the availability of antibodies which bind progesterone with specificity and preferably high avidity. Progesterone, being a hapten, is not immunogenic in its own right (in common with most molecules of molecular weight <4,000 Daltons; Kellie, Lichman, Samarajeewa, 1979) and antiserum production requires conjugation of a progesterone derivative with a carrier protein such as albumin (Erlanger, Borek, Beiser & Lieberman, 1957b) prior to immunization of the host animal.

The first generation of such steroid immunogens were fashioned by utilizing one of the primary functional groups (at C-3 or C-20) to form a derivative, usually containing a carboxylic acid group, which could subsequently be covalently conjugated by amide bond formation to the ε-amino groups of lysine in a protein (Beiser, Erlanger, Agate & Lieberman, 1959; Niswender & Midgley, 1970). Antisera raised by injection of these immunogens were generally of low specificity, however. Subsequent suggestions that a carboxylic acid (or alternative reactive residue) be introduced at a site remote to the functional group (i.e. in ring B or C: Lindner, Perel & Friedlander, 1971) to avoid steric hindrance of the distinctive features of the steroid, lead to the formation of antibodies of increased specificity (Niswender & Midgley, 1970; Kohen et al, 1975; Niswender, Nett, Meyer & Hagerman, 1975). The length of the chemical bridge between hapten and carrier protein may for similar reasons be expected to influence antiserum specificity: the optimum length would appear to be 4-6 carbon atoms (Bermúdez, Coronado, Mijares et al, 1975; Robinson, Morris, Piall, Aherne & Marks, 1975). The avoidance of conjugation of positions on the steroid which are prone to major metabolic transformation may reduce the possibility of producing antisera cross-reacting with such metabolites (Kohen et al, 1975).

Reactive derivatives of progestins suitable for conjugation to protein include oximes derived from ketonic groups, hemisuccinates and acid chlorides from hydroxyl groups and thioether alkanoic acids from halogenated or α, β-unsaturated ketones. Anti-progesterone sera have thus been raised against progesterone derivatives linked to carrier protein via O-carboxymethyloxime
at C-1 (Kohen et al., 1975) or C-3 (Niswender, 1973), carboxymethyl- or carboxyethyl thioether at C-6 or C-7 (Lindner, Perel, Friedlander & Zeitlin, 1972; Bauminger, Lindner & Weinstein, 1973), hemisuccinate, chloroformate or glucuronide at C-11 (Lindner et al., 1972; Furr, 1973; Niswender, 1973; Corrie, Ratcliffe & MacPherson, 1982), hemisuccinate at C-12 (Stupnicki & Kula, 1980) and hemisuccinate at C-20 or C-21 (Erlanger et al., 1959). Superior and comparable specificity has generally been obtained using conjugates linked via the C-7, C-11 (Kohen et al., 1975; Niswender et al., 1975) or C-12 (Stupnicki & Kula, 1980) position. The progesterone derivatives required for conjugation at C-7 and C-12 are neither cheap nor readily available. The converse is true of 11α-hydroxyprogesterone (the usual starting material for C-11 conjugation) which is a readily produced microbiological metabolite of progesterone (Peterson, Murray, Epstein et al., 1952) and so has become the most often used derivative for production of anti-progesterone sera. Generally this has been linked via a hemisuccinate bridge to the carrier protein (Thorneycroft & Stone, 1972; Lindner et al., 1972; Furr, 1973) and antisera so produced cross-react to the largest extent (>50%) with 11α-hydroxyprogesterone (Thorneycroft & Stone, 1972; Furr, 1973) which fortunately is not known to occur in significant concentrations in mammalian blood (Kohen et al., 1975). Significant (2-20%) cross-reactions against the 5α and 5β pregnane-3, 20-diones and to a lesser extent with monohydroxy pregnanes and pregnenes has been noted by some (Thorneycroft & Stone, 1972; Furr, 1973; Fotherby, 1979).

Methods used for covalently linking carboxy or amino steroid derivatives to carrier proteins have been extensively reviewed (Kellie et al., 1975; Kohen et al., 1975; Niswender et al., 1975; Butler, 1977; Pratt, 1978) and principally involve condensation with ε-amino groups of lysine residues or β or γ-carboxyl groups of aspartic or glutamic acid, respectively. The condensation of carboxylic and amino groups has been achieved by two principal methods, the mixed anhydride reaction (Erlanger, Borek, Beiser & Lieberman, 1957a) and the carbodiimide reaction (Sheehan & Hess, 1955; Kurzer & Douraghi-Zadeh, 1967).
Where chlorocarbonate derivatives are used the Schotten-Baumann reaction enables condensation with amino residues to be simply performed (Erlanger et al., 1957a).

A diverse range of carrier proteins have been used in the formation of immunogens including keyhole limpet haemocyanin, thyroglobulin and albumins from various species or synthetic macromolecules such as poly-L-lysine (James & Jeffcoate, 1974; Pratt, 1978). Although bovine serum albumin has been commonly employed it has been stressed that the latter would not be the carrier of choice if species phylogenetically similar to the bovine were being immunized (sheep or goats) since this may lead to an attenuated response related to inter-species tolerance (Lescowitz, 1972): ovalbumin provides a useful alternative carrier for these species.

Although antibodies are produced by transformed B-lymphocytes (plasma cells) in response to immunization it is important that lymphocytes are not exposed directly to the immunogen since this may give rise to tolerance (Boak, Kolsch & Mitchinson, 1969): it is preferable that exposure of the B-lymphocyte to the immunogen is achieved through presentation by the macrophage (Boak, Kolsch & Mitchinson, 1969). Slow release of immunogen such as achieved through intramuscular, subcutaneous or intradermal injection has proved particularly effective for antiserum production (see Hurn, 1974). Satisfactory anti-steroid serum production necessitates the injection of an adjuvant with the immunogen since this induces non-specific proliferation of lymphocytes derived from bone-marrow (Roitt, Greaves, Torringiani et al., 1969).

It has proved possible to raise antisera against all of the steroid hormones and many of their metabolites, although the success of the immunization procedures and the binding properties of the resultant antibodies have been highly variable. Differences between species and individual animals have been found. The adjuvant, the dose and the purity of the immunogen, its route of administration and the timing of repeat inoculations and subsequent blood samples have been found to give rise to differences in response (Hurn & Landon, 1971; Hunter, 1978; Abraham, 1974; Parker, 1976; Hunter & Corrie, 1983).
Blood taken 7-10 days after booster injection is assessed for titre, specificity and avidity of binding. Antibody titre is that dilution of antiseraum giving rise to 50% binding when incubated with a stated amount of labelled analyte alone. Low titre is not, however, indicative of a poor antiseraum; quality is assessed following an estimation of the avidity of binding, which governs the sensitivity and detection limit of the assay and the specificity of binding which indicates the extent to which structurally similar steroids may interfere with the assay.

The binding of analyte to antibody proceeds in accordance with the Law of Mass Action (see Ekins, 1974),

\[ H + Ab \rightleftharpoons H \cdot Ab \text{ at equilibrium} \]

\[ \frac{[H \cdot Ab]}{[H][Ab]} = \frac{K_a}{K_d} = K \]

Where \( H \) = hapten or analyte; \( Ab \) = antibody; \( H \cdot Ab \) = hapten-antibody complex;

\( K_a + K_d \) = rates of association and dissociation respectively of the complex;

\( K \) = the equilibrium constant or avidity of the antiseraum.

Ekins (1976) has demonstrated that the theoretical lower limit of detection in saturation analysis is of the order of \( \frac{\varepsilon}{K} \) (\( \varepsilon \) is the relative error of the response determination), thus the greater the affinity of binding the lower the detection limit. The determination of the equilibrium constant is complicated by the heterogeneity of the polyclonal antibody population but estimations may be made by applying Scatchard or Michaelis-Menton analysis (see Buller, Schrader & O'Malley, 1976) to data obtained by determining the quantity of free and bound analyte following addition of increasing amounts of labelled steroid to a fixed amount of antibody.

Tests of antiseraum specificity are performed in order to evaluate if, or to what extent, samples may require to be purified before assay and is investigated by estimation of the extent to which potential cross-reactants compete with labelled analyte for antibody binding sites. This is expressed as a percentage of the competition obtained with unlabelled analyte. Two of the more commonly employed methods of describing cross-reactions are (i) in terms of the ratio of amount of analyte to cross-reactant required to give rise to a 50% displacement of
labelled analyte \((\text{CR}_50: \text{Abraham}, 1969)\) or (ii) the displacement of labelled analyte produced by a fixed mass of analyte or cross-reactant \((\text{De Lauzon, Cittanova, Desfosses \\& Jayle, 1973})\). The fixed-mass test is to be preferred since in the first method, binding inhibition curves of the analyte and cross-reactant are not parallel \((\text{Rodbard \\& Lewald, 1970})\) and the concentrations of cross-reactant used often fall outside the physiological range of concentrations \((\text{De Lauzon et al., 1973})\). These methods require a prescience on the part of the investigator which may often be incomplete. Tests which involve addition of analyte to analyte-free samples or dilution of samples containing significant endogenous quantities of the "analyte" and subsequent evaluation of the linearity of estimates \((\text{recovery and parallelism experiments})\) provide additional information relating to the specificity of the assay in practice.

1.8.2. The Choice of Label

In saturation analysis, there is no requirement that the analyte and its labelled form be chemically identical. They do, however, have to compete for the same antibody binding sites and the labelled analyte should be capable of detection and quantitation by sensitive methods. In steroid immunoassay radiolabelling is most commonly used.

Progesterone may be labelled internally by substitution of \(^{14}\text{C}\) or \(^3\text{H}\) into the normal steroid structure \((\text{labelled analyte and analyte are chemically identical})\) or externally by conjugation with a compound, e.g. tyrosine methyl ester or histamine, which may be iodinated before or after conjugation: \(^{125}\text{I}\) has been the radionuclide most often employed \((\text{Scarisbrick \\& Cameron, 1975})\).

High specific activity radio-labelled analytes in conjunction with antibodies of high avidity enable a high degree of precision and sensitivity to be achieved in RIA. Although modern automatic and manual \(\beta\) and \(\gamma\)-radiation counters
operate at high efficiency and are easy to use there are numerous disad-
vantages to these forms of labelling. The performance of RIA is limited to
specialised licensed laboratories due to the high cost of counters and the
regulations associated with the use of radio isotopes. Shortcomings relating
to the use of β-particle emitting isotopes are the comparatively low specific
activity of $^{14}$C- and $^{3}$H-labelled analytes (long counting times required for
high precision) and the high cost of liquid scintillator required for their
determination. The $^{125}$I-labelled alternatives, however, have short half-lives
and lack stability ("decay catastrophe"). Schall and Tenoso (1981) have reviewed
the numerous alternatives to radio-labelling for use in immunoassays. These
include use of bacteriophages (Andrieu, Manas & Dray, 1975), chemiluminescence
precursors (Kohen, Kim, Lindner & Collins, 1981; Pazzagli, Kim, Messeri et al,
1981; Kohen, Lindner & Gilad, 1983), fluorochromes (Soini & Hemmilä, 1979;
Allman, Short & James, 1981; Bacigalupo, Ins, Merini et al, 1983), enzymes
(Engvall & Perlmann, 1971; Van Weemen & Schuurs, 1971; Dray, Andrieu & Renauld,
1975), coenzymes (Schroeder, Carrico, Boguslaski & Christner, 1976), enzyme
inhibitors (Finley, Williams & Lichti, 1980) enzyme substrates (Burd, Wang,
Feeney et al, 1977; Pearson, Smith & Marks, 1984), metal atoms (Cais, Dani,
Elden et al, 1977), sol particles (Cambiaso, Leek, de Steenwinkel et al, 1977)
and stable free radicals (Wei & Almirez, 1975). The widespread application of
these labels is, however, dependent upon the development of detector technology
that will enable instrumentation to become available at reasonable cost. The
main exceptions are those methods in which enzyme activity is determined: the
relative merits of such methods are described in section 1.9.

1.8.3. Distinction Between Free and Antibody Bound Analyte

The end-point of saturation analysis immunoassays involve discrimination
between and quantitation of the relative proportion of analyte which is free
and that which is bound to antibody: the practical and theoretical aspects of
the means by which this may be achieved have been reviewed by several authors
(Aubert, 1970; Rodbart & Catt, 1972; Hunter, 1973; Ratcliffe, 1978). This
usually requires separation of the two species, although in very particular circumstances (homogeneous assays) where the quantifiable properties of the label are changed as a result of binding (see Boguslaski & Li, 1982) this may not be necessary. Although homogeneous assays offer much potential for the development of simple tests, in most forms they lack the sensitivity required for the determination of steroid hormones (Schuurs & Van Weemen, 1977) particularly where samples are measured without extraction and concentration. Estimation of label is also prone to interference from components of complex biological fluids such as serum or milk since by definition no free/bound separation or wash stages are employed. For these reasons these types of procedure are not considered further here.

An ideal separation method should enable precise and complete separation of the components and should not, as a result, interfere with the equilibrium attained by the primary binding reaction nor be affected by other components in the sample. The reagents and equipment should be cheap, readily available and should enable separation to be performed quickly and easily thereby allowing large numbers of samples to be processed.

Separation techniques are varied and rely on differences in molecular weight, charge, adsorption, solubility and precipitative properties between the bound and free fractions. Methods involving adsorption and precipitation of the free hormone (e.g. by addition of dextran coated charcoal: Abraham et al., 1971; Lindner et al., 1972; Niswender, 1973; Heap et al., 1976) have often been employed in the assay of progesterone by RIA: conditions must be optimized, however, since adsorption is highly dependent upon pH, temperature and contact time and may result in some stripping of analyte from antibody binding sites if low avidity sera are used (Abraham et al., 1971; Malvano & Rolleri, 1975; Runnenbaum, 1975). Adsorption methods rely on similarities in physical properties between the labelled and unlabelled free analyte to separate them from the bound forms. Such methods are, however, not well suited to enzyme immunoassay where the molecular weight of the labelled and unlabelled hormone differ by orders of magnitude.
Immunoprecipitation methods, involving addition of a second antibody raised against the primary (analyte specific) antibody, are less prone to errors in quantitating the proportion of label in each fraction (misclassification errors) than adsorptive methods (Ratcliffe, 1983). Although this separation process used to require prolonged incubation to ensure complete precipitation, separation times may be reduced by attachment of the second antibody to a particulate solid phase (double antibody solid phase method) or by the use of an accelerator such as polyethylene glycol (Edwards, 1983). The attachment of primary antibody directly to a solid phase such as the walls of plastic test tubes (Catt & Tregear, 1967; Gowan & Etches, 1979) or to particulate material such as cellulose microgranules (Dighe & Hunter, 1974) provide perhaps the most rapid and convenient means of separation, allowing short incubation periods and providing low misclassification errors (Wide, 1978). The immunoprecipitation and solid-phase primary antibody methods are both well suited to use with the majority of forms of label including enzymes.

1.8.4. Extraction Versus Direct Assay of Progesterone

In order to eliminate potential sources of interference, both non-specific (i.e., not interfering directly with antibody-analyte binding) and from cross-reacting substances, solvent extraction procedures and chromatographic purification have traditionally been used prior to RIA. Extraction alone with a selective, non-polar solvent such as petroleum ether may be sufficient to eliminate more polar steroids which cross-react with progesterone (Dighe & Hunter, 1974). Where antisera are suitably specific to enable direct addition assays to be performed, non-specific interference such as may be caused through binding of progesterone to corticosteroid-binding globulin (in women) has been prevented either by performing the assay at reduced pH (Ratcliffe, Corrie, Dalzial & MacPherson, 1982) or by addition of reagents capable of displacing non-specifically bound progesterone: Danazol (McGinley & Casey, 1979), cortisol (Haynes, Concorran, Eastman & Doy, 1980) and 8-anilino-1-naphthalene sulphonic acid (Ratcliffe et al., 1982). Such solutions have not,
however, been found to be applicable where non-specific binding was to other serum components such as albumin or lipoprotein (Pratt, 1978). Problems associated with non-specific binding in direct addition assays of progesterone in bovine plasma (Holdsworth, 1980) or milk (Heap et al., 1973; Heap et al., 1976), however, have not proved to be so severe as in human applications and such matrix effects have generally been circumvented by preparation of assay standards in analyte free preparations of the fluid under test.

1.9. ENZYMEMMUNOASSAY

Although many forms of labelling have been considered for use in saturation analyses, the use of enzyme-labelled analyte provides the most convenient means by which the sensitivity and specificity of RIA can be brought within the reach of the small laboratory: saturation analyses using enzyme-labelled analytes are termed enzymeimmunoassays (EIA). The advantages of using enzyme as opposed to radio-label have been enumerated by several authors (Landon, Crookall & McGregor, 1975; Schuurs & van Weeman, 1977; O'Sullivan, Bridges & Marks, 1979; Blake & Gould, 1984) and include the innocuous nature, stability and low cost of the reagents and the ready availability of equipment required for end-point determinations: the potential for producing colorimetric end-points which can be assessed by eye may be particularly valuable where quantitative or semiquantitative evaluation will suffice.

The use of EIA was first reported by Engvall and Perlmann (1971) and van Weemen & Schuurs (1971) and although such assays have since been developed for many steroid hormones (Table 1.1) and the majority of hormones associated with the reproductive process (van Weemen, Bosch, Dawson, van Hell & Schuurs, 1978; Sauer, Cookson, MacDonald & Foulkes, 1982a), RIA has remained the preferred method (Schall & Fraser, 1983). The main exceptions to this are in the monitoring of drugs by homogeneous EIA (Schall & Fraser, 1983) and in screening for infectious diseases (Voller, Bartlett & Bidwell, 1978; Wardley & Crowther, 1982). In the former case this is related to the relatively simple
### TABLE 1.1 Application of EIA to the Determination of Steroid Hormones

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Enzyme Label</th>
<th>Conjugation Method</th>
<th>Hetero/Homology</th>
<th>Body Fluid Assayed</th>
<th>Direct Addition or Extraction</th>
<th>Separation Technique</th>
<th>Detection Limit (pg/tube)</th>
<th>Author</th>
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<td>Cortisol</td>
<td>(\beta)-Gal</td>
<td>MA</td>
<td>homo</td>
<td>plasma</td>
<td>E</td>
<td>DA, DASPc</td>
<td>100</td>
<td>Comoglio &amp; Celada, 1976</td>
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<tr>
<td></td>
<td>AP</td>
<td>CDI</td>
<td>hetero</td>
<td>serum</td>
<td>D</td>
<td>DA</td>
<td>1000</td>
<td>Kobayashi et al., 1978</td>
</tr>
<tr>
<td></td>
<td>(\beta)-Gal</td>
<td>MBSae</td>
<td>hetero</td>
<td>plasma</td>
<td>D</td>
<td>DA</td>
<td>200</td>
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<td>Ogihara et al., 1977</td>
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<td>-</td>
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<td>HRP</td>
<td>Glut*</td>
<td>homo</td>
<td>serum</td>
<td>E</td>
<td>SPct</td>
<td>10</td>
<td>Sadeh et al., 1979</td>
</tr>
<tr>
<td></td>
<td>HRP</td>
<td>MA</td>
<td>homo &amp; hetero</td>
<td>plasma</td>
<td>&amp; (s)</td>
<td>-</td>
<td>DA</td>
<td>-</td>
</tr>
<tr>
<td>Oestradiol (total)</td>
<td>HRP</td>
<td>MA</td>
<td>homo</td>
<td>serum</td>
<td>D</td>
<td>DASPc</td>
<td>30</td>
<td>Bosch et al., 1978a</td>
</tr>
<tr>
<td></td>
<td>AP</td>
<td>CDI</td>
<td>homo</td>
<td>serum</td>
<td>D</td>
<td>DA</td>
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<td>MA</td>
<td>homo</td>
<td>serum</td>
<td>D</td>
<td>DASPc</td>
<td>30</td>
<td>Osterman et al., 1979</td>
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<td>-</td>
<td>DA</td>
<td>15</td>
<td>Dray et al., 1975</td>
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<tr>
<td></td>
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<td>MBSae</td>
<td>hetero</td>
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<td>DASPgb</td>
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<td>Gros et al., 1978</td>
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<td></td>
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<td>?</td>
<td>homo</td>
<td>serum (p)</td>
<td>E</td>
<td>DA</td>
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<td>Johnsen et al., 1980</td>
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<td>MA</td>
<td>homo</td>
<td>plasma</td>
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<td>SPao</td>
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<td>plasma</td>
<td>E</td>
<td>DA</td>
<td>10</td>
<td>Joyce et al., 1978</td>
</tr>
<tr>
<td></td>
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<td>MA</td>
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<td>plasma</td>
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<td>DASPc</td>
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<td>Kamonpatana, 1979</td>
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<td>E</td>
<td>SPct</td>
<td>55</td>
<td>Patrie et al., 1978</td>
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<td>CDI</td>
<td>homo</td>
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<td>E</td>
<td>SPct</td>
<td>55</td>
<td>Seeger et al., 1978</td>
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<tr>
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<td>HRP</td>
<td>MA</td>
<td>hetero (s+b)</td>
<td>serum</td>
<td>E</td>
<td>SPct</td>
<td>-</td>
<td>Bosch et al., 1978b</td>
</tr>
<tr>
<td></td>
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<td>MBSae</td>
<td>hetero</td>
<td>serum</td>
<td>-</td>
<td>-</td>
<td>DA</td>
<td>-</td>
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<tr>
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<td>HRP</td>
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<td>homo</td>
<td>-</td>
<td>-</td>
<td>SPc</td>
<td>50</td>
<td>Rajowski et al., 1977</td>
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<tr>
<td></td>
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<td>CDI</td>
<td>homo</td>
<td>serum</td>
<td>E</td>
<td>DA</td>
<td>250</td>
<td>Tateishi et al., 1977</td>
</tr>
</tbody>
</table>

**ABBREVIATIONS**

- **Enzyme** - \(\beta\)-Gal, \(\beta\)-Galactosidase; AP, alkaline phosphate; HRP, horse radish peroxidase; GA, glucoamylase;
- * enzyme conjugated to antibody ('sandwich' technique or immunoenzymometric assay).
- **Conjugation Method** - Glut., glutaraldehyde; MA, mixed anhydride; CDI, water soluble carbodiimide; MBSae, maleimido benzoate active ester; NBSae, N-hydroxysuccinimide active ester.
- **Body Fluid** - Species is human except where indicated; bovine (b), equine (e) or porcine (p).
- **Direct Addition or Extraction** - D, direct addition; E, hapten extracted prior to assay.
- **Separation Technique** - SP, solid phase antibody; DA, double antibody; DASP, double antibody solid phase; PEG, polyethylene glycol precipitation; c, cellulose; s, sephadex; gb, glass balls; mt, microtitre plate; ct, coated tube; so, sepharose.
and rapid homogeneous assay procedures which are possible when analyte concentrations are relatively high (μg/mL test fluid). In the latter there was a relatively low commitment to other types of assay prior to development of enzyme-label techniques.

With respect to steroid hormone assays, few laboratories have committed themselves to EIA and the necessary reagents have not become commercially available: this is related to several factors. Many endocrinology laboratories have considerable capital invested in radioisotope counting equipment and cannot immediately justify the change. Many heterogeneous EIA procedures offer little advantage in time and convenience over RIA (particularly where ¹²⁵I-labelling is used). As a result EIA remains relatively untested and its potential advantages undeveloped. On the other hand, unspecialised laboratories which could justify the performance of steroid assays but baulk at the cost and regulatory strictures imposed by the use of isotopes may, for the above reasons, remain unconvinced that EIA procedures are simple or reliable enough to offer a viable alternative. Finally, EIA sensitivity has been poor compared with that achieved using tritium labelling because large differences may exist in the affinity of antibody binding with enzyme-labelled steroid as opposed to unlabelled steroid (van Weemen & Schuurs, 1975).

It is apparent, therefore, that to fully capitalize on the potential of EIA, assay performance (sensitivity, accuracy, precision) at least equivalent to that of RIA must be demonstrated along with a degree of practicability exceeding that of RIA. Problems associated with the attainment of these goals and how they may be circumvented are discussed below.

1.9.1. Sensitivity and Precision in Hapten EIA

It is not absolute sensitivity which is prime importance in the development of an assay but whether the assay is sufficiently sensitive to detect clinically significant changes in the concentration of analyte. The assay of ovarian steroid hormones does, however, make demands on assay sensitivity since their concentration in tissues or body fluids may fall below the limit of detection.
of a particular assay. Such demands are particularly apparent where direct addition procedures are preferred (i.e. the analyte is not extracted from the sample prior to assay and cannot therefore be concentrated) and where it may be desirable to minimize sample volume in order to limit non-specific interference.

The factors which influence sensitivity of EIA parallel those in RIA in most respects, sensitivity being essentially limited by avidity of antibody for analyte and by the magnitude and nature of errors incurred in the measurement of the response. A fundamental objective in assay design should therefore be to maximize precision of measurement through the range of analyte concentrations designated as clinically important (Ekins, 1976).

Sensitivity has been variously defined by numerous analysts and has been discussed in the context of EIA (van der Waart & Schuurs, 1977). The limit of detection of an assay may be defined as the smallest amount of analyte giving rise to a response significantly different from zero at the 95% confidence limit (Abraham, 1975). Expressions giving a useful indication of the sensitivity of the dose-response curve include the slope of the calibration curve at a defined concentration of analyte (Yalow & Berson, 1968; Ekins, 1974) and the concentration of analyte resulting in a defined reduction in binding (e.g. 50%; Feldman & Rodbard, 1971; Bolton & Hunter, 1973). An assessment of the proportion of label bound in the presence of a defined mass of analyte enables useful comparisons to be made during assay optimization where a maximal response is required at that concentration. Ekins (1976) has stressed the importance of precision of determination irrespective of which expression is used. The precision component is related to the size of the signal, manipulative errors such as occur in preparation and addition of solutions and misclassification errors associated with incomplete separation of free from bound label (Hunter, 1978).

1.9.2. The Characteristics of the Antiserum and Label: Homologous and Heterologous EIA

No reports have been found of studies investigating directly the effect of
the avidity between antibody and enzyme-labelled analyte on the sensitivity of EIA. Experience with RIA, however, would indicate that antibodies of highest avidity for analyte would enable attainment of maximum sensitivity: sensitivity would be expected to decrease when the affinity for labelled analyte was significantly higher or lower than that for unlabelled analyte (Ekins, Newman & O'Riordan, 1968; Exley, 1978). Certainly, steroid RIA utilizing internal tritium labelling has commonly been shown to give rise to assays of sensitivity (as assessed both by limit of detection and calibration curve slope) superior to EIA's employing labels formed from steroid derivatives identical to those used in producing the immunogen (i.e. homologous label). This phenomenon was first demonstrated for the EIA of oestrogens (van Weemen & Schuurs, 1975 & 1976). This reduced sensitivity is thought to arise as a result of a higher binding affinity for the enzyme-labelled analyte than for the analyte itself. This arises because the antigenic determinant of the steroid immunogen consists of not only the steroid but also the chemical bridge of its derivative and the lysine residue (and adjacent amino acids of the protein) to which it is covalently attached (Gilby & Jeffcoate, 1973; Jeffcoate, Gilby & Edwards, 1973).

Steroid-enzyme conjugates in which the same steroid derivative was used to couple with lysine residues on the enzyme as on the carrier protein would thus be expected to possess near identical antigenic determinants to those of the immunogen. As a result, a higher antibody binding affinity for the steroid-enzyme conjugate would be anticipated than for the steroid hormone and the latter would not compete so effectively for antibody binding sites. An analogous reduction in sensitivity arises from the use of steroids externally labelled with $^{125}$I (Cameron, Scarisbrick, Morris et al, 1974).

Attempts have been made to reduce affinity for the labelled analyte by attaching the enzyme to a different carbon atom on the steroid nucleus (site heterology) or at the same carbon atom but using a different bridge (bridge
heterology) or a combination of the two. These approaches have been effective in increasing sensitivity of EIA for oestradiol (van Weemen & Schuurs, 1975 & 1976; Exley & Abuknesha, 1977), cortisol (Arakawa et al, 1979) and testosterone (Bosch, Stevens, van Wijngaarden & Schuurs, 1978a; Hosoda, Yoshida, Sakai et al, 1980) although no comparisons of sensitivity were made with RIA.

Several authors have, however, noted decreased assay specificity in heterologous systems particularly when site or site and bridge heterology was used (van Weemen & Schuurs, 1975; van Weemen, Bosch, Dawson et al, 1978; Hosoda et al, 1980): this can be explained in terms of steric hindrance of important antigenic determinants on the steroid-enzyme conjugate permitting preferential binding to less specific molecules in the antibody population.

EIA's for progesterone have largely been of the homologous type (Dray et al, 1975; Joyce et al, 1977 & 1978a; Nakao, 1980): only preliminary investigations of heterologous systems have been made (Gros, Flecheux & Dray, 1978; Joyce, Read & Riad-Fahmy, 1978b).

Proof of the general applicability of particular heterologous combinations would obviously require demonstration of sensitivity comparable with that produced using RIA (tritium label) using a large number of antisera. In the particular case of progesterone EIA, the heterologous conjugate would usefully be compatible with progesterone-11β-hemisuccinate antisera since in the short term these are most widely available. Any improvement in sensitivity should not, however, be at the expense of specificity.

1.9.3. The Choice of Enzyme and Substrate

Numerous criteria have been cited as important in the selection of a particular enzyme for use in EIA (Landon et al, 1975; Schuurs & van Weeman, 1977). Of importance to assay sensitivity are enzyme specific activity and the sensitivity of detection of the product since these directly determine the size of signal at the assay end-point: the smaller the quantity of label which can be used and subsequently measured with precision, the greater will be the potential sensitivity (Yalow & Berson, 1968; Ekins, 1976). Only enzymes which
are available in a highly purified state should be used since protein impurities can also give rise to antigenic products. Of practical importance are availability, cost, the stability of the enzyme (and its conjugate), the speed and ease of estimation of enzyme activity and the safety of the reagents used. The enzyme chosen should preferably be absent from the test sample as should potential modulators of its activity. In practice, however, such sources of interference can usually be eliminated during the assay when free and bound enzyme-label are separated.

Although few enzymes fulfill all these criteria several have been employed in steroid immunoassays and these include β-galactosidase (from E. coli), alkaline phosphatase (from calf intestinal mucosa), penicillinase (from B. cereus) glucoamylase (from Rhizopus niveus) and horse radish peroxidase (van Weemen & Schuurs, 1972; Dray et al., 1975; Tateishi, Yamamoto, Ogihara & Hayashi, 1977; Kobayashi, Ogihara, Amitane et al., 1978; Joshi, Shah & Sudhama, 1979). These can all be assayed with great sensitivity of the same order of magnitude and are available commercially at high purity and reasonable cost.

For optimum end-point determination, the choice of substrate may be a matter of compromise. For alkaline phosphatase (from calf intestine mucosa) phenyl phosphate gives a higher maximum velocity than any other substrate but the product, phenol, has a relatively low extinction coefficient. Para-nitrophenyl phosphate provides greater sensitivity of detection since its product p-nitrophenol has a far higher extinction coefficient than phenol (Bowers, Kelly & McComb, 1967). Although availability of equipment may dictate that colorimetric end-points are used, β-galactosidase, alkaline phosphatase and peroxidase may also be assayed using substrates with fluorescent products. This may increase the sensitivity of enzyme detection by an order of magnitude (van der Waart & Schuurs, 1977; Ishikawa & Kato, 1978; Arakawa, Maeda & Tsuji, 1979).

1.9.4. Methods of Formation of Steroid-Enzyme Conjugates

The coupling of steroids to enzymes can be achieved using procedures identical to those used in formation of immunogens (Schuurs & van Weeman, 1977;
O'Sullivan et al., 1979; Sauer et al., 1982a). In the majority of instances attachment to the enzyme has been through formation of peptide bonds between the carboxyl group of a steroid derivative and ε-amino groups of lysine residues of the enzyme. The condensation reaction has almost exclusively involved use of the mixed anhydride or the carbodiimide method (see Sauer et al., 1982a): both methods (Fig. 1.6) have been used in the formation of progesterone conjugates with either β-galactosidase or horse radish peroxidase. β-Galactosidase linked to progesterone110-hemisuccinate using the carbodiimide method provided the label first used in progesterone EIA (Dray et al., 1975) and similar procedures have been adopted in other laboratories (Patricot, Poggi & Revol, 1978; Seeger, Thurow, Haede & Knapp, 1979; Nakao & Kawata, 1980). In contrast Joyce, Read & Fahmy (1977) used progesterone11α-hemisuccinate-peroxidase conjugate as label, prepared by the mixed anhydride procedure: their reaction conditions were subsequently refined (Dawson, Denisson & van Weemen, 1978; Joyce, Othick, Read & Riad-Fahmy, 1981) and adopted by others (Kamonpatana, van de Wiel, Koops et al., 1979).

In producing enzyme-labelled analytes to provide maximum sensitivity in heterogeneous EIA it is of utmost importance that the immunoreactivity of the analyte and the catalytic activity of the enzyme remain unaltered. These aims may be achieved by the use of conjugation methods which avoid reaction between
the condensation reagents and the enzyme (minimizing co-conjugation) and by incorporation of analyte in such a way that binding with antibody will not be subsequently sterically hindered.

It might be anticipated that the use of water soluble carbodiimides for the condensation of progesterone derivatives with enzymes would result in appreciable loss of enzyme activity since the reagent is normally used in appreciable excess (100 times the steroid concentration) and in the presence of the enzyme. Dray et al (1975), however, reported recovery of 89% of enzyme activity following conjugation of progesterone 11α-hemisuccinate with β-galactosidase: others using this method of conjugation did not report a recovery rate (Patricot et al, 1978; Seeger et al, 1979; Nakao, 1980; Nakao, & Kawata, 1980). In contrast, when a similar procedure was used for conjugation of oestradiol-17β-6-(O-carboxymethyl)oxime with β-galactosidase, Exley and Abuknesha (1977) reported appreciable loss of enzyme activity which necessitated the use of affinity chromatography for removal of denatured enzyme.

Formation of steroid mixed anhydrides on the other hand necessitates anhydrous conditions and subsequent addition of this reactive intermediate to aqueous enzyme should circumvent the possibility of "activation" and thus polymerization of the enzyme: in theory then, this method should be preferable to the carbodiimide procedure. The enzyme activity of progesterone-enzyme conjugates formed by the mixed anhydride procedure has not generally been reported, although Comoglio and Celada (1975) and Arakawa et al (1979) reported values of 80% and 40% of the original activity for conjugation of cortisol derivatives with β-galactosidase and peroxidase respectively. It is not clear which is the conjugation method of choice since both give rise to some loss of enzyme activity. Both methods would also appear to be inefficient in incorporation of steroids into the enzyme. Dray et al (1975) reported that use of a 100 molar excess of progesterone 11α-hemisuccinate resulted in conjugation of only 26% of β-galactosidase molecules by the carbodiimide method. Joyce et al (1977), using the mixed anhydride procedure, reported that only six steroid molecules were incorporated into peroxidase when a fifty molar excess of
derivative was employed. Similarly, with other hapten s, a 1000 fold molar excess of hapten was required at conjugation to produce conjugates of sufficient immuno-reactivity when the mixed anhydride procedure was used (Comoglio & Celada, 1975; Al-Bassam, O'Sullivan, Bridges & Marks, 1979). In theory, optimum immuno-reactivity and specific activity (enzyme activity/mol steroid) would be obtained by incorporation of one progesterone molecule per enzyme (Exley & Abuknesha, 1977).

For optimum sensitivity it is essential that excess unreacted analyte is removed following conjugation. With progesterone conjugates it has been widely reported that this can be adequately achieved by dialysis and/or gel filtration. The presence of unconjugated enzyme has not generally presented a problem in heterogeneous EIA since unconjugated enzyme can be washed away when free and bound analyte are separated.

1.9.5. Separation of Free from Antibody-Bound Label

Separation of free from antibody bound enzyme-label may be achieved in several ways (see Table 1.2.). The method most commonly employed in steroid EIA has been double antibody precipitation (see Table 1.1.). Although simpler to perform and involving fewer steps, solid phase procedures have proven less popular, possibly because they have been less tried and tested in RIA procedures.

Since all reagents remain in solution until the precipitating second antibody is added the double antibody method offers the advantage of better availability of primary antibody to the other reagents: as a result, antibody binding proceeds more rapidly. It has been suggested that this may result in assays of improved sensitivity (Joyce et al., 1978a) although this has not proved to be a general finding (Arakawa et al., 1979; van Weemen et al., 1979).

In the diagnosis of infectious diseases by immunoenzymometric methods (enzyme-linked immunosorbent assay, ELISA) the well walls of disposable plastic microtitre plates (96 wells per plate) have been used as the solid phase and have proved to be the most convenient and popular means of separation (Voller, Bidwell, Huldt & Engvall, 1974; Voller, Bidwell & Bartlett, 1976a,b). Although
Table 1.2 Methods of Separating Bound from Free Label

1. Polyethylene glycol precipitation (Österman et al, 1979)

2. Double antibody precipitation (Comoglio & Celada, 1976; Joyce et al, 1978a)

3. Double antibody solid-phase (Comoglio & Celada, 1976; Bosch et al, 1978b)

4. Solid-phase primary antibody:
   Polycarbonate-coated, polystyrene or nylon balls (covalent or passive binding: Miranda et al, 1977; Smith & Gehle, 1977; Hendry & Herrman, 1980).
this method had not previously been applied to the assay of haptens, it shows
great potential where large numbers of determinations are to be performed.

Problems associated with well-to-well variations in antibody binding have been
noted by a number of investigators, however (Denmark & Chesum, 1978a & 1978b;
Halliday & Wisdom, 1978; Standefer & Saunders, 1978; Burt, Carter & Kricka,
1979; Kricka, Carter, Burt et al, 1980) and these must be resolved if micro-
titre plates are to be used for the reliable quantitation of steroid hormones.

1.9.6. Non-Specific Effects and EIA Performance

Non-specific adsorption of enzyme-label, interference with specific binding
and factors interfering with determination of enzyme activity may be expected
to considerably reduce assay performance. Such influences have not generally
been reported in steroid EIA, however, since the majority of procedures involve
extraction of the analyte prior to assay (Table 1.1).

The adoption of adequate wash procedures in direct addition assays should,
however, eliminate the influence of endogenous enzyme, modulators of activity
or non-specific binding of the label to the assay tube if enzyme activity is
measured in the bound fraction (Schuurs & van Weemen, 1977). The inclusion of
detergent or high concentrations of salt or protein (gelatin or bovine serum
albumin) in buffers used during wash procedures or at the immuno-incubation stage
respectively have been reported as effective in reducing or circumventing non-
specific binding (Engvall & Perlmann, 1971; Engvall, Jonsson & Perlmann, 1971;
Engvall & Ruoslahti, 1979; Saunders, 1979). It has been long recognised that
endogenous steroid binding proteins interfere with the direct RIA of plasma
steroids by competing with specific antibody to bind the steroid (see
section 1.8.4). Although there have been few reports of direct EIA's
of steroids in plasma, problems associated with EIA of progesterone
(Riad-Fahmy, Read, Joyce & Walker, 1981) and cortisol (Ogihara, Miyai, Nishi
et al, 1977) were reported to be appreciably reduced by use of lowered pH of the
immunoreaction and prior heating of the sample, respectively.

Since milk represents a rich source of potentially interfering substances
including lipids, binding proteins (casein) and enzymes (see Renner, 1983) the
direct measurement of steroids in milk may require particularly rigorous wash
procedures. Two of the most commonly used enzymes in EIA, alkaline phosphatase
and peroxidase (catalase) are present in milk in the fat globule membrane
and in whey, respectively. The latter represents 1% of whey proteins (Renner,
1983). The concentration of both these enzymes has been reported to increase
during disorders of milk secretion (Andrews & Alichanidis, 1975; Bingham &
Kalan, 1967).

1.9.7. Limitations of Existing Progesterone EIA Methods and Potential for
Improvement

With few exceptions steroid EIA procedures reported up to the early 1980's
either represent procedures in which the steroid had been extracted prior to
assay or model systems not tested or fully validated by the assay of samples
(see Table 1.1.). Direct addition procedures for progesterone determination in
human plasma (Patricot et al., 1978) and cream (at 1/4_0 dilution) from cows milk
(Nakao & Kawata, 1980) have, however, been reported but full validation details
were not provided in either case. The data obtained for progesterone determina-
tion in cream indicated that only 50% of the progesterone content was
detected but no explanation of the discrepancy was offered.

The main shortcomings of current progesterone EIA's are related to poor
sensitivity and lack of practical advantages compared with RIA.

Although Dray et al. (1975) reported an EIA for progesterone which was
comparable in sensitivity with RIA both in terms of limit of detection and
slope of standard curve it is apparent from reports by others using the same
procedures (Gros, Flecheux & Dray, 1978; Patricot et al., 1978; Nakao, 1980;
Seeger et al., 1979) that this may have been an exception to the general case.
Typically, these homologous procedures together with that developed by Joyce
et al. (1978a) provide calibration curves with limits of detection of the same
order as those using ^3H-labelled progesterone but of much reduced slope. This
is considered to limit severely the possibility of developing reliable, simple
In previous sections, numerous economic advantages have been described which justify the investigation of EIA techniques to develop the means for performing estimations of progesterone concentrations in milk. Improvements permitting extension of its use to the veterinary practice or possibly to the farm itself would enable prediction of the time of oestrus, pregnancy diagnosis and assessment of ovarian status to be rapidly performed.

Development of microtitre plate procedures for steroid EIA would considerably simplify the speed and ease with which all stages of EIA (pipetting, separation of free from bound label and washing the bound phase) can be performed. Such procedures could be automated through the availability of multi-channel dispensers and automatic microtitre plate readers. Dramatic improvements in sensitivity through judicious use of heterologous systems may enable further extension of the areas of application if they facilitate the development of assays, the end-point of which can be assessed by eye.

1.10. OBJECTIVES OF STUDY

The objectives of this study were (a) primarily to develop a simple and reliable enzymeimmunoassay for progesterone in milk and investigate the factors affecting its sensitivity and (b) to investigate the application of such a test to the breeding of dairy cattle by artificial insemination.
CHAPTER 2:
MATERIALS AND GENERAL METHODS
2.1. MATERIALS AND REAGENTS.

All reagents were of an analytical grade unless otherwise specified.

2.1.1. General Reagents

Hydrochloric, sulphuric, acetic, citric (H\textsubscript{2}O), formic, boric and diethylbarbituric acids, sodium bicarbonate, sodium chloride, cupric sulphate (5H\textsubscript{2}O), potassium sodium (+)-tartrate (4H\textsubscript{2}O), \textit{di}-sodium hydrogen orthophosphate (12H\textsubscript{2}O), sodium dihydrogen orthophosphate (2H\textsubscript{2}O), \textit{tri}-sodium citrate (2H\textsubscript{2}O), ammonium sulphate, Folin and Ciocalteau's phenol reagent, molecular sieve (type 3A), microcrystalline cellulose and all organic solvents were obtained from BDH Chemicals Ltd (Poole). Sodium azide, 2-mercaptoethanol, sodium ethylmercurithiosalicylate (Merthiolate), 6,9-diamino-2-ethoxyacridine lactate (Rivanol), polyoxyethylenesorbitan monolaureate (Tween 20), chicken egg albumin (ovalbumin, grade V), diethanolamine and activated charcoal (untreated powder) were obtained from the Sigma Chemical Company Ltd (Poole). Bovine serum albumin (BSA, puriss) was purchased from Koch-Light Laboratories Ltd (Haverhill), gelatin andFreund's adjuvant (complete and incomplete) from Difco Ltd (West Molesey, Surrey) and Decon 90 from Scientific Supplies Ltd (London). Plastic "cling" film was supplied by Payne Scientific Apparatus (Croydon).

2.1.2. Water

Water was softened (Aldous & Stamp Ltd, Beckenham), glass-distilled and deionized (Elgastat B102, Elga Group, High Wycombe) prior to use and is subsequently referred to as deionized water.

2.1.3. Unlabelled Steroids

11α-hydroxyprogesterone 11-hemisuccinate (progesterone 11α-hemisuccinate) and 11α-hydroxyprogesterone 11-hemimaleate (progesterone 11α-hemimaleate)
were synthesized as described in sections 3.2.1a. and 3.2.1b. respectively. 11α-Hydroxyprogesterone 11-β-D glucuronide (progesterone 11α-glucuronide; Corrie et al., 1981a) was a gift from Dr. J.E.T. Corrie (MRC Immunoassay Team, Edinburgh). Progesterone (pure) was purchased from Koch-Light Laboratories (Colnbrook) and all other steroids from Steraloids Ltd (Croydon).

2.1.4. Tritium and Carbon-14 Labelled Steroids

(1,2,6,7-3H)Progesterone (80Ci/mmol) and (1,4-14C)succinic anhydride (80mCi/mmol) were obtained from Amersham International PLC (Amersham) and (14C)formaldehyde (53mCi/mmol) from New England Nuclear Ltd (Southampton). Progesterone 11α-(1,4-14C)hemisuccinate was prepared as described in section 3.2.1a.

2.1.5. Reagents for Chemical Condensation Reaction

N,N'-dicyclohexylcarbodiimide, N-hydroxysuccinimide, succinic and maleic anhydrides, sodium cyanoborohydride, cyanogen bromide and nickel (II) chloride (6H2O) were purchased from the Sigma Chemical Company, iso-butylchloroformate, pyridine, tri-n-butylamine and dimethylformamide from BDH Chemicals Ltd and N,N'-disuccinimidyl carbonate from the Aldrich Chemical Co. Ltd (Gillingham).

2.1.6. Enzymes and Enzyme Assay

E. coli β-galactosidase (grade VIII), alkaline phosphatase from calf intestinal mucosa (type VII-T), horseradish peroxidase (peroxidase, type VI) and B. cereus penicillinase (type I) and their respective substrates and chromogens o-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl phosphate, hydrogen peroxide (30%) and o-phenylenediamine (dihydrochloride) and phenoxy-methylpenicillinic acid (penicillin-V), hydrolyzed starch (potato) and iodine were all obtained from the Sigma Chemical Company.

2.1.7. Antisera

Antisera were produced using either progesterone 11α-hemisuccinate-BSA or
progesterone 11α-hemisuccinate-ovalbumin conjugate as immunogen as detailed in Table 2.1.

Table 2.1 Details of antiprogesterone sera.

<table>
<thead>
<tr>
<th>Antiserum Code</th>
<th>Species</th>
<th>Immunogen</th>
<th>Source*</th>
<th>Method of γ-globulin Preparation</th>
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<tr>
<td>G711/12</td>
<td>Goat</td>
<td>Progesterone 11α-hemisuccinate-BSA</td>
<td>NIRD\textsuperscript{1}</td>
<td>Rivanol</td>
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<tr>
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<td>Progesterone 11α-hemisuccinate-ovalbumin</td>
<td>GUILDHAY\textsuperscript{4}</td>
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<td>S1509/16</td>
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<td>CBC\textsuperscript{5}</td>
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* See Glossary for full institute and establishment names. \textsuperscript{1} - \textsuperscript{4} = Gifts from 1. Dr. G.S. Pope; 2. Mr. R.G. Holdsworth; 3. Ms. E. Watson; 4. Mr. B.A. Morris. \textsuperscript{5} = Produced as described in section 2.3.1.a) and 2.3.1.b).
\textsuperscript{δ} See section 2.3.1.e) and 2.3.1.f).

2.1.8. Buffer Solutions

All buffers and aqueous solutions were prepared in deionized water (see section 2.1.2.).

\textit{a) Phosphate based buffers.} Phosphate buffers (0.1M, pH7.0) were prepared with disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate with sodium chloride (0.9% w/v; PS buffer), sodium azide (0.1% w/v; PAS buffer) and gelatin (0.1% w/v; PAS-gelatin buffer) added. PMS and PMS-gelatin buffers were prepared in the same manner but contained sodium ethylmercurithiosalicylate (Mercithiolate; 0.01% w/v) as preservative instead of sodium azide.
b) Tris (hydroxymethyl) aminomethane (Tris). TAS buffer was prepared with Tris (0.1M or 1M) containing sodium chloride (0.9% w/v) and sodium azide (0.1% w/v) and was adjusted to pH 8.5 or pH 9.0 with HCl (conc.).

c) Diethanolamine. DAM buffer was prepared with diethanolamine (1M) and contained magnesium chloride (0.5mM) and sodium azide (0.1% w/v) and was adjusted to pH 9.8 with HCl (conc.).

d) Britton-Robinson. (See Dawson, Elliot, Elliot & Jones, 1974). A 1 litre solution of citric acid (0.031M), potassium dihydrogen phosphate (0.0286M), boric acid (0.0286M) and diethylbarbituric acid (0.0286M) was prepared and 100ml of the solution titrated against sodium hydroxide solution (0.2M) to give the required pH. The solution was diluted to provide the appropriate molarity and the pH adjusted if required.

e) Citrate. Citric acid solution (0.1M) was titrated against tri-sodium citrate solution (0.1M) to provide the appropriate pH.

f) Acetate. Sodium acetate solution (0.1M) was titrated against acetic acid (0.1M) to provide the appropriate pH. For preparing antibody solutions for coating to microtitre plate wells, a stock solution (0.1M, pH 5.0) was prepared, stored frozen and thawed as required. On dilution to the working concentration (1mM or 0.17mM) it was not generally necessary to correct the pH.

2.1.9. Substrate Solutions for Determination of Enzyme Activity

Concentrations of substrates and chromogens were optimized as described in section 4.3. All substrate solutions were prepared on the day of use.

a) β-Galactosidase. O-Nitrophenyl-β-D-galactopyranoside (10mM) was prepared in PAS-gelatin buffer, pH 7.0, containing magnesium chloride (0.01M) and mercaptoethanol (0.1M).

b) Alkaline phosphatase. p-Nitrophenyl phosphate (15mM) was prepared in DEM buffer, pH 9.8 and stored in the dark until used.
c) Peroxidase. The substrate/chromogen solution contained hydrogen peroxide (7.1 mM) and o-phenylenediamine (22.1 mM) and was prepared in PMS-gelatin buffer, pH 6.0 and stored in the dark until used.

d) Penicillinase. The substrate/indicator reagent was prepared 15 minutes before use by addition of iodine solution (15 mM, 0.75 ml), starch solution (2% w/v, 6.5 ml) and penicillin-V (12.5 mM in PS buffer, 0.65 ml) to 58.5 ml of PS buffer pH 7.0. Stock iodine solution (80 mM prepared in 3.2 M potassium iodide) was stored in an amber bottle at room temperature (20 - 22°C) and diluted in PS buffer as appropriate on the day. Starch solution (2%) was prepared fresh daily by boiling hydrolysed starch for 2 - 3 minutes in PS buffer.

2.1.10. Progesterone Standard Solutions for Immunoassays

Progesterone (100 μg/ml methanol) was added (0.1 ml/20 ml) to whole milk collected from an ovariectomised cow (ovX milk) and preserved as described in section 2.3.7. This solution was further diluted with the same milk to provide standards (milk standards) containing 0-80 ng of progesterone/ml. Buffer standards were prepared in PAS-gelatin buffer in the same manner.

2.1.11. Microtitre Plates

Microtitre plates were purchased from four sources; Falcon polyvinyl chloride (PVC) plates from Becton & Dickinson (Oxford), Flow PVC (flat bottom, high activated) and polystyrene (sterile, flat bottom) plates from Flow Laboratories Ltd. (Rickmansworth), Dynatech PVC (M29) and polystyrene (M129B) plates from Dynatech Laboratories Ltd. (Billingshurst) and NUNC polystyrene plates (Immunoplate type 1F) from GIBCO (Uxbridge).

2.2. EQUIPMENT.

2.2.1. Liquid Handling

All repetitive pipetting was performed using positive displacement
pipettes manufactured by SMI Ltd. (Alpha Laboratories, Eastleigh), Soccorex Ltd. (Camlab Ltd., Cambridge) or Brand Instruments Ltd. (Transferpettor, A.R. Horwell Ltd., London). A BCL 500 dispenser (BCL Ltd. Lewes) was used for repetitive dispensing of aqueous reagents (50 - 500μl) and a Nichiryo dispenser (Chemlab Instruments Ltd. Hornchurch) for dispensing solvents or scintillation cocktails. An 8-channel peristaltic dispenser (Micro Compu-Pet Multi-Dilutor, General Diagnostics, Abingdon) was used for addition of antibody solutions or PAS-gelatin buffer to wells of microtitre plates as appropriate.

2.2.2. Centrifugation

An MSE super minor centrifuge (MSE Ltd., Crawley) was used for the majority of procedures; larger samples (>500ml) were centrifuged using an MSE Coolspin.

2.2.3. Photometry

Absorbance determination was performed using a Cecil CE292 spectrophotometer (Cecil Instruments, Cambridge) fitted with an autosampling attachment. A flow cell (10mm path length, 70μl capacity) was installed to enable continuous monitoring of effluent from chromatography columns. A Dynatech MR580 plate reader (Dynatech Laboratories) was used for in situ absorbance measurements of solutions in microtitre plate wells: the bases of wells were wiped clean and dry with a soft absorbent tissue beforehand.

2.2.4. Freeze Drying

An SB4 Laboratory Freeze Dryer (Chemlabs Ltd., Ilford) was used for all freeze drying processes. Samples were shell-frozen where possible over a liquid nitrogen bath.
2.2.5. **Sample Mixing**

Samples were vortex mixed as appropriate either singly or in batches using a Rotamixer (Scientific Supplies Ltd.) or V100 Multivortex Mixer (Denley Instruments, Billingshurst) respectively.

2.2.6. **Dialysis**

Dialysis procedures were performed at 4°C in knotted Visking tubing (\(\frac{1}{4}''\) inflated diameter; Scientific Instruments Ltd., London) with constant stirring provided by a magnetic stirrer (Voss Ltd., Maldon) and follower.

2.3. **METHODS.**

2.3.1. **Antibody Production and Purification**

a) **Formation of immunogen.** Progesterone 11\(\alpha\)-hemisuccinate-ovalbumin conjugate was used as immunogen and was prepared by a modification of the direct active ester procedure (section 3.2.4.a). N-hydroxysuccinimide (550 \(\mu\)mol in 1ml dry dioxan) and dicyclohexylcarbodiimide (550 \(\mu\)mol in 1ml dry dioxan) were added to progesterone 11\(\alpha\)-hemisuccinate (500 \(\mu\)mol in 2ml dry dioxan), the mixture stirred for 1 hour at room temperature (20 - 22°C) and acetic acid (14 \(\mu\)l = 250 \(\mu\)mol) added. After stirring for a further 15 minutes, the insoluble by-product dicyclohexylurea was removed by centrifugation (2000g for 10 minutes). The active ester solution (3.7ml) was slowly added to a continuously stirred ovalbumin solution (500mg; 11.1 \(\mu\)mol, dissolved in 50ml PS buffer) to which 25ml distilled water and 25ml dioxan were added (to maintain solution of buffer salts and the active ester) and stirring continued for a further 3 hours. The products were shell frozen in a conical flask (250ml) and then freeze dried. Addition of deionized water (8ml) to the lyophylisate revealed that a large proportion of the conjugate was insoluble. To facilitate removal of unconjugated steriod, the insoluble component was separated by centrifugation (1600g for 10 minutes) and the
pellet washed with 3 x 100ml methanol over a glass Buchner funnel before
drying under vacuum. Purification of the soluble component was achieved
by passage through a Sephadex G25 column (swollen in distilled water, 50ml
bed volume) and elution with distilled water. The fraction eluting at the
void volume was freeze dried and mixed with the dried insoluble fraction.
The white powder (0.463g) constituted the progesterone 11α-hemisuccinate-
ovalbumin immunogen.

b) Immunization of sheep and collection of blood samples. The immunogen
was prepared for inoculation in an emulsion of sterile saline (0.9%) and
Freunds complete (primary inoculation) or incomplete (subsequent "boost"
inoculations) adjuvant in a ratio of 1:3 v/v using a Silverson laboratory
homogenizer (Silverson Ltd., Chesham) at full speed for ten minutes. Inoc­
culations (0.5ml), two per limb, were by deep intramuscular injections of
the emulsion through a 20g x 1¼" needle, (4ml in total, containing 2mg or
1mg of immunogen/animal for primary and boost inoculations respectively).
In the forelegs, the inoculation was made into the triceps muscle of the
upturned sheep and in the hindlegs, into the medial aspect of the quadriceps
femoris. Booster injections were given at intervals of one to three months
and blood (~630ml) collected 7 - 10 days later from the jugular vein, via
a 12g x 2½" needle and plastic tube, into a plastic centrifuge bottle. The
blood was allowed to clot and the clot to contract (1-2 hours at room
temperature + 1 hour at 37°C) before separation of the antiserum follow­
ing centrifugation (Coolspin, 2,600g for 1 hour).

c) Assessment of antiserum titre. Antiserum titre is defined as the
dilution of antiserum at which 50% of (³H)progesterone is bound in the
absence of competing steroid. This was determined by incubation of serial
dilutions of antiserum with (³H)progesterone in accordance with the RIA
procedure described below (section 2.3.4.).
**d) Absorption of carrier-protein antibodies from antisera.** A procedure modified from that of Thorneycroft et al (1970) was used. Aliquots of antiserum (100 μl) were placed in conical glass test tubes and ovalbumin or BSA solution (10 μl in 1M TAS buffer pH 8.5), over the concentration range 0.125 - 64mg/ml, added. The tubes were stoppered and incubated for 1 hour at 37°C and a further 3 days at 4°C before separation of the immunoprecipitate by centrifugation (15 minutes at 1600g). The supernatant was aspirated off and the pellet resuspended and washed with 0.1M PAS pH 7.0 (4ml). Following centrifugation and removal of the supernatant, the wash cycle was repeated and the protein pellet dissolved in 0.5M sodium hydroxide solution (1ml, containing 0.1% sodium azide). The concentration of carrier protein which gave rise to maximum antibody precipitation (the point of equivalence) was subsequently determined either by quantitative determination of protein concentration by the method of Lowry et al (1951), using standards prepared in 0.5M NaOH solution (section 2.3.2.) or relatively by comparison of estimates of absorbance of the NaOH solutions at 280nm. Larger batches of antiserum for use in EIA and RIA were subsequently absorbed in an identical fashion by addition of the equivalent quantity of protein. Following centrifugation the supernatant was retained and stored at -20°C until required.

**e) Preparation of the γ-globulin fraction of antisera using 6,9-diamino-2-ethoxyacridine lactate (Rivanol).** The treatment of sera with Rivanol at an appropriate pH (Thorneycroft et al, 1970) results in precipitation of the majority of serum proteins leaving the globulins in solution (Horejsi and Smetana, 1956).

Treatment of goat or sheep antiserum with 0.4% Rivanol in 0.1M PAS (pH 6, 7 and 8) or TAS (pH 7 and 8) buffer (4 volumes) was found to provide maximum recovery of antiprogesterone γ-globulin (by RIA) with the minimum of contamination by other serum proteins (assessed by polyacrylamide gel electrophoresis) when performed at pH 9.0 (pH range 6 - 10 tested) in the manner
described by Sauer, Cookson, MacDonald and Foulkes (1982a). \(\gamma\)-Globulin fractions of the carrier-protein-absorbed antisera detailed in table 2.1 were prepared by addition, whilst mixing, of 0.4% Rivanol (4 vol) in 0.1M TAS buffer (pH 9.0) at room temperature (20 - 22°C). After 0.5 hour, precipitated proteins were separated by centrifugation (3,000g, 0.5 hour) and the supernatant applied to a Sephadex G25 column (bed volume \(\sim\) 10x supernatant volume) to enable removal of Rivanol. Elution with 0.01M phosphate buffer pH 7.0 containing 0.02% sodium azide enabled collection of the \(\gamma\) -globulin fraction at the void volume as detected by monitoring absorbance at 280nm. \(\gamma\)-Globulin solutions were stored frozen (-20°C) until required.

f) Preparation of the \(\gamma\)-globulin fraction of antisera by ammonium sulphate precipitation. The method used was essentially that described by Garvey, Cremer and Sussdorf (1977). Carrier-protein-absorbed antiserum (2 vol) was dispensed into a conical test tube and the level of the meniscus marked. Saturated ammonium sulphate solution (1 vol) was added dropwise, with continuous vortex mixing, to this and the preparation further mixed at intervals of 10 minutes. After 30 minutes the \(\gamma\)-globulin precipitate was separated by centrifugation (5 minutes at 1600g) and the supernatant aspirated off. The pellet was loosened by vortex mixing, dissolved in deionized water to the mark and the precipitation procedure repeated twice. The resulting \(\gamma\)-globulin solution was stored frozen (-20°C) until required.

2.3.2. Determination of Protein Concentrations.

The method used was essentially that of Lowry, Rosebrough, Farr and Randall (1951). The following reagents were required for the assay:

Solution A: Sodium carbonate solution (2% w/v) was prepared in 0.1M sodium hydroxide containing sodium azide (0.1%) and stored at 4°C until required.

Solution B: Cupric sulphate (5H\(_2\)O) solution (0.5% w/v) was prepared fresh on
the day of use in potassium sodium (+)tartrate solution (1% w/v).

Solution C: Solution A (100ml) mixed with solution B (2ml) on the day of use.

Solution D: Folin-Ciocalteau reagent (2ml) diluted 1:2 in deionized water on the day of use.

Standard solutions of protein (BSA) in the range 0 - 100μg/ml were prepared in PAS buffer or, for the assay of sodium hydroxide-hydrolysed protein, in sodium hydroxide solution (0.5M) and stored at 4°C until required. To determine protein concentrations, 2ml of solution C was added to 0.2ml of standard solution or sample, vortex mixed and left at room temperature (20 - 22°C) for a minimum of 10 minutes. Solution D (0.2ml) was then added, with immediate mixing and the samples left at room temperature for 30 minutes. Absorbance values were subsequently determined at 750nm and protein concentrations of the samples determined by interpolation from the calibration curve calculated by linear regression.

2.3.3. Determination of Radioactivity

Radioactivity was determined using a Tricarb Model B2450 liquid scintillation spectrometer (Packard Instruments Ltd., Caversham, Berkshire) with a radium-226 external standard. Quench correction was performed automatically by the external standard channels ratio method. The radioactivity of tritium or carbon-14-labelled compounds was measured in glass scintillation vials (20ml capacity) following addition of an appropriate scintillation fluid (7ml) to each vial: the scintillation cocktails and solubilizers used were obtained from Packard Instruments Ltd. Hydrophilic radiolabels in buffer solution (0.05 - 1.0ml) were counted in Instagel (xylene-based scintillation cocktail) or, following treatment with Soluene-350 (1ml/0.01 - 0.2ml sample) for 3 hours at room temperature (21 - 23°C), in Toluene Scintillator (5g of 2,5-diphenyloxazole and 0.1g of 1,4-Di-2-(5-phenyloxazolyl)-benzene per litre of toluene). Hydrophobic radiolabels in buffer solutions (0.01 - 1.0ml) were
counted in Toluene Scintillator following mechanical shaking (1 hour minimum; shaking incubator, Mickle Ltd., Gomshall) to ensure complete partition and, in the case of progesterone RIA supernatants, dissociation of the steroid-antibody complex. Radioactivity adsorbed to bisected polyvinyl microtitre plate wells was counted in Filter Count (1, 2, 4-trimethylbenzene-based cocktail) following an incubation at room temperature for at least 24 hours: polystyrene wells were dissolved and counted in Toluene Scintillator.

2.3.4. Radioimmunoassay

RIA was performed in a manner similar to that described by Heap et al (1976). Standards (10μl, in quadruplicate) or samples (10μl, in duplicate) were pipetted into polystyrene test tubes. \(^{(3)H}\)Progesterone in PAS-gelatin buffer (200μl, \(=\) 7pg progesterone) and anti-progesterone γ-globulin (100μl, equivalent to 100μl of a 1/15,000 dilution of antiserum) in PAS-gelatin buffer were then added. The tubes were mixed and incubated overnight at 4°C. Ice-cold PAS-gelatin buffer (500μl) was added to each tube and free \(^{(3)H}\)progesterone separated from bound by the addition of dextran-coated charcoal suspension (200μl, containing 6.25mg charcoal/ml and 0.625mg dextran/ml) in PAS-gelatin buffer at 4°C. After 10 minutes at 4°C, the tubes were centrifuged at 1,600g (5 minutes), the supernatant poured into scintillation vials and the radioactivity determined following extraction into Toluene Scintillator (7ml). Concentrations of progesterone in the samples were determined by interpolation from the calibration curve fitted manually to the standard points with the aid of a flexible plastic curve.

2.3.5. Enzymeimmunoassay

Assays were based on the saturation analysis principle and were performed by addition of standard or sample and enzyme-labelled progesterone to microtitre plate wells previously coated with antibody. The competitive procedure
was performed under specified conditions and antibody-bound enzyme label quantified colorimetrically after rinsing away unbound label. Specific details are delineated in appropriate sections (Chapters 5, 6 and 7) since investigation of these procedures was a subject of this thesis.

2.3.6. **Statistics and Analysis of Data**

The variance ratio test and students "t" test (see Snedecor and Cochran, 1967) were used to establish the significance of differences between experimental data.

a) **Conventional linear regression analysis.** This was used to describe the line of best fit where appropriate but was only applied when one parameter was determined experimentally: a pre-programmed calculator (Munroe, Model 1930, New Jersey, USA) was used for this purpose.

b) **Weighted linear regression with errors in X and Y.** Conventional linear regression is inappropriate for the comparison of two methods since two critical assumptions are made. The first is that the relative size of the errors are constant for all regions of the calibration curve. The second is that errors of measurement are made in only one of the variables (Y). Munson and Rodbard (1982) have described a method using a weighted linear regression assuming error in both procedures which enables correction for variations in X and Y: this method has therefore been used in the present studies and is described briefly below. Data were processed in this manner by microcomputer (Commodore PET 2001) using a BASIC programme written by Dr. J.A. Foulkes. (See appendix 2. for full details.)

1. Calculation of corrected slope (b) and intercept (a). The method compensates for non-uniformity of variance by using statistical weighting: each point is assigned a weight based on its predicted reliability and statistical comparisons then based on the weighted sums of squares (SS_w):

$$SS_w = \sum_{i=1}^{n} W_i (Y_i - bX_i - a)^2$$  \hspace{1cm} \text{equation 1}
and $X_i$ are the i'th X and Y values, $a$ is the intercept and $b$ the slope of the best fitting line. The weighting $W_i = 1/\sigma_y^2$, where $\sigma^2$ is the variance of the appropriate Y value.

With errors in both Y and X, the appropriate weighted sum of squares ($SS_{ba}$) is given by:

$$SS_{ba} = \frac{\sum_i (Y_i - bX_i - a)^2}{\sigma_y^2 + b^2 \sigma_x^2}$$

and the slope by:

$$b = \frac{(b_2 - \lambda/b_1)^+ (4 \lambda + (b_2 - \lambda/b_1)^2)^{1/2}}{2}$$

$\lambda = \sigma_y^2 / \sigma_x^2$: where each method has equal and uniform error, $\lambda = 1$.

$b_1 =$ regression slope of Y on X

$b_2 =$ the reciprocal of the slope for the X on Y regression.

The sign of the root is determined by the sign of the correlation coefficient. In the current application the variance in any technique was calculated from its coefficient of variation.

Knowledge of the slope $b$ enables calculation of the intercept $a$:

$$a = Y - bX$$

2. Compatibility of weighted regression of methods with the line of identity. An F-ratio ($F_{2,n-2}$) based on the "extra sum-of-squares" principle was used to compare the weighted regression described by equation 2 above with the line of identity (slope = 1, intercept = 0).

$$F_{2,n-2} = \frac{(SS_{ba} - SS_{res})/2}{SS_{res}/(n - 2)}$$

$F_{2, n - 2} =$ the critical value of the F-statistic with 2 (the number of parameters fixed = a and b) and $n - 2$ degrees of freedom: the significance of F at the appropriate probability level was established by
reference to tables (Snedecor and Cochran, 1967).

\[ SS_{ba} = \text{the sum of squares for a particular value of slope and intercept} \]

\[ SS_{res} = \text{the mean square residual or minimum (residual) sum of squares.} \]

2.3.7. Milk Samples: Collection, Treatment and Storage

Unless otherwise stated, milk samples were taken from the collection jar or an appropriate vessel following agitation of the complete milking and preserved by addition of a potassium dichromate tablet (Lactab Mk III, Thompson & Capper Ltd., Runcorn; 1 tablet/20ml milk). Samples were stored at 4°C until required. Sodium azide solution (20% w/v in PAS-gelatin buffer; 5μl/ml milk), was used as preservative in earlier studies.

2.3.8. Cleaning of Glassware

Glassware was washed by immersion in Decon 90 (2.5% v/v) for 24 hours, rinsed with tap water, then immersed in dilute technical grade hydrochloric acid (1% v/v) for 24 hours before rinsing in three changes of tap water, three changes of distilled water and drying inverted in an oven.
CHAPTER 3:

CONJUGATION OF PROGESTERONE DERIVATIVES WITH ENZYMES
3.1 INTRODUCTION

Ideally, methods for the synthesis of enzyme-labelled analyte for use in EIA should produce conjugates in which all enzyme molecules are coupled with analyte. In addition, there should be no enzyme co-conjugation or reduction of catalytic activity.

The carbodiimide (Goodfriend, Levine and Fasman, 1964) and mixed anhydride (Erlanger et al., 1957a and 1957b) methods have been most commonly employed to form hapten-enzyme conjugates. Representative procedures for condensation using carbodiimides have been described by Dray et al. (1975) and Exley and Abuknesha (1977) for EIA of progesterone and oestradiol-17β respectively and using mixed anhydrides by Al-Bassam et al. (1979) and Joyce et al. (1981) for EIA of methotrexate and progesterone. To evaluate their efficacy, preliminary studies were made in which each of these methods was followed to produce homologous enzyme-conjugates with progesterone 11α-hemisuccinate. Alternative procedures (below) were found to be more effective, however, and are described in detail.

Synthesis of the N-hydroxysuccinimide or p-nitrophenol ester of steroid carboxylic acid derivatives should enable ready conjugation with primary amine groups of enzymes since such intermediates are commonly used to form amide bonds in peptide synthesis: such ester formation is achieved through use of the condensation reagent dicyclohexylcarbodiimide (Bodansky, 1960; Anderson, Zimmerman and Callaghan, 1964).

It has previously been shown that esters of N-hydroxysuccinimide in particular may be isolated from the condensation reagent in a pure form (e.g. N-succinimidyl 3-(4-hydroxyphenyl)propionate, Bolton and Hunter 1973; dithiobis(succinimidyl propionate), Lomant and Fairbanks, 1976) and conjugated in aqua with proteins at neutral pH (Lomant and Fairbanks, 1976): the
absence of condensation reagent thus potentially allows conjugation with enzymes without inducing co-conjugation or denaturation. Conjugation with protein will occur with unhindered N-terminal amino or ε-amino groups of lysine to form an amide bond, N-hydroxysuccinimide being eliminated (Bolton and Hunter, 1973).

The use of the N-hydroxysuccinimide ester (active ester) of progesterone11α-hemisuccinate for the formation of conjugates with enzymes was therefore investigated. The suggested reactions involved are shown in Fig. 3.1. Similarly, the preparation of heterologous enzyme conjugates with progesterone 11α-glucuronide, progesterone 11α-hemimaleate and progesterone 3-carboxymethyloxime was also investigated. β-Galactosidase was chosen for initial studies since it had proved successful in the EIA of progesterone (Dray et al., 1975) and oestradiol-17β (Exley and Abuknesha, 1977) and is not present in milk. In later work, alkaline phosphatase, peroxidase and penicillinase were also employed.

Although generally dicyclohexylcarbodiimide has been used for the production of N-hydroxysuccinimide esters, it has been reported to produce toxic effects when in routine use: these include sensitization of the skin and eyes (Sigma Chemical Co.). Ogura, Kobayashi, Shimizu, Kawabe and Takeda (1979) have shown that N,N'-disuccinimidyl carbonate could be more conveniently and safely used to produce N-hydroxysuccinimide esters of N-substituted amino acids and peptides. Its use for the synthesis of such esters of progesterone carboxylic acid derivatives was therefore investigated and compared with esterification using the carbodiimide procedure. An outline of the expected reaction is illustrated in Fig. 3.2.
1) SYNTHESIS OF $^{14}$C LABELLED 11β-HYDROXYPREGNOSTERONE 11-HEMISUCCINATE.

\[
\text{Pyridine} \quad 90^\circ C
\]

2) SYNTHESIS OF PROGESTERONE "ACTIVE ESTER".

\[
\text{N,N'-DICYCLOHEXYL UREA}
\]

3) SYNTHESIS OF ENZYME-PROGESTERONE CONJUGATE.

Fig. 3.1. Formation of β-galactosidase-progesterone conjugates by the 
N-hydroxysuccinimide ester method.

![Diagram of enzymatic conjugation](image)

Fig. 3.2. Proposed reaction summary for the synthesis of steroid active esters using N,N'-disuccinimidyl carbonate and subsequent formation of steroid-enzyme conjugates.

RCOOH = progesterone 11α-hemisuccinate or progesterone 11α-glucuronide.
3.2 METHODS

The organic solvents used for all chemical syntheses and conjugation procedures were dried before use by addition of molecular sieve.

3.2.1. Formation of Carboxylated Progesterone Derivatives

a) Progesterone 11α-\(^{14}\)C hemisuccinate. Since it is not available commercially, a radiolabelled derivative of progesterone 11α-hemisuccinate was formed to enable the degree of steroid incorporation into the enzyme and thus the efficiency of the reaction to be ascertained. The synthesis was performed using a modification of the method of Furr (1973): the concentrations of the reagents was increased, otherwise a poor yield resulted.

11α-Hydroxyprogesterone (330mg), succinic anhydride (100mg), \(^{14}\)C-succinic anhydride (50mCi; 80mCi/mmole) and pyridine (300μl) were heated in a loosely-capped conical glass test tube on a sandbath at 90°C for 24 hours. To decompose any residual anhydride, the tarry residue was dissolved in methanol (2ml) and the methanol subsequently evaporated under a stream of nitrogen for 1 hour at 90°C. The products were dissolved in diethyl ether (3 x 2.5ml) and extracted with sodium bicarbonate solution (5% w/v, 4 x 10ml). The pooled aqueous fractions were acidified with sulphuric acid (6M) to pH 4.0 and immediately extracted into diethyl ether. After dehydration over anhydrous sodium sulphate, the ether extract was dried down under a stream of nitrogen and progesterone 11α-\(^{14}\)C hemisuccinate subsequently crystallised from ethanol:water (70:30 v/v). On recrystallisation a yield, by weight, of 57% of the theoretical maximum was found. The melting point was determined to be 154°C.

b) Progesterone 11α-hemimaleate. 11α-Hydroxyprogesterone (1g), maleic anhydride (3.0g) and tri-n-butylamine (0.5ml) were dissolved in dimethylformamide (DMF, 1ml) and heated in a loosely-capped conical glass test-tube on a sandbath at 90°C for 24 hours. The residue was dissolved in chloroform/
methanol (9:1 v/v, 10ml), the volume reduced under a stream of nitrogen and the oily residue transferred to a separating funnel with further chloroform/methanol. The solution was washed with acetic acid (0.1M, 3 x 25ml) and the organic phase extracted with sodium bicarbonate solution (5% w/v, 3 x 25ml). The pooled aqueous phases were acidified (pH 4.0) with hydrochloric acid (3M) and extracted with chloroform/methanol (9:1 v/v, 3 x 75ml). The pooled organic phase was evaporated under a stream of nitrogen to a volume of approximately 5ml, from which white crystals of progesterone 11α-hemimaleate separated. The crystals were washed with cold acetone (5ml, -20°C) over filter paper (Whatman No. 1) and recrystallised from methanol/water (70/30 v/v): the yield by weight was 43.3% of the theoretical maximum.

3.2.2. Formation and Isolation of N-Hydroxysuccinimide Esters (Active Esters) of Carboxylated Progesterone Derivatives

a) Progesterone 11α-(14C)hemisuccinate. The crystallised progesterone 11α-(14C)hemisuccinate (43mg) was dissolved in dioxan (1ml) and N-hydroxysuccinimide (12.5mg) and dicyclohexylcarbodiimide (23.8mg) added. The mixture was stirred at 25°C for 30 minutes and the by-product dicyclohexylurea removed by washing through a sintered glass funnel with 3 x 3ml dioxan. The clear filtrate was dried under a stream of nitrogen and the residue dissolved in a minimum volume of dichloromethane at 35°C. Addition of cold diethyl-ether allowed crystallization of the N-hydroxysuccinimide ester of progesterone 11α-(14C) hemisuccinate. A melting point of 195 - 197°C was found after recrystallization, (yield 62.6% by weight of the theoretical maximum) and a molecular weight of 528.27 determined (Dr. D. Manning, NIRD, Shinfield) for the $M + 1$ ion by mass spectrometry using ammonia chemical ionization. The formula was confirmed as $C_{29}H_{37}NO_8^+$ by elemental analysis (Dr. F.B. Strauss, Microanalytical Laboratory, Oxford) and the specific activity determined as
73.2 x 10^3 dpm/mol (33nCi/mol) by liquid scintillation spectrometry (Toluene Scintillator).

b) Progesterone 11α-hemimaleate and progesterone 3-carboxymethylloxime.
The esters of these compounds were formed in a manner identical to that described for progesterone 11α-hemisuccinate. It was possible to crystallize only relatively small quantities of these products (10 - 20mg) due to the formation of stable oils and the products were not further characterized.

3.2.3. Use of Isolated (Crystallized) Active Esters to form Conjugates with β-Galactosidase and Peroxidase

β-Galactosidase solution was dialysed overnight at 4°C against PS buffer (1000 vols). Aliquots (0.1ml) of the resulting solution (5.1nmol β-galactosidase/ml) were dispensed into glass test tubes containing a magnetic follower. The steroid active ester in dimethylformamide solution was slowly added (10μl:concentration range 0.114 - 114 μmol/ml) to provide a 2 - 2000 fold molar excess of steroid. The mixture was stirred continuously for 1 hour at room temperature and after removal of excess unreacted steroid by dialysis at 4°C against PAS buffer (3 x 31 over 3 days), was chromatographed on 10 x 1cm columns of G25 Sephadex in PAS buffer. Conjugates were collected as a single peak at the void volume as observed by continuous monitoring of eluate absorbance at 280nm: a Celic spectrophotometer fitted with a flow cell was used for this purpose. Conjugates were stored at 4°C following addition of gelatin solution (10% w/v in PAS buffer) to a final concentration of 0.1% w/v. An identical procedure was used for formation of peroxidase-progesterone 11α-hemisuccinate conjugates but PMS buffer was substituted for PAS buffer.

3.2.4. Use of Active Esters without Isolation (Direct Procedure) to form Conjugates with Enzymes

a) Alkaline Phosphatase, penicillinase and β-galactosidase. Difficulties
encountered in purifying active esters of progesterone derivatives because of the formation of stable oils prompted the use of the active esters immediately following the removal of the by-product dicyclohexylurea. Since only a 10% molar excess of dicyclohexyl carbodiimide reagent was used in the formation of the active ester (commonly a 100-fold excess of reagent is used in the direct carbodiimide conjugation procedure: Dray et al., 1975) co-conjugation or denaturation of enzyme was likely to be small but was further limited by reaction of unreacted carbodiimide with acetic acid. A representative procedure for conjugating progesterone derivatives with alkaline phosphatase, penicillinase and latterly β-galactosidase is described below: for higher molar ratios of steroid:enzyme the concentration of steroid solution was adjusted appropriately.

Enzyme solution (100nmol/ml) was dialysed against PS buffer (11) for 24 hours at 4°C and adjusted to a concentration of 5mg/ml (50nmol/ml) with PS buffer prior to use. N-hydroxysuccinimide (110nmol in 20μl dry dioxan) and dicyclohexylcarbodiimide (110nmol in 20μl dry dioxan) were added to progesterone 11α-glucuronide (100nmol in 20μl dry dioxan) and stirred for one hour at room temperature. Unreacted dicyclohexylcarbodiimide was inactivated by addition of acetic acid (50nmol in 20μl dry dioxan). After a further 15 minutes, the insoluble by-product dicyclohexylurea was removed by centrifugation (2,000g for 10 minutes). One hundred per cent conversion of progesterone to "active ester" was assumed. Steroid solution (40μl) was slowly added to enzyme in PS buffer (500μl) to provide a molar steroid excess of 2:1. The mixture was stirred for 2 hours at room temperature before dialysing at 4°C against 4 changes of 3 litres of PAS buffer over 4 days. The conjugate solution was finally passed down a Sephadex G25 column, eluting with PAS buffer to remove any remaining free steroid. Gelatin solution (10% w/v in PAS buffer) was then added to a final concentration of 0.1% w/v.
Conjugate was stored at 4°C and under these conditions has been shown to be stable for at least one year. Loss of enzyme activity was less than 1% and no significant alteration in the performance of the conjugates in EIA was found before or after dialysis, indicating that minimal release of steroid by hydrolysis had occurred.

b) Use of N,N'-disuccinimidyl carbonate in the formation of active esters for direct conjugation with alkaline phosphatase. Alkaline phosphatase solution (100nmol/ml) was dialysed against PS buffer (11) for 24 hours at 4°C and the volume adjusted to a concentration of 50nmol/ml with PS buffer prior to use. Disuccinimidyl carbonate (880nmol in 20μl acetonitrile) and pyridine (880nmol in 20μl acetonitrile) were added to solutions of progesterone derivative (progesterone 11α-(14)C)hemisuccinate or progesterone 11α-glucuronide; 800nmol in 20μl acetonitrile) and stirred for 2 hours at room temperature (21°C) before addition of acetic acid (400nmol in 20μl acetonitrile): the reaction mixture was stirred for a further 0.5 hours. The active esters so formed were added slowly to aliquots of enzyme (0.16ml; 8nmol) in volumes appropriate to the molar excess required (see table 3.4) and the mixture stirred continuously for a further 2 hours. Conjugates were then purified by dialysis and gel filtration and stored in the manner described previously (section 3.2.4.a ). For comparison, a further series of conjugates were prepared in parallel using dicyclohexylcarbodiimide to form the active ester in the manner described previously (section 3.2.4.a ): progesterone 11α-(14)C)hemisuccinate was used at 5:1, 10:1 and 20:1 molar steroid excess and progesterone 11α-glucuronide at a 2:1 molar excess. The formation of active ester with time was monitored by periodic removal of aliquots (5μl or 2μl) of each reaction mixture and application to precoated silica gel TLC plates (20 x 20cm, layer thickness 0.25mm; Merck 60F254, BDH Ltd.): the adsorbent contained a fluorescence indicator.
The samples were chromatographed for 1 hour in the solvent system chloroform: ethyl acetate:methanol (40:10:50 by volume) and the components located by visualization of fluorescence quenching at 254nm (UV lamp UVSL-58; Scientific Supplies Ltd.) scraped from the plate with a scalpel and extracted into methanol (1ml, glucuronide components) or acetonitrile (1ml, hemisuccinate components). The extent of conversion to the active ester was estimated by liquid scintillation spectrometry (Toluene Scintillator, 7ml) in the case of the hemisuccinate or UV adsorption at 240nm (Perkin Elmer 552 spectrophotometer) for the glucuronide. The percentage conversion to the active ester was calculated from the total radioactivity or UV adsorbing material in the TLC components associated with steroid. The availability of crystalline reference material allowed identification of progesterone 11α hemisuccinate active ester, but the location of the progesterone 11α-glucuronide equivalent was presumed by elimination of the other known components: the reaction mixture excluding steroid was run separately for this purpose. An unidentified minor component (Rf 0.52) was formed by both procedures as a product of glucuronide active ester synthesis: this was assumed to be a steroid and was included in the quantification of total steroid (above).

3.2.5. Preparation of Peroxidase Conjugates with Progesterone 11α-glucuronide using the Mixed Anhydride Procedure

Particular difficulty was experienced in forming peroxidase conjugates with progesterone 11α-glucuronide as evinced by their poor immunoreactivity and low binding in EIA. By these criteria conjugates formed by the direct active ester procedure (section 3.2.4.a) were no more effective than those formed by a modified mixed anhydride procedure below, although higher molar excesses were required for the latter method (25, 125 and 250-fold rather
than 20 and 40-fold). Since use of the mixed anhydride procedure at a 125-fold molar steroid excess provided a conjugate sufficiently immuno-reactive to be used at a dilution which would enable a practical number of assays to be performed, this conjugate was used in subsequent comparative EIA's. The procedure described below was modified from that of Al-Bassam et al., (1979): more concentrated reagents were necessary to enable economic use to be made of the limited supply of progesterone 11α-glucuronide.

Progesterone 11α-glucuronide (2.0mg; 3.93μmol) was dissolved in dioxan (75μl), tri-n-butylamine (3.93μmol in 25μl dioxan.) added and the mixture cooled to 10°C before addition of iso-butyl chloroformate (3.93μmol in 25μl dioxan). The mixture was stirred for 30 minutes at 10°C and an aliquot (50 μl) slowly added to a stirred solution of peroxidase (12.5nmol; 0.5mg in 0.1ml of 0.1M sodium bicarbonate buffer, pH 8.2) and allowed to react for 3 hours at 10°C, then overnight at 4°C, before dialysis and gel filtration against PMS buffer and storage in the manner described previously (section 3.2.4.a)).

3.2.6. Assessment of Enzyme-labelled Steroids

a) Estimation of enzyme activity. Enzyme activity following conjugation was compared with that of the original enzyme: the relative quantity of product formed by a fixed mass of enzyme conjugate (in 10μl of appropriate buffer) was compared with that produced by the same mass of enzyme over the same period. The mass of conjugate or enzyme was estimated by determination of protein concentrations. Assays were performed in test tubes or microtitre plates using the appropriate condition described in section 4.3.

b) Performance in EIA. The performance of conjugates in EIA was evaluated by comparison of the relative degree of binding of fixed masses of conjugate with antibody adsorbed to microtitre plate wells and by assess-
ment of the degree to which binding was inhibited by the presence of competing progesterone: such tests were performed using milk standards and the protocol appropriate to the particular enzyme (see section 6.2.1.).

c) Estimation of the molar incorporation of steroid per enzyme molecule.

Molar incorporation of steroid was only assessed in specific cases. Where progesterone 11α-(¹⁴C)hemisuccinate was used, this was performed by determination of the radioactivity of aliquots of conjugate of known concentration (digested in soluene, 1ml) in Toluene Scintillator by liquid scintillation spectrometry.

For some preparations a comparison was made between conjugates by determining their potency in RIA: the ability of a fixed mass of the conjugate, relative to progesterone, to inhibit antibody binding of (³H)-progesterone enabled an estimate to be made by interpolation from an RIA calibration curve using progesterone standards prepared in buffer.

A spectrophotometric procedure based on the method described by Erlanger et al (1957a) proved useful for the determination of incorporation of progesterone 11α-glucuronide into alkaline phosphatase conjugate. Doubling dilutions of progesterone 11α-glucuronide solution were prepared (30 - 1.875μM) and the solutions scanned twice (PS buffer reference) over the wavelength range 190 - 290nm (Perkin Elmer 552 spectrophotometer): maximum absorbance was found to be at 248nm. Plotting mean relative peak heights (y, range 67 - 962mm) at 248nm against steroid concentration (x) enabled a calibration curve to be fitted (y = 31.8x + 3.771, r = 0.9999). Similarly, following adjustment of the protein concentration of progesterone 11α-glucuronide-alkaline phosphatase conjugate solutions to equal that of the alkaline phosphatase solution used for reference (see below), the conjugates were scanned, peak heights at 248nm determined and the equivalent steroid concentrations calculated by interpolation from the calib-
ration curve. The molar steroid incorporation per enzyme was calculated knowing the molar concentrations of the enzyme and steroid components of the conjugate. Since sodium azide interferes strongly with this procedure, if such estimations are to be made, dialysis and gel filtration procedures should be performed using PS buffer.

Whichever procedure was used, molar concentrations of enzyme were calculated following estimation of protein concentration by the method of Lowry et al (1951; see section 2.3.2).

3.3 RESULTS

3.3.1. The Properties of Progesterone 11α-hemisuccinate-enzyme Conjugates, Formed using Established Mixed Anhydride and Carbodiimide Procedures

The mixed anhydride procedure was used to conjugate progesterone 11α-hemisuccinate with peroxidase and β-galactosidase according to the methods described by Joyce et al (1981) and Al Bassam et al (1979) respectively. The methods of Dray et al (1975) and Exley and Abuknesha (1977) were used to form conjugates with β-galactosidase by the carbodiimide procedure.

Conjugates prepared by these procedures proved to be ineffective in EIA when applied in this laboratory and table 3.1 illustrates why this may have been the case. In the best preparations (conjugates 8 and 37) their limited ability to inhibit (3H)progesterone binding in RIA indicated that less than one in ten enzyme molecules were conjugated with progesterone despite being formed using a high molar excess of steroid (1477 and 2500 fold).

Subsequent observations substantiated the indication that incorporation of progesterone derivative into enzyme was low: using a cellulose-linked antibody system (Bolton, Dighe and Hunter, 1975) as described by Sauer, Foulkes and Cookson (1981), only conjugate 8 provided EIA calibration curves but a relatively large mass of label (200ng) was
required to provide detectable binding to antibody. Furthermore, although
loss of enzyme activity did not exceed 50%, the carbodiimide procedure of
Dray et al (1975) and Exley and Abuknesha (1977) gave rise to substantial
enzyme co-conjugation as indicated by the considerable degree of turbidity
produced at conjugation.

Table 3.1. Properties of conjugates of progesterone 11α-hemisuccinate with
β-galactosidase and peroxidase prepared following published procedure

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Conjugate No.</th>
<th>Condensation Reaction</th>
<th>Steroid Ratio (mol/mol enzyme)</th>
<th>Enzyme Activity Recovery (μg)</th>
<th>Potency of Steroid Incorporation (mol/mol enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase</td>
<td>1</td>
<td>MA (A)</td>
<td>50</td>
<td>108</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&quot; &quot;</td>
<td>100</td>
<td>80</td>
<td>1.25</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>3</td>
<td>CDI (B)</td>
<td>74</td>
<td>99</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&quot; &quot;</td>
<td>296</td>
<td>114</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&quot; &quot;</td>
<td>887</td>
<td>90</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>&quot; &quot;</td>
<td>1477</td>
<td>54</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>&quot; (C)</td>
<td>500</td>
<td>50</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>MA (D)</td>
<td>500</td>
<td>53</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>&quot; &quot;</td>
<td>1500</td>
<td>50</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>&quot; &quot;</td>
<td>2500</td>
<td>59</td>
<td>0.21</td>
</tr>
</tbody>
</table>

a) The ratio of steroid:enzyme in the reaction mixture.
b) The mass of conjugate giving rise to the same % inhibition of \(^3\)Hprogesterone
   binding as 5pg progesterone.
c) Calculated as described in section 3.2.6.c) from potency data.

Enzyme activities for peroxidase and β-galactosidase were determined as described by Joyce et al (1977) and Exley and Abuknesha (1978).

* The protocols followed were those of A, Joyce et al, 1981; B, Exley and

MA = Mixed anhydride procedure  CDI = carbodiimide procedure.
3.3.2. The Properties of Progesterone 11α-hemisuccinate-β-Galactosidase Conjugates formed using Isolated Active Ester

The first requirements in the evaluation of the conjugation procedure using isolated active ester (section 3.2.3.) were to establish the efficiency of the reaction with enzyme, its effect on enzyme activity and the value of such conjugates in EIA. No assumptions were made initially regarding the reaction efficiency or the degree of conjugation required to produce conjugates useful in EIA: a wide range of molar excess of steroid: enzymes were therefore used in this study (2:1 - 2,000:1). The characteristics of the conjugates formed are shown in table 3.2.

Table 3.2 Molar Incorporation of Progesterone 11α-hemisuccinate into β-Galactosidase and Enzyme Activity of the Resulting Conjugates

<table>
<thead>
<tr>
<th>Conjugate No.</th>
<th>Molar Ratio of Progesterone:Enzyme in Conjugation Reaction Mixture</th>
<th>Molar Ratio of Progesterone to Enzyme: Radioactivity, Determination</th>
<th>Enzyme Activity of Conjugates (% of Original Activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>0</td>
<td>0</td>
<td>103.4</td>
</tr>
<tr>
<td>52</td>
<td>2</td>
<td>2.0</td>
<td>104.4</td>
</tr>
<tr>
<td>58</td>
<td>20</td>
<td>7.9</td>
<td>111.1</td>
</tr>
<tr>
<td>59</td>
<td>100</td>
<td>48.6</td>
<td>91.6</td>
</tr>
<tr>
<td>60</td>
<td>200</td>
<td>87.7</td>
<td>82.4</td>
</tr>
<tr>
<td>61</td>
<td>500</td>
<td>104.4</td>
<td>62.6</td>
</tr>
<tr>
<td>62</td>
<td>2000</td>
<td>107.0</td>
<td>22.6</td>
</tr>
</tbody>
</table>

Low molar ratios of steroid to enzyme in the reaction mixture produced substantial incorporation as determined by radioactivity measurements and at high molar ratios approached the theoretical maximum of 120 (116 lysine ε-amino groups and 4 N-terminal amino groups: Craven et al., 1965).

Enzyme activity was not appreciably reduced by the active ester procedure except when the enzyme was highly conjugated, i.e. when the molar excess of steroid exceeded 100-fold. This may be due to alterations occurring at the
enzyme active site when incorporation occurred near this position or to allostERIC effects.

Quantities of conjugate equivalent to 5 - 15 pg of progesterone (by RIA) resulted in sufficient binding of enzyme-label to antibody for cali-

bRation curves to be produced by EIA. A comparison of curves produced using the most effective homologous β-galactosidase conjugate (prepared with a 25:1 molar excess of steroid) with that produced using liquid phase RIA is shown in Fig. 3.3.

![Comparison of homologous EIA (Δ-Δ) and RIA (□-□) calibration curves. The EIA was performed at 40°C on polyvinyl microtitre plates (G711/12 antiserum adsorbed at 1/2000 dilution) using β-galactosidase conjugate (20ng/well) prepared using a 25:1 molar excess of steroid. Progesterone standards were prepared in milk. B/B₀ = the quantity of label bound to antibody in the presence of competing progesterone (B) as per cent of that bound in the absence of competing progesterone (B₀).]

3.3.3. The Influence of Direct Conjugation Method Conditions on Enzyme Activity of Alkaline Phosphatase

a) Reaction solvent effects. Aliquots of dialysed alkaline phosphatase
solution, diluted for use in conjugation (4.2mg/ml PS buffer), were added to glass reaction vials (1ml capacity) containing magnetic followers. A range of volumes of dioxan were subsequently added and the solutions stirred for 2 hours at room temperature to reproduce conjugation conditions. The solutions were then diluted to the same final concentration of enzyme (50ng/ml) and the enzyme activity of 10µl aliquots (in octuplet), placed in microtitre plate wells and estimated in the manner described (section 4.3.5.). Volume ratios of dioxan to enzyme solution of 0:1, 1:60, 1:12, 1:3, 1:1 and 2:1 resulted in percent enzyme activity recoveries of 100 (control), 100.4, 94.5, 97.9, 85.1, and 86.4 respectively indicating that the conjugation conditions caused appreciable loss of enzyme activity only when a high proportion of dioxan was used (≥ 50%). Under the latter conditions, a precipitate of enzyme was formed (buffer controls became only slightly hazy) which readily dissolved on dilution in buffer. Clearly dioxan could be used at up to 25% v/v without appreciable loss of enzyme activity.

b) The relative concentration of reagents. As in all chemical reactions, the relative concentrations of the reagents will influence the probability of molecular interaction and thus the reaction rate and would be expected to increase with increased concentration. The use of a high proportion of organic solvent may increase the solubility of the steroid in the reaction mixture but as shown in the previous section, may also result in enzyme denaturation. The possibility of localised and therefore non-uniform conjugation of steroid with enzyme arises if the active ester solution is too concentrated or is added too quickly to the enzyme solution.

A single concentration of alkaline phosphatase (5mg/ml) was used to investigate the influence of relative active ester solution volume on subsequent formation of conjugate: the former was as concentrated as could practicably be readily reproduced, following dialysis, from commercially available alkaline phosphatase preparations. A 2:1 molar excess of
progesterone 11α-glucuronide was used throughout since this had been shown to provide calibration curves of greatest sensitivity (section 6.3.2.).

Progesterone 11α-glucuronide active ester solution was prepared in dioxan as described previously (section 3.2.4.a), diluted in dioxan to contain 50nmol in the required volumes and added slowly to stirred aliquots of alkaline phosphatase to form conjugates in the usual way (section 3.2.4a).

Table 3.3 describes the relative enzyme activity of the conjugates, their ability to bind with antibody adsorbed to microtitre plates and their performance in EIA (performed at 21°C).

Table 3.3 The Influence of the Relative Volumes of Progesterone 11α-glucuronide Active Ester and Alkaline Phosphatase Solutions on the Formation and Properties of Conjugates

<table>
<thead>
<tr>
<th>Conjugate No.</th>
<th>Vol Enzyme&lt;sup&gt;a&lt;/sup&gt;: Vol Steroid&lt;sup&gt;b&lt;/sup&gt; (μl)</th>
<th>Enzyme&lt;sup&gt;c&lt;/sup&gt; Activity Recovered (%)</th>
<th>Relative B&lt;sub&gt;d&lt;/sub&gt; ( % )</th>
<th>B&lt;sub&gt;50&lt;/sub&gt;/B&lt;sub&gt;0&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>173</td>
<td>500:5</td>
<td>96</td>
<td>100</td>
<td>44</td>
</tr>
<tr>
<td>174</td>
<td>500:50</td>
<td>91</td>
<td>102</td>
<td>43</td>
</tr>
<tr>
<td>175</td>
<td>500:125</td>
<td>87</td>
<td>91</td>
<td>44</td>
</tr>
<tr>
<td>176</td>
<td>500:250</td>
<td>87</td>
<td>79</td>
<td>43</td>
</tr>
</tbody>
</table>

<sup>a</sup>: 25nmol alkaline phosphatase (2.5mg) contained in 500μl PS buffer.
<sup>b</sup>: 50nmol progesterone 11α-glucuronide active ester in given volume (100% conversion to active ester assumed).
<sup>c</sup>: % of original enzyme activity.
<sup>d</sup>: Binding of conjugate (20ng/well) to antibody (G711/12, 1/8000 dilution) in the absence of progesterone (B<sub>0</sub>) relative to conjugate 173 (B<sub>0</sub> = 100%).
<sup>e</sup>: B<sub>50</sub> = binding of conjugate in the presence of 50pg progesterone.

Loss of enzyme activity as a result of conjugation increased with increasing proportions of active ester solution from 4% to a maximum of 13% and was greater than that associated with the solvent alone (see section 3.3.2.a ). The apparent degree of binding of conjugate to antibody in the absence of competing steroid (B<sub>0</sub>) similarly decreased as the proportion of steroid solution used for its formation increased although the sensitivity
potential \( \frac{B_{50}}{B_0} \) was unaffected. This implies that either conjugation efficiency decreased with increased dioxan volume (a higher proportion of enzyme remained unconjugated) or the apparent reduction in binding was a result of lower enzyme activity of the conjugate. The validity of the latter argument could be assessed by evaluation of the binding inhibition caused by the conjugates in RIA.

Whatever the cause of the relative differences, it would appear appropriate to prepare conjugates by addition of active ester solution to enzymes at a maximum volume proportion of 1 to 10.

3.3.4. Comparison of N,N'-Disuccinimidy1carbonate (DSC) and N,N'-Dicyclohexyl Carbodiimide/N-hydroxysuccinimide (DCC) for the Direct Formation of Alkaline Phosphatase Conjugates

Conjugates of progesterone 11α-(\(^{14}\)C)hemisuccinate and progesterone 11α-glucuronide with alkaline phosphatase were prepared using DCC or DSC to form the active esters as previously described (section 3.2.4.a) and 3.2.4.b) respectively.

TLC of aliquots of the reaction mixture during formation of the active esters provided \( R_2 \) values for progesterone 11α-(\(^{14}\)C)hemisuccinate and its active ester of 0.61 and 0.79 respectively and for progesterone 11α-glucuronide and its active ester of 0.34 and 0.62. The time courses of active ester formation are shown in Fig. 3.4. In the case of progesterone 11α-(\(^{14}\)C)hemisuccinate the DCC procedure gave rise to more rapid formation of active ester over the full period of study (100 minutes): by 100 minutes 48% conversion to the active ester was achieved compared with 34% for the DSC method. The initial rates of formation of active ester were similar for both methods and significant quantities were formed in the time between initiation of the reaction and first transfer of an aliquot of the mixture.
to the TLC plate (1 minute). Formation of the active ester of progesterone 11α-glucuronide was assessed at 1 hour by quantitative spectrophotometry and was similar for both procedures, 51% and 53% by the DSC and DCC procedures respectively.

![Conversion of steroid to active ester (%)](image)

**Fig. 3.4** The formation with time of active esters of progesterone 11α-(14C) hemisuccinate (▲, ●) and at a single time point for progesterone 11α-glucuronide (Δ, ○) using DCC (▲-▲, Δ) or DSC (●-●, ○). Components of the reaction mixture were separated by TLC and the proportion of total steroid associated with the active ester determined by use of UV spectrophotometry (240nm) for the glucuronides (Δ, ○) and by liquid scintillation spectrometry for the hemisuccinates (▲, ●).

Table 3.4 shows the characteristics of the conjugates prepared using active esters synthesized by the two methods. For both procedures, loss of enzyme activity as a result of conjugation was minimal. The immunoreactivity of the conjugates in RIA largely reflected the actual molar excess of steroid added to enzyme and thus the relative extent of active ester formation since, as previously mentioned, the molar excess was initially based
on an assumed 100% conversion of progesterone derivative to active ester. Thus, when hemisuccinate-linked conjugates were formed using a 5 or 10-fold molar excess of active ester, incorporation following use of the DCC method was approximately twice that using DSC. This difference was not apparent at a 20-fold molar excess, however, and may reflect conjugation with all the most accessible amino residues under these conditions. Assessment of the degree of conjugation by RIA binding inhibition studies or by evaluation in EIA gave rise to similar conclusions although values were less than obtained by determination of radioactivity (table 3.4): this is in keeping with findings in earlier studies using β-galactosidase (table 3.2) and the indication that steric hindrance may in part be responsible. Later studies demonstrated that the attainment of equilibrium would be unlikely in EIA under these conditions (section 6.3.4.a)) which suggests that equilibrium would be unlikely here: this would also contribute to underestimation of the steroid content of the enzyme.

In the case of glucuronide-linked conjugates incorporation was assessed only in relative terms by RIA binding inhibition and performance in EIA, although for comparison, spectrophotometric estimations have been included on conjugates prepared on a separate occasion. Data are limited to comparison of conjugates formed using a 4:1, 7:1 and 14:1 excess of active ester by DSC with a single conjugate formed at a 10:1 excess using DCC. The DCC conjugate (10:1) showed a similar degree of incorporation to that formed using DSC at a 4-fold molar excess of active ester when assessed by RIA binding inhibition, implying that at conjugation, less active ester was actually present when formed by DCC (table 3.4).

Although there was little difference between procedures in the yield of glucuronide active ester, yield was lower for the hemisuccinate active ester by the DSC procedure. It is the performance of the conjugates in EIA, however, which is most important. In this respect, the glucuronide-linked
<table>
<thead>
<tr>
<th>Conjugate No.</th>
<th>Molar Steroid Excess at Conjugation</th>
<th>Steroid Derivative</th>
<th>Method</th>
<th>Steroid Incorporation* (10^3) (mol Progesterone Equivalent/mol Enzyme)</th>
<th>Enzyme Activity Recovery (%)</th>
<th>Performance in EIA Bo (ΔOD/h)</th>
<th>B&lt;sub&gt;50&lt;/sub&gt;/Bo (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>183</td>
<td>5</td>
<td>HS</td>
<td>DCC</td>
<td>1.16</td>
<td>86</td>
<td>1.109</td>
<td>61.3</td>
</tr>
<tr>
<td>184</td>
<td>10</td>
<td>HS</td>
<td>DCC</td>
<td>2.33</td>
<td>96</td>
<td>1.555</td>
<td>64.8</td>
</tr>
<tr>
<td>185</td>
<td>20</td>
<td>HS</td>
<td>DCC</td>
<td>2.98</td>
<td>96</td>
<td>1.652</td>
<td>67.5</td>
</tr>
<tr>
<td>186</td>
<td>5</td>
<td>HS</td>
<td>DCC</td>
<td>0.66</td>
<td>100</td>
<td>0.617</td>
<td>57.9</td>
</tr>
<tr>
<td>187</td>
<td>10</td>
<td>HS</td>
<td>DCC</td>
<td>1.26</td>
<td>95</td>
<td>1.115</td>
<td>62.0</td>
</tr>
<tr>
<td>188</td>
<td>20</td>
<td>HS</td>
<td>DCC</td>
<td>3.06</td>
<td>105</td>
<td>1.211</td>
<td>64.7</td>
</tr>
<tr>
<td>189</td>
<td>4</td>
<td>Glu</td>
<td>DCC</td>
<td>-</td>
<td>105</td>
<td>0.315</td>
<td>27.6</td>
</tr>
<tr>
<td>190</td>
<td>7</td>
<td>Glu</td>
<td>DCC</td>
<td>-</td>
<td>99</td>
<td>0.572</td>
<td>34.6</td>
</tr>
<tr>
<td>191</td>
<td>14</td>
<td>Glu</td>
<td>DCC</td>
<td>-</td>
<td>97</td>
<td>1.032</td>
<td>41.9</td>
</tr>
<tr>
<td>181</td>
<td>10</td>
<td>Glu</td>
<td>DCC</td>
<td>-</td>
<td>94</td>
<td>0.614</td>
<td>34.2</td>
</tr>
<tr>
<td>195</td>
<td>2</td>
<td>Glu</td>
<td>DCC</td>
<td>-</td>
<td>91</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>196</td>
<td>10</td>
<td>Glu</td>
<td>DCC</td>
<td>-</td>
<td>86</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

For legend please see over.
Conjugates 195 and 196 were prepared in an identical manner to 183 - 185 and 181 but on a separate occasion.

+ Calculated as described in section 3.2.5.c.

- Not determined.

* EIA was performed using polystyrene plates (NUNC; antiserum G711/12 X-globulin preparation, 1/8000 dilution) in the manner described in section 7.2.2.b using 5ng of conjugate/well.

\[ B_0 = \text{amount of enzyme-labelled progesterone bound to antibody in the absence of competing progesterone. Values are expressed as optical density change/h (AOD/h).} \]

\[ B_{50}/B_0 = \text{amount of label bound in the presence of 50pg of competing progesterone as percent of } B_0 \text{ value.} \]

The conjugate formed using DSC and a 4:1 molar excess of steroid provided the most sensitive calibration curve as indicated by \( B_{50}/B_0 \) values (Table 3.4). The small differences in sensitivity between conjugates formed using the same derivative are likely to reflect differences in the degree of conjugation (see section 6.3.3.).

It can be seen that the immunoreactivity (= steroid incorporation by RIA) of the glucuronide conjugates is far lower than that of the hemisuccinate conjugates formed using the same molar excess (Table 3.4). This would be expected if bridge recognition and therefore the binding affinity was lower with the glucuronide bridge (see section 6.3.2.). Additionally, it cannot be discounted that differences in the degree of incorporation may play a minor role. Although some reservations are justified about the use of spectrophotometric measurements for absolute determinations of incorporation rate (Erlanger et al 1957a) this method showed 2 - 3 times lower incorporation for glucuronide conjugates (195 and 196) than equivalent hemisuccinate conjugates (182 and 184) assessed by radioactivity determination (Table 3.4). Spectrophotometric determinations were not performed for the latter.
3.4 DISCUSSION

The preliminary studies reported in Table 3.1 and those by others
(Dray et al, 1976; Commoglio and Celada, 1976; Exley and Abuknesha, 1977;
Joyce et al, 1977; Al Bassam et al, 1979) clearly indicate that a large molar
excess of hapten is required to produce effective conjugates using the
mixed anhydride and carbodiimide procedures even though it may be necessary
to incorporate only one per enzyme molecule.

Data from the RIA binding inhibition studies enabled comparison of the
degree of incorporation of progesterone into β-galactosidase using the
active ester procedure (Table 3.2), with that obtained previously by other
methods (Table 3.1). This indicated that conjugation proceeded more
readily using the active ester method since a higher degree of substitution
was achieved using lower molar excess. Comparison of conjugate 60 (Table 3.2)
with conjugates 7, 17 and 36 (Table 3.1) indicates that the active ester
procedure was an order of magnitude more efficient than the carbodiimide or
mixed anhydride procedures. This is in keeping with the data of Comoglio
and Celada (1975) who used the mixed anhydride procedure to form steroid
enzyme conjugates and Exley and Abuknesha (1978) who used the carbodiimide
method. These groups estimated the degree of conjugation by radioactivity
incorporation and RIA determinations on hydrolysed conjugates, respectively:
the degree of incorporation was at best one tenth that reported here using
the active ester procedure at approximately equivalent molar excess of
steroid.

Previous reports of the use of steroid active esters for this purpose
have not provided details of the efficiency of substitution into the

When used for conjugation with proteins (e.g. BSA for which a 50 fold
molar steroid excess would be usual) for the formation of immunogens,
efficient incorporation is generally anticipated with the mixed anhydride
procedure (Erlanger et al., 1957a): for particular enzymes, reduced efficiency may be related to the accessibility of ε-amino residues. Al Bassam et al. (1979) reported that a 2,500 molar excess was optimal for the formation of methotrexate conjugates with β-galactosidase.

Substantially lower molar steroid incorporation was recorded by RIA binding inhibition than by radioactivity incorporation, a phenomenon also reported by Comoglio and Celada (1975). Clearly the only progesterone molecules detected by immunoassay are those located at sites on the enzyme molecule which do not sterically hinder antibody binding.

It has been stated that optimum sensitivity in EIA is in part dependent on achieving a low (around 1:1) degree of incorporation (Exley and Abuknesha, 1977; Joyce et al., 1978a). From the findings here, however, this would only be true where all conjugated steroid was immunologically detectable.

Although EIA calibration curves produced using the homologous conjugates (hemisuccinate bridge) were markedly less sensitive than RIA (Fig. 3.3) the performance of the EIA proved adequate for preliminary application to the direct measurement of progesterone in milk and enabled corpus luteum function to be monitored in dairy cows (see section 8.3.1.).

The necessary adoption of the direct active ester conjugation procedure (as opposed to use of crystallized esters) for formation of heterologous conjugates gave rise to minimal effects on the catalytic activity of alkaline phosphatase (Table 3.3). The main disadvantage of such direct procedures (as with the carbodiimide and mixed anhydride procedures) is that the molar excess of reactive steroid added to enzyme is unknown. For conjugation procedures performed using this method 100% conversion to active ester was assumed, although clearly this was not reached in practice (Fig. 3.3).

N,N'-disuccinimidyl carbonate was demonstrated to provide a practical
alternative to dicyclohexyl carbodiimide and N-hydroxysuccinimide for the formation of active esters and may prove to be less hazardous for general use.

3.5 CONCLUSIONS

1. Use of active esters of progesterone derivatives whether isolated or added directly enabled efficient formation of enzyme-labelled progesterone with minimal loss of enzyme activity. The binding characteristics of these labelled steroids with antibody commended them for use in EIA.

2. Disuccinimidyl carbonate provided a more convenient and arguably safer alternative to carbodiimide and N-hydroxysuccinimide for the synthesis of active esters.
CHAPTER 4:
The Enzymes Used As Labels:
Optimization Of Assay Procedures
CHAPTER 4. THE ENZYMES USED AS LABELS: OPTIMIZATION OF ASSAY PROCEDURES

4.1 INTRODUCTION

Alkaline phosphatase, β-galactosidase, penicillinase and peroxidase were assessed for their usefulness in microtitre plate EIA since they have previously been applied successfully in steroid EIA (see section 1.9.3): only β-galactosidase (Dray et al., 1975) and peroxidase (Joyce et al., 1977) have been used in the assay of progesterone, however.

The properties of enzymes used in these studies are shown in Table 4.1. The enzyme preparations were of the highest purity and specific activity commercially available. Colorimetric assays for these enzymes were adapted for use in microtitre plates from test-tube procedures described by Joyce et al., (1977, peroxidase), Novick (1962, penicillinase), Exley and Abuknesha (1978; β-galactosidase) and McComb and Bowers (1972, alkaline phosphatase).

The main objective was to optimize substrate and chromogen concentrations to ensure, where possible, the attainment of zero-order kinetics (reaction velocity independent of substrate concentration) over the normal assay period. This is particularly critical for microtitre plate procedures since the volume of substrate which may be added is limited by the small size of the wells (maximum volume 0.3 - 0.4ml). Since in EIA the mass of enzyme bound in each well will depend on the degree of competition, it is also important that product formation should increase linearly with the mass of enzyme present. Maximum sensitivity in EIA should be attained when minimum concentrations of label are used and full advantage of this will be achieved when assay conditions subsequently permit substrate conversion at maximum velocity. The reactions catalysed by the four enzymes are outlined in Fig. 4.1.

In the case of alkaline phosphatase and β-galactosidase, assay optimization procedures were considerably simplified by the availability of chromogenic substrates.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>International Code</th>
<th>Source</th>
<th>M. Wt.(^a) (Daltons)</th>
<th>Enzyme Activity(^b) (pH, temp °C)</th>
<th>Substrate/Chromogen</th>
<th>No. Primary(^c) Amino Residues/Enzyme Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Penicillinase</td>
<td>EC 3.5.2.6</td>
<td><em>Bacillus cereus</em></td>
<td>30,800</td>
<td>2,000</td>
<td>Benzyl penicillin</td>
<td>21 to 31 + 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(7.0, 30°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Peroxidase</td>
<td>EC 1.11.1.7</td>
<td>Horseradish</td>
<td>40,000</td>
<td>4,500 - 5,940</td>
<td>H(_2)O(_2)/purpyrogallin</td>
<td>6 + 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(6.0, 25°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Alkaline phosphatase</td>
<td>EC 3.1.3.1</td>
<td>Calf intestine mucosa</td>
<td>100,000</td>
<td>2,000</td>
<td>p-NPP</td>
<td>53 + 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(9.8, 37°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 β-Galactosidase</td>
<td>EC 3.2.1.23</td>
<td><em>Escherichia coli</em></td>
<td>540,000</td>
<td>600 - 900</td>
<td>O-NPG</td>
<td>116 + 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(7.3, 37°C)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(p\)-NPP = \(p\)-nitrophenyl phosphate; \(O\)-NPG = \(o\)-nitrophenyl \(-β\)-D-galactopyranoside.

a) 1, Citri (1971); 2, Shannon, Kay and Lew (1966); 3, Fernley (1971); 4, Craven et al (1965).

b) Enzyme activity expressed as \(\mu\)mol product formed per mg enzyme per minute at the pH and temperature indicated.

The volumes given are those for preparations of the highest specific activity available from the Sigma Chemical Co: Penicillinase, Type I; Peroxidase, Type VI; Alkaline phosphatase type VII-T and β-galactosidase grade VIII.

c) Lysine \(ε\)-amino groups + N-terminus. 1, Citri (1971); 2, Shannon et al (1966); 3, Fosset, Chappelet-Tordo and Lazdunski, 1974; 4, Craven et al (1965).
Fig. 4.1 Outline of Reactions Catalysed by a) β-Galactosidase, b) Alkaline Phosphatase, c) Peroxidase and d) Penicillinase

a) \( o\)-nitrophenyl-\( \beta \)-D-galactopyranoside + \( H_2O \) \( \rightarrow \) D-galactose + \( o\)-nitrophenol
\( \beta \)-galactosidase

b) \( p\)-nitrophenyl phosphate + \( H_2O \) \( \rightarrow \) \( p\)-nitrophenol + inorganic phosphate
alkaline phosphatase

c) suggested reaction (see Saunders et al., 1964)
\( 3H_2O_2 + o\)-phenylenediamine \( \rightarrow \) 2,3-diaminophenazine + 6\( H_2O \)
peroxidase

d) penicillin + \( H_2O \) \( \rightarrow \) penicilloic acid
penicillinase
A number of amino and phenolic chromogens may act as hydrogen donors in the reduction of hydrogen peroxide by peroxidase (Saunders et al., 1964) and the resulting spectral shift provides a sensitive means of monitoring peroxidase activity: \( o \)-phenylenediamine (oPD) was used here.

Two chromogenic substrates have been reported for penicillinase but neither were considered to be suitable for the present application. \( N \)-(2-furyl)acryloyl penicillin (Durkin, Dmitrienko and Viswanatha, 1977) gives rise to spectral changes outside the visible range (330nm) and which could not be quantitated on the plate reader using standard optical filters. The other, \( 3-(2,4\text{-dinitrostyryl})-(6R, 7R)-7\text{-}(2\text{-thienylacetamido})\text{-ceph}-3\text{-em}-4\text{-carboxylic acid} \) (O'Callaghan, Morris, Kirby and Shingler, 1972) was shown to be hydrolysed too slowly by penicillinase from \( B. \) cereus to be of practical value. An iodometric assay developed from that described by Novick (1962) was therefore used. This procedure has also been modified by Shah and Joshi (1982) for use in a microtitre plate EIA but this involved a two step method in which the end-point was attained in a test tube: this was considered to be impractical and retrograde in the present application. The Novick method depends on the reduction of iodine by penicilloic acid but not by penicillin, as indicated by decolorization of starch/iodine reagent.

4.2 METHODS

For all assays absorbance readings were made \textit{in situ} using a "through-the-well" automatic microtitre plate reader. Use of a suitable dispenser (BCL 500) enabled substrate to be added to wells already containing enzyme solution at essentially the same rate at which the wells were read (96 wells/1.2 minutes): thus termination of the enzyme reaction was considered unnecessary.

All assays were performed in a similar manner in microtitre plate wells.
Enzyme solutions were diluted in the appropriate buffer (PAS-gelatin; for peroxidase, PMS-gelatin) to provide the desired concentrations and an aliquot (10 µl) added to the wells with a positive displacement pipette prior to addition of substrate or substrate/chromogen solution. Substrate solutions were prepared (see section 2.1.8.) over an appropriate range of concentrations: in addition, for peroxidase, a range of chromogen concentrations were investigated using a fixed substrate concentration. Each variable was estimated in quadruplicate. Substrate solutions (200 µl) were added rapidly to enzyme in the wells and the plate covered with a lid and transferred to a covered water bath at 40°C (21°C - 24°C in the dark for peroxidase). Incubations took place on a grid over which the water level was adjusted to enable immersion of the base of the wells by 1 - 2 mm. Product formation was monitored by reading the plate at appropriate intervals: values were corrected for substrate blanks. Table 4.2 summarizes assay conditions and the reagents used.

4.3 RESULTS

4.3.1. β-Galactosidase

Concentrations of o-nitrophenyl-β-D-galactopyranoside (o-NPG) below 1.6 mg/ml (5.3 mM) were found to be reaction-rate limiting (Fig. 4.2a) and a concentration of 10 mM was used for all subsequent work. The reaction rate was found to be non-linear irrespective of the mass of enzyme used (Fig. 4.2b): however, a linear relationship between reaction rate (absorbance at fixed time) and quantity of enzyme present was demonstrated (Fig. 4.2c, \( r = 0.9998, y = 0.112x + 0.025 \)). A reduction in reaction rate as a result of non-immersion of wells during incubation was clearly demonstrated (Fig. 4.2b) and arises from slower attainment of equilibrium temperature (see section 5.2.5.a).
Fig. 4.2 The assay of β-galactosidase (n = 4 for all estimations).

a) The influence of substrate concentration on reaction rate at 40°C. β-Galactosidase (3.2 ng) was incubated with o-nitrophenyl-β-D-galactopyranoside (ONPG) at the concentration indicated.

b) The reaction rate of β-galactosidase (0.8 ng, △○; 1.6 ng ▲▲; 3.2 ng ■■) incubated at 40°C with ONPG (10 mM). Plate wells were incubated either in contact with (-----) or immediately above (----) water in the bath.

c) The relationship between the mass of enzyme and relative reaction rate (15 minute incubation) with ONPG (10 mM) at 40°C.

Fig. 4.3 The assay of alkaline phosphatase (n = 4 for all estimations)

a) The influence of substrate concentration on reaction rate at 40°C. Alkaline phosphatase (0.2 ng) was incubated with p-nitrophenylphosphate (pNPP)

b) The relative reaction rate of alkaline phosphatase (0.4 ng) incubated at 23°C (●•), 37°C (○•), 40°C (△△) and 45°C (▲▲) with pNPP (15 mM).

c) The relative reaction rate of alkaline phosphatase (0.4 ng) incubated at 23°C (●●), 37°C (○○), 40°C (△△) and 45°C (▲▲) with pNPP (15 mM).
4.3.2. Alkaline Phosphatase

The maximum reaction rate was achieved using $p$-nitrophenylphosphate at a concentration of 30mM (Fig. 4.3a). Further studies indicated that the reaction rate retained linearity over a range of enzyme concentrations (Fig. 4.3b) using 5, 10 or 15mM substrate ($r = 0.998, 0.999$ and $0.999$ respectively): thus, although the latter concentration gave rise to a 12% lower reaction rate than 30mM, it was used at 15mM for all subsequent work to reduce reagent cost. Comparison of incubation temperatures between 25°C and 45°C indicated that 40°C was optimal since, for incubation periods in excess of 40 minutes, the reaction rate declined at higher temperature (Fig. 4.3c).

4.3.3. Peroxidase

The concentration ranges of $\text{OPD}$ and $\text{H}_2\text{O}_2$ chosen for the studies described here were selected on the basis of an initial "latin square" experiment using a fixed mass of enzyme (1ng). Subsequent experiments were performed using a closer range of concentrations of both chromogen and substrate and indicated that the reaction rate was maximal using 22.1mM $\text{OPD}$ (Fig. 4.4a) and 7.1mM $\text{H}_2\text{O}_2$ (Fig. 4.4b): higher concentrations of either reagent resulted in an apparent inhibition of enzyme activity. The reaction rates were non-linear, possibly as a result of limiting substrate concentrations: similar complex kinetics have been reported elsewhere (Groome, 1980; Porstmann, Porstmann and Nugel, 1981). Using the optimal concentrations, however, reaction rates did increase linearly with increasing mass of enzyme (Fig.4.4c; $y = 6.785x - 0.034$ and $r = 0.9995$ for a 40 minute incubation period).

4.3.4. Penicillinase

The initial absorbance of the reagent is dictated by the concentration of iodine and this was adjusted to provide a value of 1.2 (see section 2.1.9d).
Fig. 4.4 The assay of peroxidase. All assays were performed at ambient temperature (20 -
22°C; n = 4 for all estimations).

a) The influence of o-phenylenediamine (oPD) concentration on enzyme activity. Peroxidase
(0.5ng/well) was incubated with hydrogen peroxide (7.1mM) and the chromogen oPD at
the concentrations indicated.

b) The influence of substrate concentration on enzyme activity. Peroxidase (0.2ng/well) was
incubated with oPD (22.1mM) and hydrogen peroxide at the concentrations indicated.

c) The relationship between the mass of peroxidase and relative reaction rate. Peroxidase
was incubated with oPD (22.1mM) and hydrogen peroxide (7.1mM) for 15 minutes (■-■) or
45 minutes (■-■).

Fig. 4.5 The assay of penicillinase. All assays were performed at 40°C (n = 4 for all
estimations).

a) Reduction of starch/iodine reagent as a result of autolysis of penicillin-V. Starch/iodine
reagent, prepared as described in table 4.2, was added to aliquots (10pl) of penicillin-V
solution to provide the final concentration indicated.

b) Penicillin-V concentration and catalytic reaction rate. Penicillin-V hydrolysis by
penicillinase (20pg) at concentrations of 122μM (■-■), 245μM (■-■), 489μM (■-■) and 979μM
(■-■) was determined by reduction of starch/iodine reagent. All volumes are corrected
for autolysis. The autolysis value as % of initial absorbance (■-■) of the starch/iodine
reagent is expressed where appropriate.

c) The relationship between the mass of penicillinase and relative enzyme reaction rate.
Incubation periods at a substrate concentration of 122μM (■-■) were 30, 45 or 60 minutes,
and at 245μM (■-■) 45 minutes.
Phenoxymethylpenicillinic acid (penicillin-V) was used as substrate since this is most rapidly hydrolysed by *B. oereus* penicillinase (Citri, 1971): the reaction has been described as providing zero-order kinetics (Citri, 1971).

The main problem associated with the method was development of a substantial substrate blank at high substrate concentrations (Fig. 4.5a), a phenomenon also observed with benzylpenicillin. This prevented its use with the starch/iodine system at concentrations likely to provide zero-order kinetics.

Only substrates concentrations of 122µM and 245µM gave rise to acceptably low degrees of autolysis (at 45 minutes, 3% and 9% respectively of the initial blank value) when compared with enzyme hydrolysis (Fig. 4.5b).

Although enzyme activity was substrate-limited at these concentrations, it was considered that either would enable end-point determinations in EIA. The greater enzyme-induced reduction in absorbance using 245µM rather than 122µM was balanced against a 6% increase in autolysis and as a consequence the latter was used in subsequent studies.

A lag-phase was noted at all substrate concentrations (Fig. 4.5b): This is not associated with the enzyme but with a limiting starch/iodine reduction rate (Novick, 1962). Despite this, a linear relationship was demonstrated between reaction rate and the mass of enzyme using substrate concentrations of 122µM (r = 0.999, y = 58.78x - 0.091) and 245µM (r= 0.999, y = 48.47x + 0.012) at incubation periods of 1 hour and 0.75 hour respectively (Fig.4.5c). Lack of linearity for shorter incubation periods was assumed to be associated with the greater proportional influence of the lag-phase.

### 4.3.5. **Summary of Selected Assay Conditions**

Table 4.2 summarises the conditions selected for the assay of the four enzymes in EIA. The wavelength selected for absorbance determination was
Table 4.2 Summary of Methods Adopted for Determining Enzyme Activity in EIA

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate Buffer(^a)</th>
<th>Substrate</th>
<th>Chromogen</th>
<th>Incubation Temperature (°C)</th>
<th>Incubation Time (min)</th>
<th>(\lambda_{\text{Max}}) (nm)</th>
<th>(\lambda) of Determination (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)-Galactosidase</td>
<td>PAS gelatin pH 7.0</td>
<td>o-NPG</td>
<td>-</td>
<td>40</td>
<td>30 - 60</td>
<td>405</td>
<td>405</td>
</tr>
<tr>
<td></td>
<td>+ MgCl(_2) (0.01M) +</td>
<td>(10mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mercaptoethanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.1M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>DEM pH 9.8</td>
<td>p-NPP</td>
<td>-</td>
<td>40</td>
<td>30 - 60</td>
<td>405</td>
<td>405</td>
</tr>
<tr>
<td></td>
<td>(15mM)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Peroxidase</td>
<td>PMS gelatin</td>
<td>(\text{H}_2\text{O}_2)</td>
<td>o-PD</td>
<td>20 - 24(^+)</td>
<td>30 - 60</td>
<td>425</td>
<td>405</td>
</tr>
<tr>
<td></td>
<td>pH 6.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7.1mM)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>(22.1mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillinase</td>
<td>PS pH 7.0</td>
<td>Pen-V</td>
<td>Starch/</td>
<td>40</td>
<td>60</td>
<td>560 - 600</td>
<td>570</td>
</tr>
<tr>
<td></td>
<td>(122(\mu)M)</td>
<td></td>
<td>Iodine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.2%/170(\mu)M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The enzyme reactions do not require to be stopped if O.D. determinations are made using an automatic microtitre plate reader.

a) See section 2.1.9. for substrate preparation.

o-NPG = o-nitrophenyl-\(\beta\)-D-galactopyranoside; p-NPP = p-nitrophenylphosphate; o-PD = o-phenylenediamine; Pen-V = phenoxyethylpenicillin acid.

\(^+\) Ambient temperature.
dictated by the filters available in the microtitre plate reader and were at or close to the wavelength of maximum absorbance as determined spectrophotometrically (Cecil CE292).

Table 4.3 Comparison of the Relative Sensitivity of Detection of β-Galactosidase, Alkaline Phosphatase, Peroxidase and Penicillinase Assayed in Microtitre Plates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ΔOD/ng/h</th>
<th>ΔOD/f.mol/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galactosidase</td>
<td>0.458</td>
<td>0.247</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>2.48</td>
<td>0.248</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>9.923</td>
<td>0.397</td>
</tr>
<tr>
<td>Penicillinase</td>
<td>58.8</td>
<td>1.764</td>
</tr>
</tbody>
</table>

Values were calculated from data in Figs. 4.2.c., 4.3.b., 4.4.c. and 4.5.c.

Enzymes were assayed under conditions described in Table 4.2.

ΔOD = change in optical density. This represents the product of the enzyme specific activity and the extinction coefficient of the product.

4.4 DISCUSSION

From the data shown in Figs. 4.2.c, 4.3.b, 4.4.c and 4.5.c, it was possible to compare the sensitivity of detection of the four enzymes in terms of absorbance change per unit time for a given mass of enzyme (Table 4.3).

The low (<1 f.mol) quantities of enzyme which may be detected were within those anticipated for use as label in EIA: the quantity of tritium label used in progesterone RIA would be of the order of 10 - 50 f.mol /test (ν 3 - 15pg/test).

It is clear that penicillinase may be detected with greatest sensitivity (precision was similar for all enzymes, ν1% c.v.) when considered both in terms of mass and molar quantities of enzyme: the possibility clearly exists that the sensitivity may be further improved by use of a
substrate (preferably chromogenic) less prone to autolysis in order that
the maximum reaction velocity may be approached. Similarly, sensitivity of
detection may be further improved for the other three enzymes by use of
fluorogenic substrates (see section 1.9.3.), although these are of no
practical interest for the intended application. A system has recently
been described, however, which enables the product signal of the alkaline
phosphatase-catalysed reaction to be colorimetrically amplified by up to a
hundred-fold (Self, 1982) and this may be of value in reducing the mass of
conjugate required, the time taken to perform the assay or in providing a more
readily visualised product for semi-quantitative applications.

Enzyme detection limits should not solely dictate the preferred
enzyme for use in EIA and the properties of these enzymes when conjugated
with progesterone are considered in Chapter 6.

4.5 CONCLUSIONS

1. It was possible to assay the four enzymes in microtitre plate wells
   by addition of a single reagent: a linear relationship between reaction
   rate and enzyme mass was noted in each case.

2. Low f.mol detection limits were attained indicating the suitability of
   the enzymes for use in microtitre plate EIA.

3. The sensitivity of detection of the enzymes was in the order
   penicillinase > peroxidase > alkaline phosphatase > β-galactosidase.
CHAPTER 5:
SEPARATION OF FREE FROM BOUND ENZYME LABEL:
THE USE OF MICROTI TRE PLATES
CHAPTER 5. SEPARATION OF FREE FROM BOUND ENZYME LABEL: THE USE OF MICROTITRE PLATES.

5.1 INTRODUCTION

In preliminary studies of separation procedures in EIA, solid-phase primary antibody (covalently attached to microcrystalline cellulose or passively adsorbed to microtitre plates) were compared with double antibody precipitation techniques. At this stage of assay development methods of end-point determination all involved manual spectrophotometric procedures in the manner described by Sauer, Foulkes and Cookson (1981). It became clear at this time that use of microtitre plates, although far from perfect, presented a major advantage over the other procedures in that manipulative stages were considerably simplified. The rigid form of the 96 well plates enabled manual pipetting stages and separation and wash steps to be performed with greater speed and facility.

"Through-the-well" microtitre plate readers (colorimeters) have subsequently become available which enable sequential, rapid (96 wells/1.2 minutes) and automatic determination of absorbance in situ thereby eliminating a major rate-limiting step in EIA. It was therefore decided to concentrate on the development of microtitre plate methods.

It was apparent from other preliminary studies that major shortcomings in the microtitre plate procedure required resolution if this was to become a practicable, reliable quantitative procedure. Their major limitations were their variable and low adsorptive capacity for antibody.

The use of microtitre plate wells as immunoadsorbent in enzyme-immunometric assays has been reported to give rise to variable results (Denmark and Chessum, 1978a and 1978b). Discrepancies have been ascribed to variations in the adsorptive properties of single or groups of wells as a result of inconstancy in manufacture or in incubation conditions either at adsorption or during the immunoassay (Denmark and Chessum 1978a & b; Burt, Carter and Kricka, 1979; Kricka et al, 1980).
Measurement of precision and extent of adsorption of γ-globulin is facilitated by the use of labelled γ-globulin and avoids the introduction of errors associated with indirect procedures. Application of 125I-labelled γ-globulin for these purposes has been widely reported (Pesce, Ford, Gaizutis and Pollak, 1977; Salonen and Vaheri, 1979; Cantarero, Butler and Osborne, 1980). The introduction of a large (electron-withdrawing) atom such as iodine would be expected to induce conformational changes and alter the isoelectric point of the γ-globulin (Hunter, 1978), however and this might influence the adsorption process. The validity of using such a label in the investigation of adsorption must therefore be held in question: (14C)γ-globulin was used in the present study. This was formed at neutral pH by the reductive methylation technique applied by Jentoft and Dearborn (1980) to the labelling of BSA. This involved minimal perturbation of protein structure since the substituted methyl groups are relatively small and the net charge distribution of the protein is not altered (Jentoft and Dearborn, 1979; Tack and Wilder, 1981).

Microtitre plates are not available in plastics containing functional groups allowing the formation of chemical bonds with antibody. Reliance has therefore been placed on passive adsorption to the well surface. The use of antibody immobilized on the surface of polystyrene in this way for immunoassay was first reported by Catt and Tregear (1967). Few details of the studies involved in arriving at the conditions described as "optimal" for the passive adsorption of antibody were reported, however. The process involved incubation of plastic tubes containing antibody solution (unspecified buffer at pH 9 - 10) for a suitable period. Engvall et al (1971) reported the use of a carbonate buffer (0.1M, pH 9.8) for the same purpose: the coated tubes were subsequently used in EIA. Similar con-
ditions have become the norm in both EIA and RIA, however, despite the apparent inefficiency of the adsorption process (Parsons, 1981).

The process of protein adsorption to hydrophobic amorphous polymers such as polystyrene and PVC is thought to involve hydrophobic bonding (Morrisey, 1977). In aqueous solution hydrogen bonds, hydrophobic and ionic interactions dictate the structural conformation of proteins so that hydrophilic amino acids are presented at the protein surface and hydrophobic amino acids to the interior. During the process of adsorption, water molecules ordered around specific regions of the protein and the solid surface must be displaced and conformational changes occur in the protein to enable bonding between hydrophobic nuclei (Kochwa, Litwak, Rosenfield and Leonard, 1977). Once formed, such hydrophobic bonding is thought to be virtually irreversible (Parsons, 1981).

The relatively small surface area of microtitre plate wells (~240mm²) necessitates identification of optimum conditions if efficient use is to be made of antisera and sufficient antibody is to be adsorbed to provide a practicable assay: the investigation of such conditions are described in this chapter.

The adsorption of caprine and ovine anti-progesterone γ-globulin to PVC and polystyrene microtitre plates was investigated. PVC plates were used in the earliest studies since they provided higher and less variable adsorption properties than concurrent polystyrene plates. High performance polystyrene plates (NUNC) subsequently became available and their characteristics justified comparison with PVC plates.

Adsorbed antibody was initially quantified by subsequent assessment of (³H)progesterone binding since equilibrium was attained within a few hours and fewer incubation stages were involved in which errors could be introduced. Later studies involved use of enzyme-labelled progesterone or direct measurement of ¹⁴C-labelled γ-globulin.
The performance of assays in test tubes permits rapid and uniform attainment of particular assay temperatures since they can be immersed in a water bath at the required temperature. The manner of construction and dimensions of microtitre plates, particularly those in polystyrene, do not readily permit such procedures. Furthermore, the use of oven incubations is not recommended since heat transfer is relatively slow and may not be evenly distributed across the plate. The occurrence and extent of between-well variation in heat transfer rate was therefore investigated since this could give rise to imprecision during γ-globulin adsorption and subsequently during EIA.

5.2 METHODS

5.2.1. Carbon-14 Labelling of Anti-Progesterone γ-Globulin

The method used was adapted from methods described by Jentoft and Dearborn (1979 and 1980) for the reductive methylation of ε-amino groups of lysine residues and α-amino terminus of BSA: the reaction readily proceeds at neutral pH.

\[
\text{RNH}_2 + \text{CH}_2 = \text{NaCNBH}_3 \rightarrow \text{RNH - CH}_2\text{OH} \rightarrow \text{RN = CH}_2 \rightarrow \text{RNH - CH}_3
\]

Further reaction of the secondary amino group occurs at high molar ratios of formaldehyde to amine (e.g. 6:1): reduction using sodium cyanoborohydride then forms the dimethyl derivative.

The γ-globulin fraction of BSA-absorbed goat anti-progesterone serum (G711/12) was prepared by the Rivanol procedure (section 2.3.1.d and 2.3.1.e), dialysed against 0.1M PS buffer, pH 7.5 (1000 vol) and the protein content determined. Sodium cyanoborohydride (1.4mg, 22μmol) was weighed into a screw capped glass bottle containing a magnetic stirring bar and freshly prepared nickel chloride suspension (1M in 0.1M PS buffer pH 7.5, 10μl ≡ 10μmol), anti-progesterone γ-globulin solution (1.682mg in 0.9ml ≡ 0.011μmol
or 0.814 μmol lysine residues*) and \(^{14}\)C-formaldehyde solution (4.7 μmol: 250 μCi in 0.1 ml 0.1M PS buffer pH 7.5) added. The mixture was then stirred in the capped vessel for 2 hours at room temperature (21°C).

Following dialysis of the mixture at 4°C overnight against 0.1M PS buffer pH 7.5, it was applied to a Sephadex G25 column (bed volume 10ml). The \(^{14}\)C-labelled γ-globulin fraction was eluted with 0.1M PAS buffer pH 7.0, collected manually at the void volume following spectrophotometric detection at 280nm and stored frozen (-20°C) in 1ml aliquots.

Following determination of the protein concentration (recovery 98%) and radioactivity (5 μl aliquots counted in 10ml Instagel) of the γ-globulin fraction an incorporation of 0.77 mol \(^{14}\)C/mol lysine and specific activity of 3.01 Ci/mmole IgG were calculated.* Comparison of binding characteristics of this and the unsubstituted γ-globulin in RIA revealed no substantial differences.

5.2.2. Plate Coating Procedure

The γ-globulin fraction of the antiserum was diluted in the appropriate buffer, 200 μl aliquots added to each microtitre plate well using an 8-channel peristaltic dispenser and the plate covered with a lid. Following incubation for 3 hours either on a grid in a water bath (base of wells 1 - 2mm below water surface) at 40°C or at room temperature (in a draught free environment away from point sources of heat), the plate was emptied, tapped dry over absorbent paper and 250 μl (PVC plates) or 350 μl (polystyrene plates) of PAS-gelatin buffer added and the plate sealed with plastic film. Plates were stored for a minimum of 16 hours at 4°C before use. To facilitate incubations in water, a small hole was cut in each corner of PVC plates to prevent floating.

* For the purpose of these calculations the γ-globulin preparation was assumed to be pure IgG. Since data for caprine or ovine IgG were not available these figures are based on data for human IgG, molecular weight 153,000 Daltons (Sober, 1970) and lysine content 74 mol lysine/mol IgG.
5.2.3. The Adsorption of Caprine $\gamma$-Globulin to PVC (Dynatech) Plates

The carrier protein-absorbed $\gamma$-globulin fraction of anti-progesterone serum was used for all experiments and the final dilution based on the original antiserum volume.

a) Duration and temperature of incubation with $\gamma$-globulin. The $\gamma$-globulin fraction of the antiserum (G711/12) was diluted to 1/200 in 0.01M PAS buffer and 200$\mu$l aliquots dispensed into quadruplet wells of microtitre plates in sequence, such that all incubation periods (0.5, 1, 2, 3, 4, 5, 6, 9, 21 and 24 hours) were completed at the same time. The plates were incubated covered with a plate lid for the specified time at room temperature or in a water bath maintained at 35°C or 40°C.

b) The pH of the coating buffer. The $\gamma$-globulin solution (G711/12) was diluted to 1/200 in 0.1M PAS at pH 5, 6, 7 or 8 or in 0.1M carbonate buffer at pH 9 and 10 and 200$\mu$l aliquots dispensed into octuplet wells. The plate was covered with a plate lid and incubated for 3 hours at 40°C in a water bath.

c) The molarity of the coating buffer. The $\gamma$-globulin (G711/12) was diluted to 1/200 in acetate buffer pH 5.0 (0.001, 0.01, 0.05, 0.1 and 0.5M) and 200$\mu$l aliquots dispensed into octuplet wells. The plate was covered with a plate lid and incubated for 3 hours at 40°C in a water bath. PAS buffer was not used in this study since phosphate ions do not provide effective buffering at pH 5.0.

In experiments a, b and c, plates were emptied after the incubation period, tapped dry, PAS-gelatin buffer (250$\mu$l) added to all wells and the plates left covered overnight at 4°C. Plates were then emptied, washed once with PAS-gelatin buffer (250$\mu$l) and ($^3$H)progesterone (15pg $\equiv$ 8,000dpm in 200$\mu$l PAS-gelatin buffer) added to each well. The plates were incubated at 4°C for 24 hours (a) or 40°C for 3 hours (b and c). Wells were
then emptied, washed with PAS gelatin buffer (x3), separated using scissors and the radioactivity bound determined by liquid scintillation counting following extraction of each well in Toluene Scintillator (7ml).

5.2.4. The Adsorption of Ovine γ-Globulin to Polystyrene (NUNC) or PVC (Dynatech) Plates using a Universal Buffer

The γ-globulin fraction of ovine antiserum S10823/24, prepared by ammonium sulphate precipitation (see section 2.3.1.f)), was diluted (1/800) in Britton/Robinson buffer over a range of pH values (2.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 or 10.0) or at pH 4.0 over a range of molarities (0.5, 1.6, 4.0, 13, 40 or 120mM, total of all solute molecules) and 200μl aliquots added to quadruple wells. γ-Globulin diluted in 1mM acetate buffer at pH 4.0 and pH 5.0 was used to prepare control wells. The γ-globulin was adsorbed following incubation for 3 hours at 40°C in the manner described in section 5.2.2. The degree of adsorption was subsequently determined following incubation (3 hours at 40°C) with progesterone 11α-glucuronide-alkaline phosphatase conjugate (5ng: prepared with a 50:1 molar excess of steroid) in the presence of ovX milk (10μl) in the manner described for EIA (section 7.2.2.b)).

5.2.5. Sources of Variability

a) Between-well variations in temperature equilibration rate. Microtitre plates containing 200μl of PAS gelatin buffer per well were cooled to 4°C prior to transfer to a water bath fitted with a lid. A thermocouple (nickel chrome/nickel aluminium; Radiospares, London) was placed into the specified well at the start of the incubation period and the plate allowed to attain equilibrium temperature (40°C). The plates were arranged such that the base of each well was either just above (2-3mm) or just below (2-3mm) the water surface. The attainment of 40°C in the
"above water" wells necessitated an increase in water bath temperature to 43°C. Temperature changes were monitored by a chart recorder which was calibrated to enable determination of the time taken to raise the well contents from 20°C to within 0.5° of 40°C.

b) The adsorption of $^{14}$C-labelled $\gamma$-globulin: the influence of detergent pre-wash. Dynatech, Flow and Falcon PVC microtitre plates were washed at room temperature with either Tween 20 (0.05% v/v for 1 hour) or Decon 90 (2.5% v/v, overnight), rinsed in tap water, then soaked in dilute HCl (1% v/v for 1 hour), rinsed (x 3 each) with tap water then distilled water and finally dried in air. $^{14}$C Anti-progesterone $\gamma$-globulin (G711/12, 716ng/ml at 1/4000 dilution, 200μl in 1mM acetate buffer pH 5.0) was added at the appropriate dilutions ($\equiv 1/4000$, 1/8000, 1/16,000), each to 30 wells of a plate. Coating buffer alone was added to two wells in each group to provide blanks. The plates were covered with lids and incubated at 40°C for 2 hours in a water bath such that the base of each well was 1 - 2mm below the water surface. The wells were then emptied, tapped dry, washed with distilled water (250μl), tapped dry, PAS-gelatin buffer (250μl) added to half of the wells in each group and the other half left dry; the plates were stored covered at 4°C overnight (20 hours). They were then emptied, tapped dry, each well separated and bisected with scissors and placed in a scintillation vial. The mass of $\gamma$-globulin adsorbed to each well was determined by liquid scintillation counting after additions of Filter Count scintillator (7ml) as described (section 2.3.3.).

c) The sources of polystyrene and PVC plates. Polystyrene microtitre plates manufactured by Dynatech, Flow and NUNC, and PVC microtitre plates by Dynatech, Falcon and Flow were used to assess between-brand variability in precision of adsorption of caprine $\gamma$-globulin to wells. Two plates of each type were assessed. PVC plates were washed in Decon 90 prior to use (see section 5.2.5.b).
Adsorption of anti-progesterone γ-globulin (G711/12, diluted 1/4000 in 0.17 mM acetate buffer, pH 5.0) to the 96 wells of each plate was performed at room temperature (21°C) for 3 hours in the manner described in section 5.2.2. Assessment of adsorption was performed following incubation (3 hours at 21°C) with progesterone 11α-glucuronide-alkaline phosphatase conjugate (20ng, prepared with a 2:1 molar excess of steroid) in the manner described for EIA (section 7.2.2.b)). The four corner wells of each plate were used to provide a mean substrate blank value (no conjugate added).

5.3 RESULTS

5.3.1. The Adsorption of Caprine γ-Globulin to Plate Wells

a) PVC plates: the influence of time, temperature, pH and buffer molarity. The influence of these variables on the adsorption process are shown in Fig.5.1. The study of the influence of buffer pH employed buffer systems (pH 9 and 10) similar to those used by Catt and Tregear (1967) and adapted for use in EIA by Engvall et al (1971) and Voller et al (1976a).

![Graphs showing the effect of incubation time, temperature, pH, and buffer molarity on adsorption of caprine γ-globulin.](image-url)

**Fig.5.1.** Effect of A, incubation duration and temperature (25°C, o-o; 30°C, ▲-▲; 35°C, ■-■; 40°C, □-□); B, pH and C, buffer molarity on adsorption of caprine γ-globulin (G711/12) on to PVC (Dynatech) microtitre plate wells. Values are mean of 4 determinations (A) or 8 determinations (B and C) ± sd.
Table 5.1  Comparison of Adsorption of Caprine Anti-progesterone γ-Globulin to Polystyrene (NUNC) or PVC Microtitre Plates

From Water or Buffers of Low Molarity at pH 5.0

<table>
<thead>
<tr>
<th>Buffer Molarity (mM)</th>
<th>POLYSTYRENE</th>
<th>OPTICAL DENSITY (405nm)</th>
<th>PVC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
<td>Citrate</td>
<td>B.R.</td>
</tr>
<tr>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.17</td>
<td>0.736 ± .031</td>
<td>0.647 ± .031&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.614 ± .027&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.50</td>
<td>0.621 ± .032&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.794 ± .211&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.632 ± .043&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.50</td>
<td>0.484 ± .044&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.555 ± .015&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.504 ± .036&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Anti-progesterone γ-globulin (G711/12, 1/8000 dilution) was adsorbed to plate wells in the manner described in section 5.2.2. (room temperature incubation) following dilution in the appropriate medium. The degree of adsorption was determined indirectly by assessment of binding following incubation (3 hours at 40°C) with progesterone 11α-glucuronide-alkaline phosphatase conjugate (20ng, prepared with a 2:1 molar excess of steroid) in the presence of ovX milk in the manner described for EIA (section 7.2.2.b). Optical density values are mean ± sd of 4 determinations.

DI = Deionized water adjusted to pH 5.0. B.R. = Britton/Robinson buffer.  a, b, c = binding significantly lower than for 0.17mM acetate buffer; a, p < 0.05; b, < 0.01; c, < 0.001.  d = s.d. significantly different from that using 0.17mM acetate buffer (p < 0.01) and consequently excluded from further comparison.
It was clear from these studies that the adsorption process was virtually complete within 1 hour and that a small increase in the total adsorbed could be achieved by incubating above ambient temperature (Fig. 5.1.a). For all subsequent studies the adsorption process was performed for 3 hours at 40°C in a water bath or at room temperature: although less γ-globulin was adsorbed, the latter procedure was chosen for subsequent use since it enabled large numbers of plates to be prepared. Maximal adsorption was obtained at pH 5.0 (Fig. 5.1b) in 1mM acetate buffer (Fig.5.1c).

b) PVC and polystyrene plates: the influence of different buffer ions.

The higher degree of adsorption achieved using acetate rather than phosphate buffer suggested a buffer ion effect: this was investigated by comparing the efficacy of 3 buffer systems at pH 5.0 in promoting antibody adsorption to both polyvinyl (Dynatech) and polystyrene (NUNC) plates as described in table 5.1. Adsorption using acetate buffer was highest at the lowest molarity used (0.17mM) and at this molarity was appreciably greater than that achieved using the other buffer systems at any molarity (see table 5.1 for significance).

5.3.2. The Adsorption of Ovine γ-Globulin to Polystyrene (NUNC) or PVC (Dynatech) Plates using a Universal Buffer

The adsorption of ovine γ-globulin was examined in a similar manner to that for caprine γ-globulin since the possibility of between-species differences could not be ignored. A universal buffer (Britton/Robinson buffer, effective pH range 2.5 - 12.0) was used to investigate the influence of pH since this eliminated the possible variable effect of different solute molecules on the process. A similar pattern emerged to that with caprine γ-globulin (Fig.5.1), maximum adsorption occurring at pH 5 for polystyrene plates and at pH 4 - 5 for PVC plates (Fig.5.2a). At the lowest buffer molarity used (0.5mM solute, pH 4.0; Fig.5.2b) adsorption was higher than
achieved with acetate (1mM) at pH 4.0 or 5.0 for both types of plate. The apparent reduction of adsorption seen at pH 2.5 may have been a result of antibody denaturation.

Using both ovine (Fig.5.2) and caprine (table 5.1) γ-globulins under identical conditions, a higher degree of adsorption was achieved with polystyrene (NUNC) than PVC (Dynatech) plates.

Fig. 5.2 The influence of pH (a) and buffer molarity (b) on the adsorption of ovine anti-progesterone γ-globulin (S10823/24) from Britton/Robinson buffer to polystyrene (----, NUNC) or PVC (---, Dynatech) plate wells.

For b, controls were included on the polystyrene (o,●) and polyvinyl (▲,▲) plates consisting of the γ-globulin diluted in 1mM acetate buffer at pH 4.0 (●,▲) and pH 5.0 (o,▲).

5.3.3. Sources of Variability

a) Between-well variations in temperature equilibration rate. The time
Table 5.2  Time Taken to Raise the Temperature of Plate Well Contents (200µl) from 20°C to Equilibration at 40°C

<table>
<thead>
<tr>
<th>Well Location</th>
<th>POLYSTYRENE</th>
<th>PVC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>above water</td>
<td>immersion</td>
</tr>
<tr>
<td>A1</td>
<td>14 ± 1.7</td>
<td>6.95 ± 0.3</td>
</tr>
<tr>
<td>F1</td>
<td>17.2 ± 2.2</td>
<td>8.2 ± 1.3</td>
</tr>
<tr>
<td>F2</td>
<td>15.5 ± 1.16</td>
<td>8.4 ± 0.44</td>
</tr>
<tr>
<td>F4</td>
<td>18.5 ± 1.1</td>
<td>9.7 ± 0.76</td>
</tr>
<tr>
<td>mean time</td>
<td>16.3 ± 1.96</td>
<td>8.33</td>
</tr>
</tbody>
</table>

The plates were located in a covered water bath with the base of the wells just above (2 - 3mm) or just below (2 - 3mm) the water surface. The time taken represents the mean ± sd of 4 determinations. The location represents the 8 x 12 well matrix (A - H, 1 - 12) of the microtitre plate.
taken to raise the temperature of buffer (200μl) contained in plate wells from 20°C to 40°C was monitored using a thermocouple. It was seen that the time to equilibrate varied considerably between wells and that immersion of the wells to a depth of 2 – 3mm reduced the mean time taken by 49% for polystyrene plates and by 86% for PVC (table 5.2). The latter took considerably less time to reach equilibrium since the PVC plate wells are substantially thinner.

PVC plates were subsequently used to investigate the precision of adsorption of γ-globulin since they enabled rapid temperature equilibration and initial studies indicated the attainment of better overall precision.

b) The adsorption of 14C-labelled γ-globulin: the influence of detergent prewash. In preliminary studies pre-washing of PVC plates with detergent (Tween 20, 0.05% v/v) to remove possible impurities on the surface of the wells caused an insignificant reduction (4%) in adsorption of γ-globulin to wells but lowered (F = 4.8, p <0.01) the coefficient of variation in EIA (cv = 2.9%, n = 288) compared with untreated plates (cv = 6.0%, n = 288) when assessed by use of progesterone 11α-hemisuccinate-β-galactosidase label. A comparison of the adsorption of(14C)γ-globulin to three types of PVC plate pre-washed with Tween 20 or Decon 90 was therefore made.

A highly significant increase in adsorption (p< 0.001) was seen in all cases when plates were pre-washed with Decon 90 rather than Tween 20 (Fig.5.3) accompanied by an overall improvement in imprecision of adsorption from 3.9 to 1.7%, 3.3 to 1.2% and 5.4 to 2.1% (Tween 20 vs Decon 90) for Dynatech, Flow and Falcon plates respectively. The subsequent degree of desorption of γ-globulin following the standard addition of PAS-gelatin buffer (gelatin is included to block unoccupied adsorption sites) for 16 hours was low for all types of plate but was higher for Decon 90 treated than Tween 20 treated plates: this effect was more marked at higher antibody dilutions. The
Table 5.3  Mean Quantity of Caprine \((^{14}\text{C})\gamma\text{-Globulin\)} Adsorbed (% of Total Added) to PVC Plate Wells (Dry Storage) and the Proportion Desorbed Following Storage (20 hours at \(4^\circ\text{C}\)) with PAS-Gelatin Buffer

<table>
<thead>
<tr>
<th>(\gamma\text{-Globulin Dilution} )</th>
<th>1/16,000</th>
<th>1/8000</th>
<th>1/4000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean: % Adsorbed</td>
<td>Mean: % Desorbed</td>
<td>Mean: % Adsorbed</td>
</tr>
<tr>
<td>Pre-Wash Medium</td>
<td>Dry</td>
<td>PAS-gelatin</td>
<td>Dry</td>
</tr>
<tr>
<td>Tween 20</td>
<td>70.8±2.5</td>
<td>69.4±1.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Decon 90</td>
<td>89.6±4.2</td>
<td>84.4±4.1</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Values represent mean ± sd values for the three types of plate (Dynatech, Flow and Falcon, \(n = 3 \times 15\)) for the given condition and antibody dilution.
Fig. 5.3 Comparison of adsorption of $^{14}$C anti-progesterone $\gamma$-globulin to Dynatech, Flow or Falcon PVC plates following a pre-wash with Tween 20 (---) or Decon 90 (—). Following $\gamma$-globulin adsorption, plates were emptied and stored empty (0) or containing 250 $\mu$l of PAS gelatin buffer (A) for 20 hours at $4^\circ$C before assessment of the degree of adsorption by liquid scintillation spectrometry. Values represent the mean ± sd dpm (n = 15).

Table 5.4 Comparison of the Adsorption of Caprine $\gamma$-Globulin to Polystyrene and PVC Microtitre Plates from Various Sources

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Plate No.</th>
<th>PVC PLATES</th>
<th>POLYSTYRENE PLATES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dynatech</td>
<td>Falcon</td>
<td>Flow</td>
</tr>
<tr>
<td>Mean</td>
<td>1.</td>
<td>0.847</td>
<td>1.051</td>
</tr>
<tr>
<td>Absorbance (cv%)</td>
<td>4.7</td>
<td>(6.0)</td>
<td>(5.9)</td>
</tr>
<tr>
<td></td>
<td>2.</td>
<td>-</td>
<td>1.230</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>(6.1)</td>
<td>(5.1)</td>
</tr>
</tbody>
</table>

Anti-progesterone $\gamma$-globulin (G711/12) was adsorbed to the wells of two plates of each type and the extent and precision of adsorption to each type determined as for EIA following subsequent incubation with progesterone $11\alpha$-glucuronide-alkaline phosphatase conjugate. Absorbance values represent the mean for 92 wells. = not performed.
mean desorption values ranged from 0.1 to 5.2% of the total adsorbed (table 5.3). The proportion of γ-globulin adsorbed decreased with increasing concentration and at a dilution of 1/16,000 (PAS-gelatin treated wells) was 69.4 ± 1.9% and 84.4 ± 4.1% of the total added for Tween 20 and Decon 90 pre-washed plates respectively (table 5.3). The results were further analysed to establish if there was a difference between adsorption to wells on the edge of the plate compared with those interior. When mean binding for four edge wells was compared for each treatment and antibody dilution with that of the block of 12 adjacent interior wells (e.g. wells 1A - 1D vs the matrix of wells 2B - 2D to 4B - 4D), no significant difference was found for any position.

a) The sources of polystyrene and PVC plates. The degree and precision of adsorption of caprine γ-globulin to various plate types was compared indirectly by incubation with progesterone 11α-glucuronide-alkaline phosphatase conjugate (table 5.4) subsequent to adsorption. Only polystyrene plates manufactured by NUNC produced higher and more precise adsorption than the washed PVC plates: only NUNC plates were available with a manufacturers maximum specification for C.V. for adsorption (not to exceed ± 10%).

5.4 DISCUSSION

The dependence of the adsorption process on the buffer system used as well as the pH and molarity demonstrated the importance of establishing optima if efficient use is to be made of γ-globulin preparations. The literature has revealed little comparable data on this subject.

For solid phase RIA, Crosignani, Nakamura, Hovland and Mishell (1970) showed a similar relationship between buffer molarity and the adsorption of rabbit antiserum to polypropylene discs at pH 9.2 to that found here. An influence of pH, dependent upon the buffer system used, was also demonstrated (maximum adsorption at pH 7.8) although a limited pH range
(7 - 9.8) was investigated.

The study of Oreskes and Singer (1961) was limited to comparison of γ-globulin adsorption (human) at pH's 3.98 and 9.01, making general conclusions regarding pH optima difficult. Adsorption was higher at pH 9.01, close to the isoelectric point of human γ-globulin. They therefore argued that such conditions would be conducive to maximum binding since intermolecular repulsion would be minimized and allow closer packing on the plastic surface.

Isoelectric point determinations were not performed here on sheep and goat γ-globulins and such data were not found in the literature. It would be anticipated, however, that a net positive charge would be induced on γ-globulin from most species at the pH optimum 4 - 5 found here: this may enable coulombic attraction to the net negative charge on the plastic surface (the negative component of the surface electrical double layer; Leininger, Cooper, Epstein, Falb and Grode, 1966; Fuerstenau, 1971) as a preliminary to formation of stronger hydrophobic bonds. The probability is that, at particular pH values, different portions of the γ-globulin or γ-globulin sub-classes will have differing net charges and thus may influence whether, for instance, the $F_c$ or $F_{ab}$ portion of the antibody attaches to the plastic surface. Attachment of the $F_{ab}$ end may result in a subsequent reduction of antigen binding due to steric hinderance.

Incubation at room temperature for 3 hours was found to provide a practical compromise for adsorption of ovine or caprine γ-globulin to large numbers of plates.

Use of 0.17mM acetate coating buffer pH 4 - 5 ensured maximum binding of γ-globulin for both polystyrene and PVC plates and enabled antisera to be used at dilutions (e.g. 1/4000 for G711/12) of the same order as for RIA (1/15,000; see section 2.3.4.). It was clear, therefore, that the carbonate/bicarbonate buffer (pH 9.6) widely adopted and recommended for
ELISA procedures by Voller et al (1976a) is not appropriate for optimal adsorption of all protein preparations. This presumably reflects the lack of comprehensive data published on this subject.

No evidence was found to indicate that differences occurred in the amount of γ-globulin adsorbed to edge wells compared with interior wells. It was therefore concluded that the adsorption achieved for PVC plates was acceptable both in terms of the extent and precision when a Decon 90 pre-wash was used. Subsequent desorption of γ-globulin on storage was minimal, in keeping with the findings of Cantarero et al (1980). The mechanism by which this wash procedure (a standard procedure for glassware, recommended by the detergent manufacturers) induced increased adsorption is not clear. It is possible that putative inhibiting substances are removed from the plastic surface or that trace quantities of detergent remain adsorbed on the plastic and facilitate exposure of hydrophobic regions of the γ-globulin to the plastic surface.

Although it can be seen from table 5.4 that it is necessary to be selective about the type of plates used, it is clear that precision of the order of 5% (cv) can be attained under conditions appropriate to EIA. This was considered acceptable for the current application of EIA of progesterone in milk.

The precision and degree of adsorption was highest with NUNC polystyrene plates: these are γ-irradiated at manufacture and this may be responsible for the apparent enhancement of adsorption compared with other brands of polystyrene plate. Since, additionally, these did not require pre-washing, they were used for the EIA procedure finally adopted (see chapter 7.).

5.5 CONCLUSIONS

1. The highest degree and precision of γ-globulin adsorption was achieved
with NUNC polystyrene plates: although pre-washed Dynatech PVC plates attained a similar degree of precision the total γ-globulin adsorbed was lower.

2. Adsorption of γ-globulin to PVC and polystyrene plates was maximised by use of acetate buffer of low molarity and pH, enabling economic use to be made of valuable antisera.

3. Incubation of microtitre plates above ambient temperature gave rise to marked between-well differences in the time taken for equilibrium temperature to be reached. Differences for PVC plates were minimal when the bases of wells were below the water surface of the water bath.
CHAPTER 6:
INFLUENCES ON MILK PROGESTERONE EIA SENSITIVITY
CHAPTER 6. INFLUENCES ON MILK PROGESTERONE EIA SENSITIVITY

6.1 INTRODUCTION

Practical considerations dictate the sensitivity and working range of calibration curves required for microtitre plate EIA of progesterone. The concentration of progesterone in whole milk ranges from less than 1ng/ml during the follicular phase of the oestrous cycle to 20 - 40ng/ml in the luteal phase or during pregnancy.

The limited capacity of microtitre plate wells (300 - 400μl) dictates that the size of the milk sample assayed must be restricted. A volume of 10μl has commonly been used in RIA (Heap et al, 1976) and this has been used throughout these studies. This indicates a requirement for a limit of detection for EIA of 10pg/well (≈ 1ng/ml) or better.

The majority of applications of EIA for progesterone in milk (see section 1.6) require that samples from cows in the follicular-phase of the oestrous cycle be readily distinguishable from those of the luteal phase or pregnancy. A discriminatory level of 4.5ng/ml milk has been determined by RIA (Holdsworth et al, 1979). Visual classification of samples following EIA requires maximization of the absorbance difference between the end-point for samples containing between 0 - 1ng/ml and those containing 4.5ng/ml.

Two main measures of sensitivity have been used to allow conditions influencing sensitivity to be compared: 1) the $B_{50}/B_0$ value, the quantity of labelled progesterone bound in the presence of 50pg progesterone (10μl of milk containing 5ng/ml) per well ($B_{50}$) compared with that bound in the absence of progesterone ($B_0$) and 2) the $ED_{50}$, the dose of progesterone effective in reducing binding of label in the absence of progesterone ($B_0$) by 50%. The influence of milk on antibody binding of progesterone dictated that EIA performance was in all cases assessed in the presence of ovX milk or with progesterone standards prepared in milk.

The Law of Mass Action dictates that the limit of detection in EIA, as in RIA, is proportional to the mean equilibrium constant of the antibody
population: antisera with the highest avidity for progesterone will thus provide the most sensitive assays. Maximum sensitivity will only be achieved, however, where the binding affinity for progesterone and labelled-progesterone are similar (Ekins et al., 1968).

It has been shown in RIA that equilibrium constants may increase with decreasing temperature (Berson and Yalow, 1959; Abraham, Swerdloff, Tulchinsky and O'Dell, 1971; Malvano and Rolleri, 1975). For this reason, RIAs have traditionally been performed at 4°C: the low molecular weight of steroids ensures that equilibrium is attained within a few hours. Keene et al. (1976) have shown, however, that this increase in equilibrium constant would only be expected where the binding reaction involved a large enthalpy component. This indicates the importance of establishing the appropriate incubation temperature for a given antiserum: clearly, particular antisera may be used at higher temperature without loss of sensitivity and the assay thereby performed more quickly.

It has long been assumed that the binding affinity of antisera for homologous enzyme-labelled steroids are generally higher than those for the free steroid and that assay sensitivity may be reduced as a result: equilibrium constants for the binding of the former have not, however, been determined. The molecular weight of enzyme-labelled progesterone may be 100 - 1000 fold greater than progesterone and this together with the use of solid-phase antibody would be expected to reduce the reaction rate. In the majority of current studies microtitre plate EIAs have been performed at temperatures above 4°C to increase binding rates.

To study assay equilibration times at various temperatures and the influence of incubation time and temperature on assay sensitivity, the time-course of label binding to antibody coated plates in the presence and absence of competing progesterone at various temperatures was investigated. The former was to enable determination of equilibrium constants in subse-
sequent experiments. Conjugates of progesterone 11α-glucuronide with alkaline phosphatase, β-galactosidase and peroxidase were used for this study.

The γ-globulin fraction constitutes about 11% of plasma protein (Armstrong, Budka and Morrison, 1947) and it might be expected that of these only 1% would be specific for a particular antigen (Parsons, 1981). The small surface area of microtitre plate wells (≈ 240mm²) therefore dictates that the antibody fraction be isolated prior to adsorption in order to ensure that specific antibodies constitute a reasonable proportion of the total protein adsorbed. This enables more efficient use to be made of antisera and may allow utilization of previously unusable low titre antisera. Two simple procedures were used here enabling much extraneous protein to be removed; a) absorption of carrier-protein antibodies by titration against the carrier protein and b) isolation of the γ-globulin fraction using ammonium sulphate or Rivanol (see section 2.3.1.). In addition to removal of some γ-globulin which is not specific for progesterone, the absorption process a) has been shown to bring about a substantial reduction in non-specific binding in EIAs using peroxidase as label (Riad-Fahmy, Read and Turkes, 1983). It is not clear whether this applies to other enzyme systems.

The necessity for this absorption process was therefore investigated with alkaline phosphatase conjugates using four anti-progesterone sera from which γ-globulin had been prepared with and without prior absorption of carrier-protein antibodies.

6.2 METHODS

6.2.1. Standard EIA Procedure

PVC microtitre plates (Dynatech) were pre-washed with Decon 90 (see section 5.2.5b) and coated with the γ-globulin fraction (as section 2.1.7.) of the particular antiserum in the manner described previously (section 5.2.2.).
EIA incubation stages were performed in a water bath with the base of the wells immersed 2 - 3mm below the water surface.

Microtitre plates previously coated with antibody were emptied, shaken dry and blotted. Whole milk standards were allowed to come to room temperature and thoroughly mixed before use. Standards (10μl, containing 0 - 5000pg progesterone) were pipetted into replicate wells and conjugate, diluted as appropriate in PAS-gelatin* buffer (200μl), added at ambient temperature. Blank values were determined by addition of buffer alone to duplicate wells containing 10μl of the zero progesterone standard. The plates were covered with a plate lid and incubated at 40°C in a covered water bath for 3 hours before gently agitating, emptying and washing 3 times with PAS-gelatin* buffer (250μl/well). The appropriate substrate solution (see section 2.1.9.) was added to each well (200μl) and the plates incubated for 1 hour at 40°C (20°C for peroxidase) in a water bath. The absorbance of each well was recorded directly at the appropriate wavelength (see section 4.3.5., table 4.2) using an automatic plate reader.

6.2.2. The Characteristics and Selection of the Enzyme-Conjugates Used

All enzyme-steroid conjugates were prepared using a range (minimum 3) of molar excesses of steroid (2 - 250 fold). The progesterone 11α-hemisuccinate and 11α-hemimaleate conjugates with β-galactosidase were prepared using the isolated active-ester procedure (section 3.2.3 ) and the progesterone 11α-glucuronide-peroxidase conjugates were prepared using the mixed anhydride procedure (section 3.2.5.). All other conjugates with penicillinase, alkaline phosphatase, β-galactosidase and peroxidase were

* PMS-gelatin buffer was used for peroxidase conjugates.
produced by the direct active ester procedure (section 3.2.4a). For each type, the conjugate with the degree of steroid incorporation providing maximum sensitivity was selected. Its working dilution was established by screening for sensitivity (B_{50}/B_0) over a range of concentrations (5 - 1000μg conjugate/ml buffer) using microtitre plates coated with serial dilutions of the particular anti-progesterone γ-globulin (at between 1/800 - 1/25,000 dilution of original serum in 1mM or 0.17mM acetate buffer pH 5.0; see section 5.2.2.). EIA was performed as described in section 6.2.1. Since all antisera used in these studies were raised against progesterone 11α-hemisuccinate conjugated to carrier protein (section 2.1.7.) enzyme labels other than progesterone 11α-hemisuccinate-enzyme are termed heterologous.

6.2.3. The Characteristics of the Antiserum and its Condition of Use

a) Binding kinetics and the influence of time and temperature. PVC (Dynatech) microtitre plates, pre-coated with anti-progesterone γ-globulin (G711/12 at 1/8000 dilution, section 5.2.2.), were emptied, tapped dry and refilled with PAS-gelatin (or PMS-gelatin for peroxidase EIA) buffer (200μl) and allowed to attain the required temperature (4°C, 21°C and 40°C) in a water bath or a cold room: a single plate was used for each conjugate at the specified temperature. Buffer was aspirated from triplicate wells at time intervals appropriate to the incubation period required (0 - 26 hours or 0 - 7 days, longest incubation first): following addition of milk standard (10μl, containing 0 - 300pg progesterone) and conjugate (200μl) the wells were re-sealed with plastic film and the incubation period continued. At the end of the study period (26 hours at 40°C or room temperature or 7 days at 4°C), all wells were emptied simultaneously, washed and enzyme activity determined as described (section 6.2.1.).

The long incubation periods at elevated temperatures involved in this
study necessitated an investigation of antibody and enzyme conjugate stability over the relevant periods. There was no significant loss of binding capacity of antibody coated microtitre plate wells following incubation with PAS-gelatin buffer under appropriate conditions of time and temperature (determined following subsequent incubation with progesterone 11α-glucuronide-alkaline phosphatase conjugate in the normal manner). Loss of enzyme activity of the conjugates, whilst appreciable at the longest incubation period at 21°C and 40°C (6 - 11%) was not considered sufficient to influence the outcome of the experiment.

b) The purity of the antibody preparation. γ-Globulin preparations of absorbed and non-absorbed antisera were prepared by the methods outlined in section 2.1.7. and 2.3.1. and on dilution, were equivalent in terms of the original antiserum volume. Absorbed and non-absorbed antisera were tested on the same plate, a different plate being used for each antiserum. Non-specific and specific binding studies (i and ii below) were performed simultaneously on the same plate, using a half plate for each.

i) Non-specific binding. Absorbed and non-absorbed anti-progesterone γ-globulin preparations of G711/12 (1/2,000 dilution), AF18/3 (1/3,000 dilution) HP/S/645111C (1/3,000 dilution) and R54/10/5 (1/3,000 dilution) were adsorbed onto microtitre plate wells (3 rows of 8 wells for each preparation). Alkaline phosphatase (10, 20 or 40ng in 200μl of PAS-gelatin buffer) was added to 6 (2 blank wells in the row) or 8 wells for each antibody preparation and the plates treated as for EIA (see section 6.2.1.).

ii) Specific binding. Absorbed or non-absorbed anti-progesterone γ-globulin preparations of G711/12 (1/2,000, 1/4,000 and 1/8,000 dilution), AF18/3, HP/S/645111C and R54/10/5 (each at 1/3,000, 1/6,000 and 1/9,000 dilution) were adsorbed onto plate wells (n = 8 for each preparation and dilution). Alkaline phosphatase conjugate (prepared with a 50:1 molar excess of steroid) was added to each well (10ng/200μl) and the plates treated as for EIA.
For both i) and ii) the incubations to determine enzyme activity were performed for 30 minutes or in one case (R54/10/5 antiserum), due to high binding activity, for 12 minutes.

6.3 RESULTS

6.3.1. The Method of Producing Enzyme-Progesterone Conjugates

The necessity for using efficient condensation reactions such as the "active ester" procedure which apparently minimizes formation of enzyme-enzyme conjugates or enzyme denaturation has been discussed in chapter 3.

In addition to circumventing the formation of denatured conjugated enzyme it is necessary to ensure that the enzyme-labelled progesterone is free from unconjugated progesterone derivative since this will reduce assay sensitivity. For all conjugates described here this has been achieved by extensive dialysis (3 - 4 changes of buffer) and subsequent gel filtration. Although it was still possible to remove free progesterone derivatives with further dialysis, the quantity detected by RIA in the dialysate indicated that this would be unlikely to interfere with the performance of the EIA at the working dilution of the conjugate. This suggests that the free progesterone derivative might bind to the enzyme to some extent and this limits its rate of elimination during gel filtration and dialysis.

Simpson and Wright (1977) have reported that dialysis is less effective at removal of free steroid from protein conjugates than gel filtration, possibly due to reduced membrane permeability to hydrophobic molecules. The use of extensive gel filtration rather than dialysis may therefore be appropriate.

6.3.2. Heterologous Systems and the Influence of the Enzyme and the Antiserum

As predicted by other studies (van Weemen and Schuurs, 1975) antisera derived using progesterone 11α-hemisuccinate-BSA as immunogen gave rise to
Fig. 6.1 Comparison of EIA calibration curves using homologous and heterologous conjugates. Calibration curves produced using homologous progesterone 11α-hemisuccinate conjugates with β-galactosidase (●-●) are compared with heterologous progesterone 11α-hemimaleate (○-○), progesterone 3-carboxymethylxoxime (□-□) and progesterone 11α-glucuronide (■-■) conjugates. Values are mean ± sd of four determinations. Conjugates were prepared using a 25 molar (A) or 250 molar (B; 200 molar for hemimaleate) excess of steroid. γ-Globulin from antiserum G711/12 was used at 1/2000 dilution: the mass of conjugates used are detailed in Table 6.1.

* Excluded from calibration curve mean cv determinations (see text) since this value was within 2sd of the mean substrate blank.

Table 6.1 The Influence of Site and Bridge Heterology and the Degree of Steroid Incorporation on the Binding of Progesterone-β-Galactosidase Conjugates to Anti-Progesterone 11α-Hemisuccinate γ-Globulin

<table>
<thead>
<tr>
<th>Conjugate No.</th>
<th>Bridge Position and Type</th>
<th>Molar Ratio Steroid:Enzyme at Conjugation</th>
<th>Mass Conjugate used in EIA (ng/well)</th>
<th>B₀ Value Absorbance Units</th>
<th>ED₅₀ pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>104</td>
<td>11α-hemisuccinate</td>
<td>25:1</td>
<td>12</td>
<td>0.320</td>
<td>875</td>
</tr>
<tr>
<td>105</td>
<td>11α-hemisuccinate</td>
<td>250:1</td>
<td>10</td>
<td>0.487</td>
<td>1220</td>
</tr>
<tr>
<td>101</td>
<td>11α-hemimaleate</td>
<td>25:1</td>
<td>21</td>
<td>0.389</td>
<td>380</td>
</tr>
<tr>
<td>90</td>
<td>11α-hemimaleate</td>
<td>200:1</td>
<td>40</td>
<td>0.412</td>
<td>216</td>
</tr>
<tr>
<td>110</td>
<td>3-carboxymethylxoxime</td>
<td>25:1</td>
<td>136</td>
<td>0.217</td>
<td>100</td>
</tr>
<tr>
<td>111</td>
<td>3-carboxymethylxoxime</td>
<td>250:1</td>
<td>11</td>
<td>0.247</td>
<td>230</td>
</tr>
<tr>
<td>114</td>
<td>11α-glucuronide</td>
<td>25:1</td>
<td>102</td>
<td>0.109</td>
<td>56</td>
</tr>
<tr>
<td>115</td>
<td>11α-glucuronide</td>
<td>250:1</td>
<td>10</td>
<td>0.156</td>
<td>87</td>
</tr>
</tbody>
</table>

EIA was performed in the manner described in Fig. 6.1 and ED₅₀ values interpolated from the calibration curves.

ED₅₀ = dose of progesterone effective in reducing label binding by 50%. 
Insensitive EIA calibration curves when used with homologous (progesterone 11α-hemisuccinate) enzyme conjugates (section 3.3.2.), presumably as a result of bridge recognition (Fig. 6.1). The maximum progesterone concentration anticipated in cow's milk during the luteal phase or pregnancy (about 40ng/ml, 400pg/10μl) would only reduce conjugate binding by 40% (Fig. 6.1a; progesterone 11α-hemisuccinate conjugate). Furthermore, only one of several antisera tested (G711/12) provided sufficient sensitivity to be useful in EIA (Table 6.2). The properties of enzymes conjugated with four progesterone derivatives (structures shown in Fig. 6.2) were therefore compared to assess the ability of heterologous systems to improve assay sensitivity (Fig. 6.1) and to reduce reliance of EIA on the availability of exceptional antisera (Table 6.2).

Fig. 6.2 Comparison of structures of progesterone derivatives used to form enzyme conjugates: A, progesterone 11α-hemisuccinate; B, progesterone 11α-hemimaleate; C, progesterone 3-carboxymethyloxime and D, progesterone 11α-glucuronide.

The different slopes and ED$_{50}$ values of calibration curves using bridge (hemimaleate or glucuronide) or bridge and site heterology (3-carboxymethyl-oxime) are demonstrated in Fig. 6.1 and Table 6.1. Since coefficients of variation (cv) throughout the calibration curves fell within the same range (mean cv's 3.6% - 5.8%) it is clear that with this antiserum (G711/12),
Table 6.2 The Influence of Heterology, the Enzyme Label and the Antiserum on the Degree of Label Binding ($B_Q$) and Assay Sensitivity ($B_{50}/B_0$)

<table>
<thead>
<tr>
<th>BRIDGE</th>
<th>5-GALACTOSIDASE</th>
<th>ALKALINE PHOSPHATASE</th>
<th>PEROXIDASE</th>
<th>PENICILLINASE</th>
<th>($^3$H) PROGESTERONE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HS</td>
<td>GLU</td>
<td>HS</td>
<td>GLU</td>
<td>HS</td>
</tr>
<tr>
<td>Mass Label/Well (ng)</td>
<td>6.4</td>
<td>33</td>
<td>21</td>
<td>136</td>
<td>5</td>
</tr>
<tr>
<td>ANTISERUM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R54/10/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$B_0$</td>
<td>0.323</td>
<td>0.197</td>
<td>0.588</td>
<td>0.280</td>
<td>0.729</td>
</tr>
<tr>
<td>$B_{50}/B_0$ (%)</td>
<td>94</td>
<td>86</td>
<td>105</td>
<td>87</td>
<td>94</td>
</tr>
<tr>
<td>HP/66/45111C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$B_0$</td>
<td>0.352</td>
<td>0.137</td>
<td>1.180</td>
<td>0.172</td>
<td>0.822</td>
</tr>
<tr>
<td>$B_{50}/B_0$ (%)</td>
<td>103</td>
<td>84</td>
<td>104</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>AF18/3</td>
<td></td>
<td></td>
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<tr>
<td>$B_0$</td>
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<td>0.196</td>
<td>0.684</td>
<td>0.132</td>
<td>0.838</td>
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<td>64</td>
<td>91</td>
<td>83</td>
<td>91</td>
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<tr>
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<td>0.276</td>
<td>0.205</td>
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<td>$B_{50}/B_0$ (%)</td>
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<td>S150/16</td>
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<td>$B_{50}/B_0$ (%)</td>
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<td>-</td>
<td>83</td>
<td>90</td>
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<td>S263/10</td>
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<td></td>
</tr>
<tr>
<td>$B_0$</td>
<td>-</td>
<td>0.126</td>
<td>-</td>
<td>0.165</td>
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<td>$B_{50}/B_0$ (%)</td>
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<td>-</td>
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<td>91</td>
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<tr>
<td>S263/10</td>
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<td></td>
</tr>
<tr>
<td>$B_0$</td>
<td>-</td>
<td>0.247</td>
<td>-</td>
<td>0.398</td>
<td>0.999</td>
</tr>
<tr>
<td>$B_{50}/B_0$ (%)</td>
<td>-</td>
<td>83</td>
<td>-</td>
<td>79</td>
<td>88</td>
</tr>
</tbody>
</table>

EIA was performed at 40°C (section 6.2.1.)

Data derived in the manner described in section 6.2.2: $B_0$ (optical density) values are mean ± sd of 4 determinations.

$B_0$ values were normalized to $A$ optical density/h. For RIA, $B_0$ represents % of total label bound.

HS, GLU, HM and CMO signify bridge types 11α-hemisuccinate, 11α-glucuronide, 11α-hemimaleate and 3(0-carboxymethyl)oxime.

- not tested.
assay sensitivity was improved through use of heterology in the order glucuronide > 3-carboxymethyloxime > hemimaleate > hemisuccinate. With the exception of the hemimaleate conjugate, greater assay sensitivity was achieved with conjugates prepared with a 25 rather than a 250 molar excess of steroid. Conjugates produced using the lower excess necessitated use of a greater quantity of conjugate to provide an equivalent degree of binding (table 6.1). Comparison of ED\textsubscript{50} values provided by hemisuccinate and glucuronide-linked conjugates emphasize the improvement in sensitivity obtained irrespective of the steroid:enzyme ratio used at conjugation: hemisuccinate conjugates required at least ten times the quantity of competing progesterone to provide an equivalent drop in label binding.

Although these results clearly demonstrate the ability of the glucuronide bridge to substantially improve assay sensitivity, the general applicability of this approach would require that the conjugates be tested with several antisera.

Progesterone derivatives conjugated with β-galactosidase, alkaline phosphatase, peroxidase and penicillinase were examined by EIA using seven antisera raised against progesterone II\textsubscript{HSA}-hemisuccinate conjugated with BSA or ovalbumin (see section 2.1.7.). The sensitivity (B\textsubscript{50}/B\textsubscript{0} values) for conjugates of each type at the dilution of γ-globulin providing maximum sensitivity are shown in table 6.2. A minimum B\textsubscript{0} value of 0.10 OD was required to ensure that discrimination was maintained between the B\textsubscript{50} value and the substrate blank. Irrespective of the enzyme label, the glucuronide conjugates provided sensitivity comparable with or often better than that of RIA. The exceptions to this were antisera R54/10/5 and HP/S/645IIIC which gave low sensitivity in RIA and poor sensitivity in EIA.

β-Galactosidase was the only enzyme conjugated with each of the progesterone derivatives: bridge recognition was least evident with the glucuronide conjugate, irrespective of the antiserum used. Glucuronide-
linked conjugates of peroxidase and alkaline phosphatase likewise gave
greater sensitivity than the homologous conjugates.

6.3.3. The Molar Ratio of Steroid:Enzyme Required at Conjugation and the
Mass of Conjugate Required for EIA

The data in Table 6.2 were derived following screening of conjugates
produced using steroid molar excesses ranging from 2:1 to 250:1 (see section
6.2.2.). In each case the conjugate selected was that giving rise to the
greatest sensitivity: the optimum steroid:enzyme ratios at conjugation
varied widely. It was clear, therefore, that the optimum excess of a
particular derivative required at conjugation was largely dependent on the
enzyme and to a lesser extent on the derivative used.

A smaller mass of homologous conjugate was generally required to pro-
duce similar or greater label binding than the heterologous equivalent
and was associated with lower apparent binding affinity for the latter.
In the case of the progesterone 11a-glucuronide conjugates of peroxidase
and β-galactosidase, a large excess of steroid was required to produce con-
jugates giving an acceptable B₀ without requiring large (microgramme)
quantities of conjugate.

For all conjugates there was an inverse relationship between assay
sensitivity and both the molar excess of steroid used to form the conjugate
and the mass of conjugate required for EIA. This is demonstrated by data
derived using progesterone 11α-glucuronide-alkaline phosphatase conjugates
(Fig. 6.3).

6.3.4. The Characteristics of the Antiserum and its Conditions of Use

a) Binding kinetics and the influence of time and temperature. Fig. 6.4
shows label binding in the absence of competing progesterone over a period
of 0.5 hours to 26 hours at 21°C and 40°C and 4 hours to 7 days at 4°C.
Fig. 6.3 The influence of quantity of conjugate and degree of conjugation on the sensitivity of microtitre plate EIA for progesterone in whole milk.

Microtitre plates were coated with anti-progesterone γ-globulin (G711/12 at 1/4000 dilution) and EIA performed using a 3 hour incubation at 40°C. Progesterone 11α-glucuronide was conjugated with alkaline phosphatase using a molar steroid excess of 2:1 (▲-▲), 10:1 (■-■) or 50:1 (●-●) at conjugation. Sensitivity was expressed as $\frac{B_{50}}{B_0}$ (%). Values are mean ± sd of 3 determinations.

Binding appeared to be biphasic, with a rapid initial uptake of label.

There was, however, no consistent indication that equilibrium had been attained after the longest incubation period; the possible consequences of this in EIA are discussed in section 7.4. The question of determination of equilibrium constants was not therefore pursued further.

Assay sensitivities at different incubation times and temperatures are shown in Fig. 6.5. Evidence for an effect of incubation period on assay sensitivity was only seen for assays using peroxidase label, in which sensitivity apparently decreased with increasing time. This difference may arise as a result of the impractically low degree of binding attained (compare absorbance values in Fig. 6.4).
Fig. 6.4 The binding kinetics at 4°C (□-□), 21°C (▲-▲) or 40°C (○-○) of progesterone 11α-glucuronide conjugated with a) β-galactosidase (10ng/well), b) alkaline phosphatase (10ng/well) and c) peroxidase (50ng/well) to anti-progesterone γ-globulin adsorbed on PVC microtitre plates: conjugates were prepared with a 50:1, 2:1 and 125:1 molar excess of steroid respectively. Optical density values are the mean of 3 determinations and represent binding in the absence of competing progesterone.

Fig. 6.5 The influence of incubation time and temperature (4°C, □-□; 21°C, ▲-▲; 40°C o-o) on the sensitivity of EIA (B/B₀) using a) β-galactosidase b) alkaline phosphatase and c) peroxidase as label. Conjugates were prepared as described in Fig. 6.4. Values are the mean ± sd of three determinations.
For alkaline phosphatase and β-galactosidase labels the sensitivity was greatest at 4°C and lowest at 40°C irrespective of the period of incubation (Fig. 6.5): peroxidase label was not tested at 4°C. At 4°C, however, unacceptably long incubation periods were required to obtain a satisfactory degree of binding (Fig. 6.4). Performance of the EIA at room temperature (21°C) represented a compromise between the two and enabled good sensitivity (Fig. 6.5) and an acceptable degree of binding (Fig. 6.4) to be achieved within a reasonable incubation period (3 hours) and indicated the possibility of performing the assay without the use of an incubator or water bath.

b) The purity of the antibody preparation. The total recovery of progesterone antibodies following absorption and preparation of the γ-globulin fraction was evaluated through their ability to bind 3H-labelled progesterone in RIA and following adsorption to plate wells, alkaline phosphatase-labelled progesterone 11α-glucuronide in EIA. Non-specific binding was assessed by measuring binding of unconjugated alkaline phosphatase to anti-progesterone γ-globulin. RIA indicated that loss of specific antibody during absorption was minimal since there was no significant difference between antibody dilution curves obtained using absorbed and non-absorbed γ-globulin.

Binding of alkaline phosphatase (10ng) to protein-absorbed anti-progesterone γ-globulin (Fig. 6.6) and expressed as % of specific binding (Fig. 6.7) of alkaline phosphatase conjugate (10ng) was low for all preparations (G711/12, 3.5%; AF18/3, 5.4%; HP/S/645IIIC, 3.4% and R54/10/5, 2.1%) but generally higher than for non-absorbed γ-globulin preparations (G711/12, 2.4%; AF18/3, 3.4%; HP/S/645IIIC, 2.6% and R54/10/5, 1.5%). Non-specific binding values (%) were corrected to account for a 33% difference between the specific activity of the conjugate and the subsequently purchased unconjugated enzyme.

6.3.5. The Choice of Enzyme as Label

The enzyme assay optimization data detailed in section 4.3 (table 4.3)
Fig. 6.6 Binding of unconjugated alkaline phosphatase (non-specific binding) to absorbed (△-△) and non-absorbed (○-○) γ-globulin preparations. Values represent means ± sd optical density (n = 8 or, when 10ng enzyme was used, n = 6).

The significance of differences between non-specific binding (expressed as % of specific binding of conjugated alkaline phosphatase at the same antibody dilution, from Fig. 6.7) for protein absorbed and non-absorbed antisera was calculated for wells containing 10ng enzyme. 1 = t significant, p < 0.01; 2 = t significant, p < 0.05; 3 = F significant, p < 0.01; 4 = F significant p < 0.005.

Fig. 6.7 Binding of progesterone 11-γ-glucuronide-alkaline phosphatase conjugate (10ng) to protein absorbed (△-△) and non-absorbed (○-○) γ-globulin preparations. Values represent mean ± sd optical density (n = 8).
demonstrated that the sensitivity of detection of the enzymes is in the order penicillinase > peroxidase > alkaline phosphatase > β-galactosidase. Using data derived from EIA using enzyme conjugates with progesterone 11α-glucuronide (table 6.2), it is clear that for particular antisera penicillinase generally produced the highest sensitivity (lowest $B_{50}/B_0$ values) and β-galactosidase the lowest sensitivity. Alkaline phosphatase and peroxidase performed in a similar manner to each other. The generally poor sensitivity obtained using conjugates with other progesterone derivatives indicated that comparisons between enzymes using these conjugates would be of little interest.

6.4 DISCUSSION

The problems of achieving the maximum sensitivity possible with a particular antiserum owing to bridge recognition were introduced in a previous section (1.9.2.). Although only limited investigations have been made in this area in the context of EIA of progesterone (Joyce et al, 1978b; Gros et al, 1978), research demonstrating its importance in attaining sensitive RIA's using $^{125}$I-labelling has been extensive (Cameron et al, 1974; Allen and Redshaw, 1978; Corrie et al, 1982). Similarly, in the EIA of oestrogens (van Weemen and Schuurs, 1975 and 1976; Exley and Abuknesha, 1977), corticosteroids (Arakawa et al, 1979; Hosoda, Kobayashi, Miyairi and Nambara, 1981; Hosoda, Kobayashi and Nambara, 1983) and testosterone (Bosch et al, 1978a; Hosada, Yoshida, Sakai, Miyairi and Nambara, 1980) this phenomenon has been widely investigated although no hard and fast rules relating to the general applicability of heterologous combinations has emerged.

Despite this problem, anti-progesterone sera have been produced which have either provided sensitive homologous assays or enabled specific heterologous combinations to be effective (see Riad-Fahmy et al, 1981): there have, however, been no investigations published demonstrating the suitability
of particular EIA systems for more than one antiserum. The results presented here indicate the suitability of the \(11\alpha\)-hemisuccinate (immunogen bridge)/\(11\alpha\)-glucuronide (label bridge) heterologous combination for the EIA of progesterone. This confirms the findings of Corrie et al (1981b) who used the same combination in an RIA using \(^{125}\)I-labelled progesterone \(11\alpha\)-glucuronide-tyramine.

Prior preliminary investigations by Gros et al (1978) indicated that improvements in progesterone EIA sensitivity may be achieved using the \(11\alpha\)-hemimaleate bridge. Although their findings were confirmed here, the increase in sensitivity achieved was relatively small and only applied to a few of the antisera tested: Joyce et al (1978b) reported minimal details of their investigations of bridge and site heterology but indicated that use of hemiphthalate, dimethylsuccinate and hexahydrohemiphthalate bridges only gave rise to minor improvements in sensitivity. This group have reported the use of sensitive homologous EIA procedures (Joyce et al, 1981) but their method implies that a pre-incubation period was required, as with the heterologous procedure of Gros et al (1978), prior to addition of label in order to attain the required sensitivity: no comparison was made between EIA and RIA calibration curves. The more recent report of Munro and Stabenfeldt (1984) confirms the finding (Fig. 6.1) that the carboxymethylxoxime bridge could be usefully employed.

Van de Wiel and Koops (1982) have provided the only example of a highly sensitive heterologous EIA using antibody raised against a progesterone derivative (7\(\alpha\)-hydroxyprogesterone-7-carboxyethylthioether) other than progesterone \(11\alpha\)-hemisuccinate. This was used in conjunction with \(6\beta\)-hydroxyprogesterone-6-hemisuccinate-peroxidase.

It is not clear why the glucuronide bridge is recognized less than others by the antisera tested: being cyclic and relatively hydrophilic in nature, many structural dissimilarities to the hemisuccinate are apparent. The RIA
work of Corrie, Ratcliffe and Macpherson (1982) indicates that branching of the bridge in close proximity to the steroid may be responsible. In an investigation of twelve bridge-heterologous EIA systems for 11-deoxycortisol, Hosoda et al (1983) concluded that an important characteristic for sensitivity was that the enzyme label bridge should be shorter than that of the immunogen: EIA's lacked sensitivity if longer bridges were used. The former relationship would also apply to the hemisuccinate/glucuronide combination. Of possibly greater importance in the present study are the characteristic groups near the steroid structure. The hemimaleate bridge was well recognized by the antisera and has an ester link, common to the immunogen, for attachment to the steroid: in contrast, the glucuronide is joined by a hemiacetal group (see Fig. 3.2). This view is supported by the findings of Nordblom, Counsell and England (1984) who employed an ether link for the immunogen and an ester for the 125I-label in a bridge heterologous (position 11β) RIA for androstenedione.

Differences in the degree of incorporation of steroid may make some small contribution to differences in sensitivity seen between different heterologous conjugates. Since it was not possible to accurately determine incorporation for the heterologous conjugates, however, no conclusions could be drawn on this point: radio-labelled derivatives were not available and spectrophotometric determinations (see section 3.2.6c) or estimations of unconjugated ε-amino residues (Fields, 1971) were found to be insufficiently reliable to detect small differences in incorporation.

The inverse relationship between assay sensitivity and both the relative degree of incorporation of steroid per enzyme and the mass of conjugate used in the assay (Fig. 6.3) are to be wholly expected since increasing either the mass of conjugate or its degree of derivitization will increase the relative probability of label interaction with the antibody. As a consequence, a high concentration of unlabelled progesterone would be required to
give an equivalent degree of binding inhibition. The former relationship has been a common finding in both RIA (Berson and Yalow, 1968) and EIA (Turkes, Turkes, Joyce, Read and Riad-Fahmy, 1979; Van de Wiel, Kamonpatana, Ngramsurijaroy, Koops and Singhajan, 1982). The increased sensitivity achieved with dilution is restricted by the ability to detect the label and was a limiting factor for many of the conjugates assessed using \( B_{50}/B_0 \) estimates.

The limit of detection of the assay is restrained in a more fundamental way by the equilibrium constant of the particular antiserum and in theory is proportional to the concentration \( 1/K \) (Ekins, 1976). The significance of this in EIA remains obscure since equilibrium constants have not been reported for such applications elsewhere. They could not be determined here because the study performed (Fig. 6.4) failed to demonstrate that equilibrium had been attained.

Clearly, the speed with which a non-equilibrium assay may be performed (see section 6.3.4.) will be dictated in part by the time taken for a detectable degree of label binding to be achieved over the range of analyte concentrations. Thus, although faster assays may be achieved either by increasing assay temperature, the mass of conjugate used or the molar incorporation ratio this would be at the sacrifice of sensitivity.

Fig. 6.8 describes events which may explain the slow nature of conjugate binding to antibody in EIA in the presence of milk. Initially, the conjugate would have ready access to antibody binding sites but enzymes, such as alkaline phosphatase, being membrane associated (see Renner, 1983) and having steroid moieties attached, would be predisposed to incorporation into the fat globule: the fat globules would, in due course, rise to the surface of the medium to form a lipid layer apart from the antibody. Similar events would occur with free progesterone but its faster diffusion rate would enable more rapid binding with antibodies before separation into the lipid
The issue is further complicated by the association of progesterone with milk proteins (Heap et al., 1975; Pope et al., 1976).

Fig. 6.8 Schematic representation of equilibria probably occurring during microtitre plate EIA of progesterone in milk. See text for further explanations.

- = progesterone molecules; O = enzyme-progesterone conjugate; □ = fat globule;

□□□□ = lipid layer; = antibody molecule; = wall of microtitre plate well.

Non-specific binding was found to be low (≤ 5%) for the alkaline phosphatase EIA: the small changes caused by carrier-protein absorption of the four antisera were of no practical significance (Fig. 6.6). Further studies (see section 7.3.4c) indicated that the degree of non-specific binding of alkaline phosphatase to anti-progesterone γ-globulin was of a similar order to binding of conjugate to "normal" γ-globulin. These findings contrast with the data of Riad-Fahmy et al. (1983) who found that absorption reduced non-specific binding of progesterone 11α-hemisuccinate-peroxidase to solid phase antibody preparations by 90% or more to levels similar to those observed here with or without absorption: their findings run contrary to accepted views concerning antibody specificity and remain unexplained.
It would be anticipated that further improvements in assay performance may be attained by use of affinity purified antibody preparations as suggested by Ishikawa, Imagawa, Yoshitake et al (1982) since this should enable isolation of the specific antibody population. Limited investigation of this approach were unsuccessful, however, due to continuous leakage of free steroid from the immunoadsorbent (progesterone 11α-hemisuccinate or progesterone 11α-hemimaleate linked to Sepharose).

The highest sensitivity with antiserum G711/12 was achieved by performing EIA at 4°C (Fig. 6.5). Incubation at 21°C (ambient temperature) proved more practical, however, and provided adequate sensitivity. Improved EIA sensitivity at reduced incubation temperature would similarly be anticipated for other conjugate/antibody combinations investigated and described in table 6.2. In the original homologous EIA (Fig. 3.3) a high incubation temperature (40°C) was required to ensure adequate binding of enzyme label to antibody-coated microtitre plates, the binding rate being temperature dependent. The use of ambient temperature incubations subsequently became a practicable proposition when optimized procedures increased adsorption of antibody to microtitre plate wells (see chapter 5). These studies also revealed that under the majority of conditions the EIA was a non-equilibrium procedure: the literature implies, by omission, that hapten EIAs will attain equilibrium within a similar period to RIA. Consideration of the size of the label alone, however, would argue against this.

Two factors contribute to the differing EIA sensitivities achieved for the four enzymes tested; the detection limit and the molecular weight of the enzymes (30,000, 40,000, 100,000 and 540,000 Daltons for penicillinase, peroxidase, alkaline phosphatase and β-galactosidase, respectively). Since diffusion rate is inversely proportioned to molecular weight (Graham's Law) it would be expected that the enzyme-conjugate of lowest molecular weight would
give rise to the highest proportion of label bound to antibody per unit time. Together, these factors would allow a smaller mass of label to be used in EIA and thus greater sensitivity to be achieved. The high molecular weight and lower specific activity of β-galactosidase conjugates may explain the necessity to use a relatively large mass in EIA (Table 6.2). The findings using the heterologous peroxidase conjugates may appear contrary to this connection between sensitivity and enzyme molecular weight/detection limit: the large mass of conjugate required in this case (Table 6.2) must, however, be viewed in the light of its apparently poor immunoreactivity which may have resulted from inefficient coupling (see section 3.2.5.). For this reason and because of the potentially mutagenic or carcinogenic nature of the chromogens used for its determination (Voogt et al., 1980; Yolken, 1982) peroxidase was not considered further.

Progesterone 11α-glucuronide-alkaline phosphatase conjugate provided more than adequate sensitivity and was used in all further validation studies of the milk progesterone EIA. This was largely because the assay was simpler to perform than that using the more sensitive penicillinase conjugates. Penicillinase may, however, prove to be well suited for assays where greater sensitivity is a pre-requisite: obviously the availability of a suitable chromogenic substrate would greatly facilitate its application.

6.5 CONCLUSIONS

1. The heterologous combination of 11α-hemisuccinate (immunogen bridge)/11α-glucuronide (label bridge) greatly reduced the problem of bridge recognition and several antisera provided EIA's of comparable sensitivity to RIA.

2. Performance of EIA at low temperature (4°C) gave rise to highest sensitivity but incubation at ambient temperature (21°C) proved more practical and provided sufficient sensitivity. Binding equilibrium was not attained under the conditions investigated.
3. Convenience and high sensitivity of detection recommended alkaline phosphatase as the label for further development and EIA validation, although penicillinase provided greater sensitivity.
CHAPTER 7:
THE ADOPTED HETEROLOGOUS EIA:
THE VALIDATION OF EIA USING
PROGESTERONE 11α-GLUCURONIDE-ALKALINE PHOSPHATASE AS LABEL
CHAPTER 7. THE ADOPTED HETEROLOGOUS EIA: THE VALIDATION OF EIA USING PROGESTERONE 11α-GLUCURONIDE-ALKALINE PHOSPHATASE AS LABEL

7.1 INTRODUCTION

Two of the microtitre plate EIA procedures described in the previous chapter were applied to the measurement of progesterone in milk samples. The first was an homologous assay using the 11α-hemisuccinate bridge between the steroid and protein in both the immunogen and the enzyme (β-galactosidase)-labelled steroid. This assay lacked the sensitivity and precision required for widespread use and so is not further considered in this chapter: it did, however, provide a useful means of evaluating the practicality of EIA for routine assessment of reproductive status in the dairy cow. This aspect is considered in chapter 8.

The second procedure, an heterologous assay, was developed to overcome these shortcomings and employed progesterone 11α-glucuronide-alkaline phosphatase as label: the full validation of this assay is described here.

Direct RIA of progesterone in milk is widely accepted for monitoring luteal functions in dairy cattle. Although it provides a useful reference method against which direct EIA can be judged, there is little published data relating specifically to its validation. Generally accepted validation procedures, including establishing recovery of added progesterone, linearity of response on dilution of the sample and comparison with a method involving isolation of the analyte prior to assay have not all been performed in any single study. Investigation of recovery and linearity of response have involved small numbers of samples and so provide no clear indications of between-sample variation in non-specific effects noted in some laboratories (Heap et al., 1976, Pennington et al., 1976; Holdsworth et al., 1979; Stevens et al., 1981): comparative studies involving chromatographic isolation of progesterone prior to RIA have not been reported.

It is a well established principle of assay validation that estimations
by new procedures should be compared with an established method, this preferably being based on a different principle of analysis. For steroid immunoassays, it has been suggested that the combined procedures of gas chromatography and mass spectrometry (GC/MS) attain suitable specificity and sensitivity to provide a reference method (Gaskell, Brownsey and Groom, 1984) although such facilities are only available in highly specialised laboratories.

A practical alternative reference procedure to GC/MS was developed for the assay of progesterone in milk. This involved the extraction of steroids from milk and the use of HPLC to separate progesterone in these extracts from steroids, especially those of similar chemical structure to progesterone. RIA, using antiserum of high and known specificity, allowed quantitation of the isolated progesterone. The procedure enabled combination of the specificity of HPLC and RIA without loss of sensitivity and comparison of results with those obtained by EIA.

Methods have been described for the extraction of progesterone from whole milk by partitioning into organic solvents including petroleum spirit (Hoffmann and Hamburger, 1973) or diethyl ether (Heap et al, 1975; Nuti et al, 1975): to avoid gel formation pre-treatment with ethanol (95%) may also be used to precipitate protein prior to extraction (Estergreen et al, 1977). These methods were assessed initially and compared with a solid phase partition procedure using octadecylsilane-bonded silica (ODS). ODS has been applied to the extraction of steroids from urine (Shackleton and Whitney, 1980; Heikkinen, Fotsis and Adlercreutz, 1981) and plasma (Heikkinen et al, 1981; Cannel, Galligan, Mortimer and Thomas, 1982) but only a model outline procedure has been described for its use in the extraction of milk (Axelson and Sahlberg, 1981). ODS extraction offers the possibility of achieving a high degree of concentration of steroids from milk without the requirement for large volumes of solvent.
ODS extraction was used by Axelson and Sahlberg (1981) to achieve efficient recovery of steroids (92% for progesterone) and their conjugated metabolites from large volumes of milk (2 - 5ml). The procedure was simplified to facilitate the handling of large numbers of samples with minimal processing and without the use of elaborate reservoirs to maintain sample temperature (64°C) during extraction.

Although there are much published data regarding the separation of progesterone from broad groups of steroids by HPLC (Allenmark, Sonberg, Hammar and Lindström, 1981; Walters, Foster and Cottrell, 1981; Kessler, 1982; Sugara, Yamanaka, Takeda and Tsuruta, 1982; Huang, Ke, Hwang and Lo, 1983; Eibs and Schoneshofer, 1984) there are relatively few which demonstrate resolution of closely related steroids (Lin, Heftman and Hunter, 1980; Purdy et al, 1980; Purdy, Durocher, Moore and Rao, 1981): this is necessary to ensure the specificity essential to this application. Purdy et al (1980; 1981) described the separation of ten pregnane and pregnene derivatives by gradient elution using a Chromegabond Diol (silica with bonded dihydroxylated carbon chains) column but the system failed to resolve 5β-pregnane-3, 20-dione from progesterone. Using a silica column with isocratic elution (0.2% ethanol in dichloromethane) good resolution was shown between 5α and 5β-pregnane-3, 20-dione and progesterone although other steroids were not tested (Purdy et al, 1981). The isocratic system described by Lin et al (1980) used a silica column which provided excellent resolution of progesterone from eight closely related steroids and formed the starting point for the method developed here.

7.2 MATERIALS AND METHODS

7.2.1. Extraction and Isolation of Progesterone from Milk

a) Milk samples. Milk from an ovariectomized cow (ovX milk) was used in the development of solvent and ODS extraction procedures following addition
of progesterone (to concentrations of 0 - 50ng/ml) as previously described (section 2.1.10). For recovery purposes \(^3\text{H}\)progesterone (0.9ng in 50\(\mu\)l methanol: \(5 \times 10^5\) dpm) was added to aliquots (15ml) of these and all samples used in subsequent HPLC studies (section 7.3.4h) and allowed to equilibrate at 4\(^\circ\)C overnight before use.

\textit{b) Solvents.} All solvents were of analytical reagent grade with the exception of dichloromethane (HPLC grade). HPLC mobile phases were degassed before use by sonication (Sonicleaner Type 6441, Dawe Instruments Ltd., London) under reduced pressure.

c) \textit{HPLC equipment.} System A comprised an ACS 351 pump and a Pye Unicam PU 4020 (8\(\mu\)l flow cell) UV detector (Applied Chromatography Supplies, Luton) and System B, a Spectroflow 400 pump and 757 (12\(\mu\)l flow cell) UV detector (Kratos Instruments Ltd., Manchester). Samples were introduced either via a Rheodyne 7125 (20\(\mu\)l sample loop) or, when column backflushing was used, a Valco C10U (100\(\mu\)l sample loop) injection valve (Alltech Associates, Carnforth). The 5\(\mu\)m Partisil columns (300 x 2mm) used in preliminary studies were packed by Mr. L. Howells (Central Veterinary Laboratory, Weybridge). The two columns (125 x 5mm) adopted for later use were packed with 3\(\mu\)m spherical silica and performed in a near identical manner: the RoSiL (Alltech Associates) column was packed by Mr. L. Howells and the Hypersil column was packed commercially (Hichrom Ltd., Reading).

Elution of steroids from the column was monitored at 280nm with the aid of a potentiometric chart recorder (Servoscribe RE 541.2, Smith Industries Ltd). Where appropriate, precise retention times were determined using an integrator/plotter (CI-10B LDC Milton Roy, Stone) to monitor the detector signal (minimum time interval registered 0.01min).

d) \textit{Extraction into organic solvents.} Aliquots (200\(\mu\)l) of ovX milk containing \(^3\text{H}\)progesterone were dispensed in triplicate into glass test tubes (4ml capacity), petroleum spirit (40\(^\circ\) - 60\(^\circ\)C b.p.), diethyl ether or methanol
(2ml or 3ml) added (Transferpipettor, A.R. Horwell Ltd.; London) and the tubes closed with polyethylene stoppers. The tubes were mixed for 10 minutes (Multi-vortex mixer).

Samples extracted with methanol were centrifuged (1600g for 10 minutes), the supernatant decanted into glass scintillation vials, dried at 60°C under nitrogen and PAS buffer (0.5ml) added. The aqueous phase of the other tubes was frozen in solid carbon dioxide/industrial methylated spirit and the supernatant poured into glass scintillation vials and evaporated at 60°C under nitrogen. Scintillator (Instagel, 7ml) was added to all vials and after shaking for 1 hour, the radioactivity in the extract determined in the manner described previously (section 2.3.3.).

e) Extraction using octadecylsilane bonded silica (ODS) columns.

Commercially-available Baker-10 ODS columns (Linton Products Ltd., Harlow, UK) consisting of a polypropylene syringe barrel pre-packed with ODS (0.1g for 1ml columns: 0.5g for 6ml columns) sandwiched between 20μm porosity polyethylene sinters were employed: these columns obviated the need for a separate sample reservoir. Their use in conjunction with a Baker 10 manifold (Linton Products) enabled 10 columns to be run simultaneously using reduced pressure to control the flow rate.

Columns were primed by passage of 2 x 2ml (1ml columns) or 2 x 5ml (6ml columns) of methanol and the same volumes of deionized water prior to use: care was taken to ensure that they were not dried after water addition since this markedly reduces subsequent flow of aqueous samples. The maximum total volume of hot milk (heated at 64°C for a minimum of 10 minutes) passed through each 1ml and 6ml column was 3ml and 15ml at a maximum flow rate of 0.5ml/min or 1.0ml/min respectively. After passage of milk samples, columns were washed with 2 x 2ml or 2 x 5ml hot water (64°C) and finally sucked dry prior to elution of progesterone into either scintillation vials (for direct radioactivity determination) or into conical glass test tubes with 1ml
(1ml columns) or 3 x 0.5ml (6ml columns) of methanol. Elution was shown to be complete by determination of (\(^3\)H)progesterone (in Instagel or Toluene Scintillator, see section 2.3.3.) in the milk sample, extracted milk and milk extract and examination of further fractions following elution with chloroform/methanol (1:1 v/v, 1ml). Tubes containing methanol extract were left overnight at -20°C and the lipids which precipitated were separated by centrifugation (1600g, 10 minutes). The extracts were aspirated into screw-capped glass vials (2ml capacity), dried down under nitrogen, redissolved in the HPLC mobile phase (ethanol, 1% v/v in dichloromethane; 0.7ml) and stored at -20°C until required. The recovery of progesterone was determined by comparison of radioactivity (Toluene Scintillator) in duplicate aliquots of milk sample (X dpm in 0.1ml) with those in the extract (Y dpm in 5\(\mu\)l). For an extract of a 15ml milk sample dissolved in 0.7ml mobile phase, 

\[
\text{recovery} = \frac{Y \times 140}{X \times 150} \times 100\%
\]

f) Isolation of progesterone from milk extracts by HPLC. Isolation of progesterone from milk sample extracts (100\(\mu\)l) was achieved using system A and the 3\(\mu\)m RoSiL column set up for column back-flushing. The mobile phase (1% v/v ethanol in dichloromethane) was used at a flow rate of 2ml/minute. Following collection of the progesterone fraction into screw-capped vials (2ml capacity), in accordance with its pre-determined retention time (\(t_R\) 2.17 minutes, collection period 1.97 minutes to 2.47 minutes), the column was backflushed for 6 minutes before injection of the next sample. The progesterone fraction was dried down at 60°C under a stream of nitrogen, redissolved in PAS gelatin buffer (0.7ml) and stored at -20°C until required for progesterone assay by RIA (section 2.3.4.) or heterologous EIA: buffer standards were used in each case. Determination of (\(^3\)H)progesterone in aliquots (5\(\mu\)l) of the fraction allowed percent recovery to be calculated (see section 7.2.1e)). Progesterone concentrations were compared with those obtained by direct RIA (see section 2.3.4.)
and EIA (section 7.2.2b) of the milk samples.

7.2.2. Enzyme Immunoassay

a) Preparation of antibody-coated microtitre plates. Polystyrene plate wells (NUNC plates) were coated with anti-progesterone γ-globulin (G711/12, 1/8000 dilution in 0.17mM acetate buffer, pH 5.0) at room temperature as described in section 5.2.2.

b) EIA procedure. Progesterone 11α-glucuronide-alkaline phosphatase conjugate (No. 151) was used. This was prepared with a 2:1 molar excess of steroid at conjugation as described in section 3.2.4.a.

Whole milk standards and samples were allowed to come to room temperature (20 - 22°C) and thoroughly mixed before assay. Standards (10μl, containing 0 - 800pg progesterone) or samples (10μl) were added to duplicate wells. Conjugate diluted in PAS-gelatin buffer (200μl; 20ng/well) was then added at ambient temperature. Blank values were determined by addition of buffer alone to duplicate wells containing 10μl of the zero progesterone standard. The plates were covered with a plate lid and incubated at ambient temperature for 3 hours in a covered plastic box lined with damp paper before emptying and washing 3 times with PAS-gelatin buffer (350μl/well) at 40°C delivered from an 8-channel peristaltic dispenser. Substrate (p-nitrophenylphosphate, 15mM in DEM buffer) was added to each well (200μl) and the plates were incubated for 1 hour at 40°C in a waterbath on a grid at water level. The absorbance (405nm) of each well was recorded directly using an automatic plate reader. The progesterone concentration in each sample was estimated by interpolation from the calibration curve derived from each plate.

c) Statistical analysis of results. When assay techniques were compared, weighted linear regressions were calculated assuming error in both procedures: slopes and intercepts were corrected after estimating the
variance in each technique from their coefficients of variation and their compatibility with a line of identity (slope = 1, intercept = 0) using an F-ratio based on the "extra sum-of-squares" principle (Munson and Rodbard, 1982) as described in section 2.3.6.

7.3 RESULTS

7.3.1. Extraction of Progesterone by Solvent Partition

The recovery of \(^3\text{H}\)progesterone following extraction from milk using methanol, petroleum spirit or diethyl ether is shown in table 7.1.

Table 7.1 The Recovery of \(^3\text{H}\)Progesterone (8877 ± 85dpm: mean ± s.d. = 16pg) Added to Triplicate Milk Samples (0.2ml) Following Extraction or Partition with Methanol, Petroleum Spirit or Diethyl Ether

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Methanol</th>
<th>Petroleum Spirit</th>
<th>Diethyl Ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent Volume</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>% Recovery</td>
<td>90.2 ± 1.8</td>
<td>92.7 ± 0.3</td>
<td>43.4 ± 2.1</td>
</tr>
</tbody>
</table>

Gel formation arose with extraction using 2ml of petroleum ether but this was considerably reduced with 3ml of solvent. Since gel formation was minimal with diethyl ether, however, there would be no advantage in using methanol precipitation of proteins prior to extraction.

7.3.2. Extraction of Progesterone using ODS Columns

a) Effect of flow rate. In preliminary studies milk samples (containing 50,000dpm \(^3\text{H}\)progesterone ≈ 90pg and 50ng progesterone/ml milk) were diluted 1:3 with 0.15M sodium chloride solution (at 64°C) as described by Axelson and Sahlberg (1981). For the passage of a single 1ml aliquot of diluted milk through 1ml columns at approximate flow rates of 0.5, 1.0 and 2.0ml/minute, progesterone recovery in the methanol eluate was found to be
89.9%, 80.7% and 74.5% respectively: the remainder was found in the extracted milk (11.0%, 20.4% and 20.3% respectively). Similarly, for extraction of undiluted milk (1ml) at a flow rate of 0.5ml/minute, 92% recovery was achieved in the methanol eluate with 8.8% remaining unextracted.

b) Adsorptive capacity of columns. To estimate the efficiency of extraction of larger volumes of milk, duplicate milk samples (progesterone concentration 38ng/ml), either diluted 1:3 as in the previous section (9 x 1ml aliquots/column) or undiluted (3 x 1ml aliquots/column), were passed through 1ml columns (mean flow rate 0.5ml/minute) and residual \(^3\text{H}\)progesterone recovered in each aliquot determined after extraction. Reduced extraction was noted with successive aliquots. For undiluted milk, mean unextracted \(^3\text{H}\)progesterone represented 3.5%, 4.1% and 8.5% of the total in the three respective fractions and the mean recovery on elution with methanol (1ml) was 88%. Similarly, for the 9 successive aliquots of diluted milk, 4.5%, 4.8%, 4.4%, 4.6%, 8.1%, 6.3%, 5.8%, 9.0% and 10.3% (means) respectively remained unextracted, with 88.5% of \(^3\text{H}\)progesterone recovered on elution with methanol.

Scaling up by use of 6ml ODS columns for the extraction of 15ml of undiluted milk at a flow rate of approximately 1ml/minute gave rise to a mean \(\pm\) sd recovery (n = 5) of 77.4 \(\pm\) 5% and 79.1 \(\pm\) 2.8% for samples containing a concentration of 0 and 50ng/ml respectively of added unlabelled progesterone.

c) Use of ODS-extracted ultra high temperature-treated (UHT) milk for the preparation of progesterone standards. Progesterone standards, prepared in the manner described previously (section 2.1.10.) using ovX milk, UHT milk from a commercial source and UHT milk previously extracted by the ODS method, were assayed in quadruplet by EIA. The calibration curves produced are shown in Fig. 7.1 and indicate that endogenous progesterone concentrations were lowest in the extracted milk: interpolation from the extracted milk calibration curve indicated that the "0" standard for the ovX milk and UHT
milk contained 1 ng progesterone/ml. (10 pg/well) and 16 ng progesterone/ml (160 pg/well) respectively.

Fig. 7.1 Comparison of EIA calibration curves using progesterone standards prepared in ovX milk (●-●), UHT milk (○-○) and ODS-column extracted UHT milk (▲-▲). Values are mean ± s.d. of 4 determinations.

7.3.3. The Isolation of Progesterone from Steroid Mixtures by HPLC

a) Mixtures in solvent. The separation of progesterone from seven structurally related steroids (5α- and 5β-pregnane-3, 20-dione; 3β-hydroxy-5-pregnen-20-one; 3β-hydroxy-5α-pregnan-20-one; 20β-hydroxy-4-pregnen-3-one; 11β-hydroxy-4-pregnene-3, 20-dione and 17-hydroxy-4-pregnen-3, 20-dione) prepared in 1% ethanol in dichloromethane on a 3μm spherical silica column (system B) was investigated using four solvent systems comprising of 0.5%, 1%, 2.5% and 5% ethanol (v/v) in dichloromethane at a flow rate of 1 ml/minute. As shown in Fig. 7.2, only the lower polarity systems (0.5% and 1% ethanol) provided good resolution; base line resolution of all the
Fig. 7.2 Separation of steroids by HPLC (system B; 3 μm Hypersil column). Using ethanol in dichloromethane as eluting solvent at concentration a) 0.5%, b) 1%, c) 2.5%, d) 5% and a flow rate of 1 ml/min. Peaks represent 1) 5α-pregnane-3, 20-dione; 2) 5β-pregnane-3, 20-dione; 3) progesterone; 4) 3β-hydroxy-5-pregnen-20-one; 5) 3β-hydroxy-5α-pregnan-20-one; 6) 17-hydroxy-4-pregnen-3-one; 7) 20β-hydroxy-4-pregnen-3-one; and 8) 11β-hydroxy-4-pregnen-3, 20-dione.

Steroids were dissolved in 1% ethanol in dichloromethane at concentrations of: 1 - 2, 25 µg/ml; 3 - 6, 50 µg/ml; 7, 100 µg/ml; 8, 42.5 µg/ml. The sample size was 20 µl. Chromatogram e) demonstrates use of 1% ethanol in dichloromethane at a flow rate of 2 ml/minute.
steroids was attained with 0.5% ethanol. Ethanol at 1% was chosen as optimal for the isolation of progesterone, however, since it ensured high resolution from the 5α- and 5β-pregnane-3, 20-diones and 3β-hydroxy-5pregnen-20-one in less time than with 0.5% ethanol; retention times for progesterone were 2.9 minutes and 5.9 minutes respectively (Figs. 7.2a and 7.2b). The nature of the additional peaks x and y (Fig. 7.2a) found using 0.5% ethanol is unknown: they may represent the summation of small quantities of impurity present in some or all of the steroid preparations, since neither was detected in substantial quantity when steroids were chromatographed individually. Resolution was little affected by use of 1% ethanol at a flow rate of 2ml/minute compared with that at 1ml/minute: elution profiles were virtually superimposable when plotted at chart speeds of 10mm/minute (Fig. 7.2e) and 5mm/minute (Fig. 7.2b) respectively. Clearly it was possible to isolate progesterone within 2 minutes of injection of sample on to the column.

b) Mixtures in milk extract. To take advantage of the rapid separation capability of this system for the isolation of progesterone from milk extracts a column backflushing system was used in subsequent experiments. This arrangement enabled reversal of eluate flow across the column (3μm RoSiL) alone immediately the progesterone fraction was eluted and collected: samples could thus be rapidly chromatographed whilst minimizing carry over of slow running components to the next sample. Comparison of the retention times (mean ± s.d.) for progesterone when 100μl of the 8 steroid mixture (described in the legend of Fig. 7.2, at a tenth concentration) prepared either in mobile phase or ODS extract of ovX milk was injected on to the column (flow rate 2ml/minute) revealed no appreciable difference: values were 2.22 ± 0.06 min (n = 3) and 2.17 ± 0.02 min (n = 4). U.V. absorbing (280nm) substances were eluted from the backflushing column in less than three times the retention time of progesterone. Chromatography
of the milk extract (the steroid mixture) containing $^{3}$Hprogesterone (30,000dpm/100μl = 54pg) enabled an estimation of recovery of progesterone to be made using the backflush procedure. Following injection of 100μl of extract three fractions were collected, from 0 - 2min, 2 - 2.4 min (progesterone fraction) and during backflushing from 2.4 - 7 min: mean ± s.d. (n = 3) $^{3}$Hprogesterone recovered in these fractions represented 5.8 ± 0.5%, 85 ± 1.5% and 8.24 ± 1.4% respectively for samples containing the steroid mixture and 5.5 ± 0.3%, 86.1 ± 2.8% and 11.9 ± 1.2% respectively for samples without the mixture added.

7.3.4. Validation of EIA

a) Assay sensitivity. The sensitivity of the RIA and EIA procedures are defined in Table 7.2 in terms of both limit of detection (Abraham, 1969) and the mid-point of the calibration curve (Feldman and Rodbard, 1971). Values shown were from calibration curves using standards prepared in milk or PAS-gelatin buffer (Figs. 7.3a and 7.3b).

![Fig. 7.3 Calibration curves for EIA (a) and RIA (b) using standards prepared in milk (a) or buffer (b). For EIA, mean B/B₀ values ± s.d. and CV (- -) for 8 (a) or 6 (b) consecutive assays are shown. Values for a single RIA calibration curve are included for comparison. Mean absorbance values in the absence of competing progesterone (B₀) fell within the ranges 0.659 - 0.894 (a) and 0.624 - 1.017 (b).](image-url)
Table 7.2 Assay Sensitivities

<table>
<thead>
<tr>
<th>Standards</th>
<th>EIA Limit of detection* (confidence limit)</th>
<th>EIA Calibration curve mid-point†</th>
<th>RIA Limit of detection* (confidence limit)</th>
<th>RIA Calibration curve mid-point†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk</td>
<td>5 pg (95%)</td>
<td>24 pg</td>
<td>5 pg (99%)</td>
<td>40 pg</td>
</tr>
<tr>
<td>PAS-gelatin</td>
<td>2 pg (99%)</td>
<td>10 pg</td>
<td>5 pg (99%)</td>
<td>20 pg</td>
</tr>
</tbody>
</table>

Data were derived from the calibration curves described in Fig. 7.3. * Smallest quantity of progesterone measured which gives rise to a response significantly different from zero (Abraham, 1969). Confidence limits were derived from the appropriate $t$ statistic. † The mass of progesterone required to produce a 50% reduction in label binding (Feldman and Rodbard, 1971).

b) Precision of adsorption of antibody to plate wells. The enzyme-labelled steroid conjugate, diluted in PAS-gelatin buffer (20ng/200μl), was added to 88 wells on each of 6 plates previously coated with anti-progesteroneγ-globulin: 8 wells in the centre of the plates received buffer alone to provide blank values. The plates were treated as described for EIA and the standard deviation (sd) of the optical densities of the 88 wells calculated for each plate. The mean within-plate coefficient of variation (CV) for the 6 plates was 5.1% (range 4.1 - 7.9%). The mean optical density calculated for the wells at the outside of each of the 6 plates did not differ significantly from the mean value for the remaining wells.

c) Blank values. The binding of enzyme-progesterone conjugate to "non-immune" γ-globulin and enzyme to anti-progesterone γ-globulin was tested to investigate non-specific binding in the microtitre plate system. Each of 2 plates was coated with both anti-progesterone γ-globulin (2 x 4 rows of 8 wells) and non-immune γ-globulin (4 rows of 8 wells). Milk samples (10μl; $n = 8$) containing high (>15ng/ml) concentrations of progesterone were added in quadruplicate to each group of 4 rows on one plate and milk samples (10μl; $n = 8$) containing low (<5ng/ml) concentrations of progest-
erone to the other plate. Alkaline phosphatase in PAS-gelatin buffer (40ng/200μl) was added to one group of wells containing anti-progesterone γ-globulin on each plate and conjugate in PAS-gelatin buffer (40ng/200μl) to the remaining groups containing anti-progesterone or non-immune γ-globulin. The plates were treated as described for EIA and the non-specific binding of conjugate or enzyme expressed as a percentage (± sd) of the specific binding seen in the presence of the high or low concentrations of progesterone in milk. Conjugate binding to the non-immune γ-globulin was 4.4 ± 1.2% and 2.15 ± 0.61% in the high and low samples respectively and enzyme binding to the specific antibody was 6.1 ± 1.2% and 2.5 ± 0.8%.

\[ d) \ EIA \ drift. \] Repeated duplicate estimations (1 - 12) were made of the progesterone concentration in each of 3 milk samples arranged in sequence across a microtitre plate. The slope and intercept obtained by least squares regression analysis of the progesterone concentrations determined (y) for each sample against order of application to the plates (x = 1 to 12) were compared with 0 slope and intercept at the mean sample concentration by the F-ratio test. At means ± sd of 2.6 ± 0.13ng/ml, y = 0.015x + 2.505 (F = 0.619); at 7.8 ± 0.4ng/ml, y = 0.021x + 7.970 (F = 0.150); and at 28.4 ± 3.1ng/ml, y = -0.360x + 30.758 (F = 1.026). There was no significant difference for any sample.

\[ e) \ Within \ and \ between \ assay \ precision. \] Milk samples were assayed by EIA and 9 samples, found to contain between 15 and 30ng progesterone/ml of milk, were combined to form a "high" quality control. Nine samples found to contain <5ng/ml were similarly combined ("low" quality control). Equal volumes of the 2 pools were mixed to provide a "medium" quality control. The 3 quality controls were assayed in duplicate at 5 different positions on each of 8 microtitre plates during the course of a routine assay and the mean and standard deviation calculated for each to allow the within-assay coefficient of variation (CV) to be determined.(Table 7.3).
A mean duplicate determination from each quality control on each plate was selected using random numbers tables and the mean between-assay CV calculated by repeating the exercise a further 4 times (Table 7.3). The between-assay precision (CV) of calibration curves obtained using either milk or PAS-gelatin standards are shown in Figs. 7.3a and 7.3b respectively.

f) Precision of RIA. The RIA (section 2.3.4.) was performed in batches of 56 or 64 tubes to enable synchronisation of charcoal addition and centrifugation. Replicate estimates of quality control samples assayed in duplicate within each batch (n = 5) or between batches (n = 8) allowed within- and between-batch CV to be calculated (Table 7.3).

Table 7.3 Within- and Between-Assay Precision for EIA and RIA and for RIA Performed at the Milk Marketing Board (MMB) at Low (L), Medium (M) and High (H) Concentrations of Progesterone in Milk

<table>
<thead>
<tr>
<th>Quality control</th>
<th>Mean within-assay precision</th>
<th>Mean between-assay precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EIA (n=40)</td>
<td>RIA (n=5)</td>
</tr>
<tr>
<td></td>
<td>ng/ml CV (%)</td>
<td>ng/ml CV (%)</td>
</tr>
<tr>
<td>L</td>
<td>3.2 11.0</td>
<td>1.6 14.0</td>
</tr>
<tr>
<td>M</td>
<td>11.2 16.7</td>
<td>6.0 7.2</td>
</tr>
<tr>
<td>H</td>
<td>17.7 9.7</td>
<td>12.8 12.2</td>
</tr>
<tr>
<td>Overall mean CV</td>
<td>12.5 11.1</td>
<td>11.9 11.1</td>
</tr>
</tbody>
</table>

For comparison, data were also derived from RIAs run routinely by the Milk Marketing Board (MMB). Milk samples submitted to them for pregnancy testing in a 1 week period and found to contain >9ng progesterone/ml were pooled to provide a quality control sample. Eight assays, each including 3 quality control determinations, were selected using random numbers tables from 34 assays conducted in 1 week and the mean within-assay CV calculated (Table 7.3). The between-assay CV was calculated from 40 values selected at random from assays performed during the same period (Table 7.3).
g) Cross-reactivity. Cross-reactivity was defined as the molar quantity of steroid, relative to progesterone (mol progesterone/mol steroid) required to reduce label binding by 50% (Abraham, 1969). Values for various steroids (dissolved in PAS-gelatin buffer) with the antiserum (G711/12) are presented in Table 7.4.

Table 7.4 Specificity of Antisera

<table>
<thead>
<tr>
<th>Steroid*</th>
<th>RIA†</th>
<th>EIA†</th>
<th>RIA‡ at MMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Pregnene-3,20-dione</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4-Androstene-3,20-dione</td>
<td>&lt;0.03</td>
<td>0.04</td>
<td>&lt;0.30</td>
</tr>
<tr>
<td>5a-Pregnane-3,20-dione</td>
<td>1.30</td>
<td>2.80</td>
<td>2.00</td>
</tr>
<tr>
<td>5β-Pregnane-3,20-dione</td>
<td>6.80</td>
<td>8.00</td>
<td>—§</td>
</tr>
<tr>
<td>17-Hydroxy-4-pregnene-3,20-dione</td>
<td>3.60</td>
<td>11.3</td>
<td>0.40</td>
</tr>
<tr>
<td>3β-Hydroxy-5-pregnene-20-one</td>
<td>0.05</td>
<td>0.40</td>
<td>1.50</td>
</tr>
<tr>
<td>11β,17α,21-Trihydroxy-4-pregnene-3,20-dione</td>
<td>&lt;0.03</td>
<td>0.06</td>
<td>&lt;0.30</td>
</tr>
<tr>
<td>11β,21-Dihydroxy-4-pregnene-3,20-dione</td>
<td>1.60</td>
<td>0.80</td>
<td>&lt;0.30</td>
</tr>
<tr>
<td>3β-Hydroxy-5-androsten-17-one</td>
<td>0.03</td>
<td>0.03</td>
<td>—§</td>
</tr>
<tr>
<td>11α-Hydroxy-4-pregnene-3,20-dione</td>
<td>75.80</td>
<td>—§</td>
<td>44.00</td>
</tr>
<tr>
<td>21-Hydroxy-4-pregnene-3,20-dione</td>
<td>—§</td>
<td>5.80</td>
<td>4.00</td>
</tr>
<tr>
<td>17β-Hydroxy-4-androsten-3-one</td>
<td>—§</td>
<td>—§</td>
<td>0.35</td>
</tr>
<tr>
<td>20α-Hydroxy-4-pregnene-3-one</td>
<td>—§</td>
<td>—§</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Cross-reactivity is defined as the molar quantity of steroid, relative to progesterone (mol. progesterone/mol steroid) required to reduce label binding by 50%.

* IUPAC-IUB (1967) revised tentative rules for steroid nomenclature.
† Antiserum G711/12.
‡ Antiserum AF18/3 (data of Sheldrick, Mitchell & Flint, 1980).
§ Not tested.

h) Comparison of EIA of milk and HPLC isolates. Whole milk samples (n = 25) were selected using a random numbers table from samples collected on a routine basis. (3H)Progesterone was added to each sample prior to extraction and HPLC as described in section 7.2.1.

The mean ± s.d. recovery of the progesterone tracer after ODS column extraction of the 25 milk samples examined was 78.8 ± 4.5%. The recovery after HPLC and redissolving the progesterone fraction in PAS-gelatin buffer was 52.8 ± 10.4%.

Progesterone concentrations were determined both before and after extraction and purification and were corrected for procedural losses. Comparisons of results from EIA with those from RIA before (r = 0.933) and
Fig. 7.4 Linear regression of progesterone concentrations in milk determined by EIA and RIA a) before and b) after extraction and isolation by HPLC and c) by EIA and d) RIA before and after extraction and purification by HPLC. — = line of regression, ——— = line of identity.

Fig. 7.5 Progesterone concentrations determined by RIA and EIA in 4 luteal-phase milk samples after dilution with ovX milk, for which regressions differed significantly from a line of identity (---).
after \((r = 0.969)\) extraction were highly correlated and the slopes and intercepts did not differ significantly from 1 and 0 respectively (Figs. 7.4a and 7.4b) when the F-ratio test was applied. Linear regressions for EIA before and after extraction \((r = 0.910)\) and RIA before and after extraction \((r = 0.952)\) were also not significantly different from a line of identity (Figs. 7.4c and 7.4d).

\(i\) Linearity of response. Ten milk samples shown to contain progesterone concentrations consistent with the luteal phase \((\geq 10\, \text{ng/ml})\) by EIA were taken at random and serially diluted with milk from an ovariectomised cow. The diluted samples were assayed by both EIA and RIA and linear regressions calculated for each set of results against dilution. Correlation coefficients fell within the range \(r = 0.984\) to 0.999 for the RIA and \(r = 0.946\) to 0.997 for the EIA. Correlation coefficients following linear regression of the results from EIA on those obtained by RIA were in the range \(r = 0.946\) to 0.998. For 6 of the dilution series, analysis of the F-ratio showed no significant deviation from slope = 1 and intercept = 0; significant differences were found in 4 of the series (Fig. 7.5) although good correlations were still exhibited \((r = 0.967\) to 0.997).

\(j\) Analytical recovery of progesterone added to milk samples. Progesterone was added to milk samples taken from 6 different cows during the oestrous period (endogenous progesterone <1.0\, \text{ng/ml} by EIA), in the manner described for the preparation of progesterone standards (section 2.1.10), to provide concentrations of 20\, \text{ng/ml}. The samples were diluted in milk from an ovariectomised cow to provide a range of concentrations (20, 16, 12, 8, 4, 2 and 1\, \text{ng/ml}). The samples were assayed by RIA and EIA and the mean concentrations and percentage recoveries of progesterone are shown in Table 7.5. Linear regressions of mean concentrations measured \((y)\) on the values expected \((x)\) gave \(y = 1.010x - 0.784\) and \(y = 1.026x - 0.718\) for EIA \((r = 0.993)\) and RIA \((r = 0.995)\) respectively.
Table 7.5 Mean Concentration (ng/ml milk) and Recovery of Progesterone Added to Milk Samples (n = 6) from Oestrous Cows

<table>
<thead>
<tr>
<th>Expected conc. of progesterone</th>
<th>EIA Mean ± s.d.</th>
<th>Recovery (%)</th>
<th>CBC RIA Mean ± s.d.</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>20.6 ± 4.5</td>
<td>103</td>
<td>20.9 ± 1.0</td>
<td>105</td>
</tr>
<tr>
<td>16</td>
<td>14.5 ± 3.4</td>
<td>91</td>
<td>14.6 ± 0.8</td>
<td>91</td>
</tr>
<tr>
<td>12</td>
<td>10.4 ± 2.3</td>
<td>87</td>
<td>11.3 ± 1.0</td>
<td>94</td>
</tr>
<tr>
<td>8</td>
<td>7.3 ± 0.8</td>
<td>91</td>
<td>7.2 ± 0.5</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>3.3 ± 0.8</td>
<td>83</td>
<td>3.5 ± 0.3</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>1.8 ± 0.6</td>
<td>92</td>
<td>1.8 ± 0.1</td>
<td>90</td>
</tr>
</tbody>
</table>

7.4 DISCUSSION

A central objective of this study was to confirm that progesterone was the sole or major component measured in the EIA (and RIA) systems. This was achieved by comparison of progesterone concentrations measured before and after isolation by extraction and HPLC.

Both the solvent-partition and ODS column methods were efficient in the extraction of progesterone from milk. The former was only suitable for extraction of samples of relatively low volume due to the large relative volume of solvent required if gel formation was to be avoided. For routine purposes, the ODS column extraction procedure provided a simple and practical alternative to solvent partitioning for relatively large volume (> 1ml) samples in large numbers, 15ml of milk being reduced to 1.5ml of methanol extract. The reduced recovery (77 - 79%) and increased variability (CV 3.5 - 6.4%) of extraction attained on scaling up the procedure to accommodate 15ml of milk was associated with difficulties in achieving uniform and steady flow rates between columns when ten columns were employed simultaneously: this effect could be considerably reduced by pre-filtration of the milk samples.

A further application of the ODS extraction method was found in the
preparation of essentially progesterone-free milk from commercially available sources. This provided a suitable matrix in which to prepare milk standards (Fig. 7.1) and circumvented the necessity for using ovX milk or selected milk samples from cows at oestrus. Axelson and Sahlberg (1981) indicated that the quantitatively-important components of milk such as proteins and lipids remain essentially unaltered by ODS extraction.

The HPLC system described by Lin et al (1980) could not be readily adopted since the column used resulted in high back-pressures which exceeded the working range of the equipment available.

This practical problem necessitated that an alternative system be contemplated. Based on data from commercially available columns of 5mm diameter (Hichrom Ltd.), the total potential efficiency N of the two Partisil columns used by Lin et al (1980) would approximate to 6,000 - 7,000 theoretical plates, \( N = \alpha \left( \frac{V_r}{W} \right)^2 \), where \( V_r \) is the retention volume of the peak, \( W \) is the peak width in volume and \( \alpha \) is a constant dependent on the height at which peak width is measured. This calculation excludes any reduction in efficiency incurred by use of columns of such low (2mm) sub-optimal diameter (Knox, 1978). The type of column employed in the present study provided total column efficiencies of 10,000 - 12,000 theoretical plates and enabled full resolution of progesterone from all the steroids tested.

The main advantage of this system compared with that of Lin et al (1980) was the speed of separation: the retention times for progesterone were 2.17 minutes and 45 minutes respectively. Purdy et al (1981) have described a similar system which provided good resolution of progesterone from 5\( \alpha \)- and 5\( \beta \)-pregnane-3, 20-dione and 3\( \alpha \)-hydroxy-5\( \alpha \)-pregnan-20-one but again progesterone exhibited a long retention time (\( \sim \)21 minutes). When used in the column backflush system it was possible to isolate progesterone and prepare the column for injection of the next sample within 10 minutes,
while minimizing the possibility of carry-over of slow running components from one sample to another.

The presence of milk has been shown to cause a significant depression in antibody-antigen binding and sensitivity in both RIA (Heap et al., 1976) and EIA (Sauer et al., 1981; Chang and Estergreen, 1984) of progesterone and in recognition of this, standards are often prepared in milk. Indeed, variations in non-specific effects between samples have been reported (Heap et al., 1976; Pennington et al., 1976; Holdsworth et al., 1979; Stevens et al., 1981) although many descriptions of immunoassays for progesterone have made no reference to such an occurrence. This problem was illustrated in the present study when 10 samples shown to contain luteal-phase concentrations of progesterone were diluted with milk from an ovariectomized cow. All samples showed linear correlations with dilution when measured by RIA or EIA and when these results were compared. Four of the samples, however, showed significant variation from a line of identity between EIA and RIA (Fig. 7.5), indicating that the sample matrix had affected the concentration of progesterone perceived by one technique or the other. A similar effect was noted when progesterone was added to 6 milk samples taken from cows at oestrus. Although the mean recoveries of progesterone after further dilution with milk from an ovariectomized cow were close to those expected (Table 7.5), greater variation in the recovery of progesterone from individual milk samples was seen with EIA than with RIA. In comparison, Chang and Estergreen (1984) reported recovery values between 80 and 88% but a high degree of variability (s.d. 26 - 71%). Although comparable recovery data for RIA are scarce or limited (Heap et al., 1976), recovery ranges have been reported by some laboratories and vary considerably, e.g. 97 - 110% (Bulman and Lamming, 1978), 104 - 126% (van de Wiel et al., 1978); and 100 - 136% (Batra, Pahwa, Suri and Pandey, 1980).
An explanation for the variation seen in EIA in particular might lie in the composition or structure of milk since the label used could interact with particular milk samples. The investigation of non-specific binding in 16 milk samples containing high or low concentrations of progesterone showed no evidence of individual non-specific binding effects and mean values of 2.15% and 4.4% were recorded in the presence of follicular and luteal-phase samples respectively: clearly endogenous alkaline phosphatase also had little influence on the system. Equivalent studies with RIA have produced variable values as high as 20% (Holdsworth et al, 1979; Stevens et al, 1981).

Differences in cross-reactivity between RIA and EIA could produce similar discrepancies in values measured: the occurrence of progestagens in milk has been investigated by Darling, Laing and Harkness (1974) but since the oxidative procedures employed would only allow broad classification, the influence of specific steroids is hard to discern. Cross-reactivity studies were conducted using the criteria of Abraham (1969) but no large differences were found between EIA and RIA (Table 7.4) although Van Weemen and Schuurs (1975) and Van Weemen, Bosch, Dawson, Van Hell and Schuurs (1978) have shown that the use of heterologous assay systems can increase cross-reactivity. Steric considerations, however, suggest that this is less likely to occur when the heterology involves the bridge itself, rather than the site of attachment of the bridge to the steroid. Darling et al (1974) and Purdy, Durocher, Moore and Rao (1980) showed that 5α- and 5β-pregnane-3, 20-diones occur in milk but it is the suggested presence of progestagen conjugates (Heap, Henville and Linzell, 1975) that should give rise to concern since these are not defined and were not tested. The probable preponderance of conjugated metabolites in the aqueous phase of milk would enhance their interaction with the antibody in comparison with progesterone and their greater availability would
be accentuated by the non-equilibrium nature of EIA (section 6.3.4a; Sauer et al, 1982b; Morino, Nakao, Tsunoda and Kawata, 1984; Munro and Stabenfeldt 1984). The importance of the "first come, first-served" principle (Pratt and Woldring, 1976) in non-equilibrium RIA and EIA has been demonstrated by a number of other investigators (Van Weemen et al, 1978; Vining, Compton and McGinley, 1981).

With these considerations in mind, it was necessary to demonstrate that progesterone was the milk component being measured by EIA. After correction for procedural losses, progesterone concentrations determined in 25 milk samples before and after extraction and isolation by HPLC were highly correlated for both EIA and RIA ($r = 0.910$ and $0.952$: Figs. 7.4c and 7.4d). In addition, results from the two techniques were closely correlated before (Fig.7.4a; $r = 0.933$) and after (Fig. 7.4b; $r = 0.969$) extraction and, most importantly, none of the regressions differed significantly from a line of identity, indicating that progesterone was the major component in milk measured by both RIA and EIA.

The use of microtitre plates to provide both a solid-phase support for the antibody and a vessel to contain the assay greatly facilitates its application in unsophisticated laboratories. Previous studies (Kricka et al, 1980) have found evidence for variation in the adsorption of antibody to plates ($CV = 5.2 - 30\%$). Although similar problems were originally encountered with both polyvinyl and polystyrene plates (see chapter 5), the use of plates specifically manufactured for immunoassay and attention to detail in ensuring a stable environment during antibody coating and incubation resulted in acceptable precision ($CV = 5.1\%$). In particular, no evidence was seen of preferential binding to wells at the edge of the plate (see Kricka et al, 1980). The within- and between-assay precision of EIA were 12.5 and 14.8% respectively and compared favourably with values for RIA both in this study (Table 7.3) and others (Heap et al, 1976; Holdsworth
et al., 1979; van de Wiel, Kalis and Shah, 1979; Batra et al., 1980). Bulman and Lamming (1978), however, have given details of an RIA with greater precision (CVs = 8.9% and 10.3%).

If standards and 40 samples are assayed in duplicate on a microtitre plate, about 15 minutes will elapse between introduction of the first sample and addition of the conjugate. Munro and Stabenfeldt (1984), using 50 μl of antibody per well to coat the plate and a 50 μl sample, found that this resulted in a systematic variation in the value determined for a sample depending on the time of addition to the plate ("drift"), presumably because the earlier the addition, the greater the time for contact between the sample and antibody. Since the microtitre plate EIAs described do not attain equilibrium (Sauer et al., 1982b; Munro and Stabenfeldt, 1984), this presents a potential source of error. The use of 200 μl of antibody to coat the plate and only 10 μl of the sample apparently prevented drift in the present application, however.

CONCLUSIONS

1. Rapid extraction of progesterone from milk samples was achieved by use of ODS columns. Milk extracted in this way provided a suitable matrix for the preparation of EIA standards.

2. HPLC of milk extracts using a 3 μm silica column and isocratic elution enabled separation of progesterone from steroids of similar chemical structure.

3. The precision, sensitivity and accuracy of EIA was shown to rival that of RIA. Its simplicity and safety in use commend its application to the solution of practical problems in the management of reproductive performance of dairy cattle.
CHAPTER 8:
APPLICATIONS OF PROGESTERONE EIA
8.1 INTRODUCTION

The value of measuring milk progesterone concentrations by RIA in determining causes of reproductive failure is widely established and has been discussed (see section 1.6.6.). However, the use of radioisotopes restricts RIA services to licensed specialized laboratories and severely limits its practical value. The only routine application of this approach to the management of reproductive performance has been a postal pregnancy-testing service (Booth and Holdsworth, 1976). The development of EIA procedures described in previous chapters has removed these restrictions and the present study exploits some of the possibilities revealed by RIA.

Two EIA procedures have been used in the present studies. The relatively insensitive homologous assay, with progesterone 11α-hemisuccinate-β-galactosidase as label, was used to investigate the value of daily measurements of progesterone concentration in cow's milk as a means of predicting the appropriate time for AI. The use of a second, sensitive heterologous EIA, employing progesterone 11α-glucuronide-alkaline phosphatase as label was investigated as a simple means of pregnancy testing and for monitoring corpus luteum function during hormone treatment for oestrus synchronization.

Monitoring corpus luteum function during hormone administration for oestrus synchronization enabled the efficacy of treatment to be evaluated. The progesterone-releasing intravaginal device (PRID) used here has been promoted for oestrus synchronization and may initiate ovarian activity in the post-partum anoestrus animal: their administration for twelve days has found acceptance as a means of reducing days to conception and improving pregnancy rates (Drew, 1982). Using this treatment, however, Gyawu et al (1980) found that where the devices were fitted late in the oestrous cycle no conception occurred. This may result from prolonged exposure of the developing follicle to progesterone, leading to abnormalities of the
ovum (Van Niekerk and Belonge, 1970) or to early embryonic mortality (Wishart and Young, 1974). Further, the oestradiol benzoate capsule normally attached to the PRID may not be luteolytic (Seguin, 1979). The object of this particular study was to assess whether a seven-day administration of PRID with a single intra-muscular injection of cloprostenol 24 hours before PRID removal could achieve precise synchronization and a satisfactory conception rate.

8.2 MATERIALS AND METHODS

8.2.1. Application of the Homologous EIA to Oestrus Prediction and the Timing of AI

a) Preparation of antibody-coated microtitre plates. PVC plates (Dynatech) were coated with anti-progesterone γ-globulin (G711/12, 1/4,000 dilution, 1mM acetate buffer pH 5.0) at 40°C as described in section 5.2.2.

b) Homologous EIA. Progesterone 11α-hemisuccinate-β-galactosidase conjugate (No. 98) was used, prepared as described in section 3.2.3, with a 20:1 molar excess of steroid at conjugation. All pipetting stages were performed at 4°C in a cold room. Milk progesterone standards were preserved using sodium azide (0.1%).

Standards (10 μl) or milk samples (10 μl) from animals under test were drawn up using a diluter/dispenser (IMV, L'Aigle, France) and washed into the wells of the plate with conjugate (190 μl in PAS-gelatin buffer, 10ng/well). Blank values were determined by addition of buffer alone to duplicate wells containing zero progesterone standards. The plates were covered with a lid and after incubation for 3 hours at 40°C in a water bath, the wells were emptied and washed three times with PAS-gelatin buffer (250 μl). The degree of binding of enzyme conjugate to the antibody-coated wells was determined by addition of freshly-prepared substrate (O-nitrophenyl-β-D-galactopyranoside, 200 μl; 3mg/ml) in PAS-gelatin buffer containing 0.01M
magnesium chloride and 0.1M mercaptoethanol. Following an incubation period of 1 hour at 40°C, the optical density at 405nm was measured directly through the wells using an automatic plate reader. The progesterone concentration in each sample was estimated by interpolation from the calibration curve derived from each plate.

c) Cattle and the collection of milk samples for EIA. Cows (164 in total) which were at least 42 days post-calving were identified and randomly assigned to two similar sized groups (experimental and control) on each of four commercial farms. Morning milk samples were collected daily over a 30 day period from animals in the experimental group (identified by use of tail tape) and transported to the laboratory for progesterone determination on the same day by EIA. The stockmen were instructed to agitate the milk jars before taking the representative sample (15 to 20ml) into polystyrene vials (Dilu-vial; Elkay Products Ltd., Basingstoke), pre-labelled with the cow's number.

d) AI of cattle. Milk progesterone concentrations were determined by the afternoon following collection and results for each cow plotted daily. When two successive low values (below 5ng progesterone/ml milk) had been noted, the farm was contacted by telephone and arrangements made for the cow to be inseminated the following day by Cattle Breeding Centre inseminators. Cows in the control groups were similarly inseminated following observation of behavioural oestrus by the stockmen.

e) Pregnancy testing. Pregnancy testing was performed by EIA using milk samples taken from animals in both experimental and control groups 24 days after insemination. Progesterone concentrations greater than 10ng/ml were taken to indicate pregnancy and those below to indicate a return to oestrus.

8.2.2. Use of the Heterologous EIA to Monitor Corpus Luteum Function Following Hormone Treatment for Oestrus Synchronization

a) EIA. NUNC polystyrene microtitre plate wells were coated with
γ-globulin from antiserum G711/12 (see section 7.2.2a ) for use in EIA.
EIA was performed as described in section 7.2.2b .

b) Cattle and hormone treatments. A group of 29 Friesian cows of 1st, 2nd and 3rd lactation was housed in winter and fed a conventional diet. The average interval from previous calving to day 0 of treatment was 122.0 ± 27.1 days. Progesterone-releasing intravaginal devices (PRID, CEVA Ltd.), from which the gelatin capsules containing 10mg oestradiol benzoate had been removed, were introduced into the vagina of each cow (day 0). Six days later (day 6) each cow was injected intramuscularly with 500μg cloprostenol (a synthetic analogue of prostaglandin F₂₃, ICI plc.). The devices were removed from the cows 24 hours later (day 7). AI was carried out using semen from a single ejaculate from a progeny-tested bull 48 hours (day 9) and 72 hours (day 10) after PRID removal. All procedures were carried out between 10am and noon.

c) Collection of milk samples for EIA. Milk samples were taken during the morning milking (0700 - 0900 hours) at 2 - 3 day intervals during the 21 days preceding the introduction of the PRID and daily from the day before treatment began until day 16. Further milk samples were taken at 21, 22, 24 and 25 days after AI to confirm pregnancy. The milk samples were taken from individual milk jars after agitation, preserved using Lactab Mk III (section 2.3.7.) and stored at 4°C until assayed by EIA. EIA data were processed automatically by interfacing the plate reader with a microprocessor (Commodore PET 2001): progesterone concentrations were interpolated from calibration curves calculated using a 4-parameter logistic function (Rodbard, 1978). The BASIC programme was provided by Mr. A. Thomas (see appendix 3.).

8.2.3. Heterologous EIA and Pregnancy Testing of Dairy Cows: Comparison with Other Methods

a) EIA. NUNC polystyrene microtitre plate wells were coated with
γ-globulin from antiserum G711/12 (see section 7.2.2a) for use in EIA. EIA was performed as described in section 7.2.2b.

b) Comparison with RIA. Milk samples (200) submitted to the MMB by commercial farms for pregnancy testing 22 - 26 days after service or by veterinary surgeons investigating herd fertility were assayed by the RIA in routine use at the MMB Veterinary Laboratory (Holdsworth, Chaplin and Booth, 1979) before transporting for re-assay by RIA (as section 2.3.4.). The majority (168) were milk samples taken after agitation of the whole milking in the collecting jar; however, 32 were fore-milk samples collected by hand.

c) Confirmation by reference to calving data. A further 110 whole milk samples were obtained from cows in four commercial herds 24 days after service and progesterone concentrations determined by EIA. The conclusions were subsequently verified from calving data.

d) Confirmation by palpation per rectum. The 29 cows involved in the oestrus synchronization experiment described previously (section 8.2.2.) were tested for pregnancy by progesterone determination (EIA) of milk samples taken 21 - 25 days after first AI and subsequently at 8 weeks by palpation per rectum by a veterinary surgeon.

8.3 RESULTS
8.3.1. The Timing of AI: Comparison of Observed Oestrus with that Predicted by Homologous EIA

A comparison of pregnancy rates following predicted and observed oestrus are shown in table 8.1. Examples of typical profiles of milk progesterone concentrations (farm B) determined by EIA and which formed the basis of the timing of AI in the experimental group are shown in Fig. 8.1. Of 82 cows in the experimental group, 80 were inseminated during the 30 day period of the trial and 50 gave positive pregnancy test results 24 days after insemination. Of the two animals not served, one was anoestrus and the other
failed to be inseminated although this was requested. In the control group, 58 of the 82 cattle were observed at oestrus and inseminated during the same period: 39 were diagnosed pregnant at 24 days. There was thus a gain in the number of cows in calf following intervention on the farms.

Table 8.1 Number of Cows Served and Diagnosed Pregnant in Experimental and Control Groups on Each Farm. Mean Calving to First Service Intervals are given in days.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Experimental group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Served</td>
</tr>
<tr>
<td>A</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>C</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>D</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>80</td>
</tr>
</tbody>
</table>

(97.5%) (62.5%) (70.7%) (47.5%) 

The intervals between calving and first service were recorded for each cow: mean values for each herd showed significant decreases in the experimental groups after logarithmic transformation to reduce the distortion resulting from long intervals in the control group.

Departures from the experimental protocol instigated by the stockmen necessitated re-allocation of animals before analysis of fertility data. Of the 80 cows served in the experimental group, two were served twice as the stockman saw them bulling on a different day to that predicted: these were excluded from further analysis. Ten cows in the experimental group were served at the stockman's instigation and these were included with the control group for determination of conception rates resulting from insemination at either predicted or observed oestrus. One cow was served at predicted oestrus outside the 30-day study but was included for the purpose of comparing fertility (table 8.2).

Thus, of the 69 cows served at the time predicted from progesterone
Table 8.2 Number of Cows Diagnosed Pregnant Following Service at Predicted or Observed Oestrus

<table>
<thead>
<tr>
<th>Herd</th>
<th>Served</th>
<th>Pregnant on day 24</th>
<th>Days to service</th>
<th>Served</th>
<th>Pregnant on day 24</th>
<th>Days to service</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18</td>
<td>13</td>
<td>86.1</td>
<td>25</td>
<td>17</td>
<td>90.2</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>15</td>
<td>83.3</td>
<td>14</td>
<td>12</td>
<td>78.9</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>11</td>
<td>72.7</td>
<td>19</td>
<td>11</td>
<td>85.4</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>5</td>
<td>64.5</td>
<td>9*</td>
<td>5</td>
<td>87.8</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>44 (63.8%)</td>
<td>78.9</td>
<td>67</td>
<td>45</td>
<td>86.2</td>
</tr>
</tbody>
</table>

* A tenth cow was served but was excluded from the analysis because the 24-day pregnancy diagnosis result was not available.

Fig. 8.1 Progesterone concentrations by homologous EIA determined in whole milk samples taken from individual cows on farm B. ¥ Cow judged pregnant according to milk progesterone concentration 24 days after AI.
profiles, 44 (63.8%) were found to be pregnant by milk progesterone analysis; of the 67 cows inseminated at observed oestrus 45 (67.2%) were found to be pregnant (Table 8.2).

8.3.2. Use of the Heterologous EIA to Monitor Corpus Luteum Activity of Dairy Cows Following Treatment with Progesterone Releasing Intra-vaginal Devices (PRID) and Cloprostenol

All cows showed patterns of milk progesterone concentration consistent with normal cyclic ovarian activity before treatment began (Fig. 8.2). The day of the cycle on which treatment began was defined retrospectively with the day of oestrus as the second day after the first measurement of a progesterone concentration below 5ng/ml milk. Profiles of progesterone concentrations suggested that during treatment luteolysis occurred at the normal time: progesterone levels were maintained at between 5 to 15ng/ml until the day after PRID removal (day 8), however, after which they declined within 24 hrs to levels consistent with the follicular phase (less than 2ng/ml) in all cows (Fig 8.2. Milk progesterone levels were confirmed to be low for all cows on both days (9 and 10) on which AI was performed and for at least the following 3 days. This was followed by rises in milk progesterone in 28 out of 29 cows. On the basis of milk pregnancy testing at 21 to 25 days, 18/29 (62%) cows were judged to have conceived to AI following synchronization. The positive pregnancy tests were confirmed by rectal palpation 8 weeks after AI in 17 of the 18 animals; the remaining animal was sold. The pregnancy rate was 11/17 (64%) where PRID was inserted before day 11 of the cycle and 7/12 (58%) where treatment was begun after day 12 of the cycle. The pattern of progesterone rise in the 7 days following AI for cows which failed to conceive was similar to the pattern observed for the other animals and was consistent with the occurrence of ovulation and subsequent formation of a corpus luteum (Fig. 8.2).

*Concentrations >5ng/ml were taken to be indicative of pregnancy.*
Fig. 8.2 Progesterone concentrations in milk from individual cows following initiation of treatment (day 0) at various stages of an oestrous cycle. ▼ PRID in, ▲ PRID out, ▼ cloprostenol injection and ◇ estimated day of cycle at initiation of treatment.

8.3.3. Heterologous EIA and Pregnancy Testing of Dairy Cows

a) Comparison with RIA. Progesterone concentrations determined in the 200 milk samples by EIA and RIA and by RIA performed at the MMB were compared using linear regression equations calculated and corrected for error in both techniques as described previously (section 2.3.6): scatter was particularly notable when high values were recorded. This was true when results from either the RIA or EIA were compared with those from the MMB RIA (r = 0.890 and 0.833 respectively; Table 8.3). The MMB used 4.5ng/ml and 1.0ng/ml in whole milk and fore-milk respectively to discriminate between samples from pregnant and non-pregnant animals at 22 - 26 days post-insemination. The comparisons above with concentrations determined at the MMB allowed equivalent discrimination points to be determined for the RIA and EIA.
Table 8.3 Regression of Results from EIA and RIA of 200 Milk Samples

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>Regression</th>
<th>F†</th>
<th>Discrimination point ± 2 s.d.‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk (n = 168)</td>
<td>0.901</td>
<td>y = 0.832x + 1.72</td>
<td>13.1</td>
<td>5.46 ± 1.62</td>
</tr>
<tr>
<td>EIA on MMB RIA</td>
<td>0.838</td>
<td>y = 0.699x + 3.21</td>
<td>32.8</td>
<td></td>
</tr>
<tr>
<td>RIA on MMB RIA</td>
<td>0.905</td>
<td>y = 1.140x - 1.63</td>
<td>7.4</td>
<td>3.50 ± 0.69</td>
</tr>
<tr>
<td>Fore-milk (n = 32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIA on MMB RIA</td>
<td>0.662</td>
<td>y = 1.579x - 0.555</td>
<td>15.6</td>
<td>1.02 ± 0.31</td>
</tr>
<tr>
<td>EIA on RIA</td>
<td>0.768</td>
<td>y = 1.365x + 0.815</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>RIA on MMB RIA</td>
<td>0.901</td>
<td>y = 1.023x - 0.286</td>
<td>0.27*</td>
<td>0.74 ± 0.15</td>
</tr>
<tr>
<td>All samples (n = 200)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIA on MMB RIA</td>
<td>0.890</td>
<td>y = 0.831x + 2.00</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>EIA on RIA</td>
<td>0.833</td>
<td>y = 0.702x + 3.36</td>
<td>38.6</td>
<td></td>
</tr>
<tr>
<td>RIA on MMB RIA</td>
<td>0.908</td>
<td>y = 1.133x - 1.45</td>
<td>8.2</td>
<td></td>
</tr>
</tbody>
</table>

†Calculated for the deviation from a line of identity; *not significantly different (P > 0.25).
‡Calculated from 4.5 ng/ml (whole milk) and 1.0 ng/ml (fore-milk) in the MMB RIA and standard deviations from the mean overall coefficients of variation observed in the individual assays (Table 3).

(Table 8.3). Results falling within two standard deviations of the discriminating point were considered inconclusive. Excluding these, 129 samples were classified by the MMB RIA as indicating pregnancy and 63 non-pregnancy. Conclusions from the EIA results coincided for 122 and 52 samples respectively, with 7 values in each case falling within 2 standard deviations of the discriminating value. The figures from the RIA were 126 and 56, with 2 and 5 results inconclusive. Both the EIA and the RIA produced 4 results that were at variance with conclusions drawn from the MMB RIA and these are summarised in Table 8.4: three (no's 145, 149, 154) were fore-milk samples. Veterinary investigation of three of the five apparent anomalies provided an indication of which conclusions were correct (Table 8.4).

b) Comparison with palpation per rectum. In a separate experiment described in section 8.3.2., the milk progesterone determinations at 21 to 25 days post-AI showed 18 of 29 cows (62%) to have conceived following oestrus synchronization. The positive pregnancy tests were confirmed by
Table 8.4 Progesterone Concentration (ng/ml) in Milk Samples Submitted to the MMB for which Different Conclusions were Reached on Reproductive Status.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>MMB RIA</th>
<th>EIA</th>
<th>RIA</th>
<th>Veterinary examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>81</td>
<td>1·9 (−)</td>
<td>11·0 (+)</td>
<td>4·3 (+)</td>
<td>Oestrus</td>
</tr>
<tr>
<td>108</td>
<td>3·9 (−)</td>
<td>7·3 (+)</td>
<td>5·1 (+)</td>
<td>Luteal</td>
</tr>
<tr>
<td>145†</td>
<td>0·7 (−)</td>
<td>2·1 (+)</td>
<td>1·3 (+)</td>
<td>EIA</td>
</tr>
<tr>
<td>149†</td>
<td>0·6 (−)</td>
<td>1·6 (+)</td>
<td>1·0 (?)</td>
<td>Pregnant</td>
</tr>
<tr>
<td>154†</td>
<td>3·6 (+)</td>
<td>1·2 (?)</td>
<td>0 (−)</td>
<td></td>
</tr>
</tbody>
</table>

†Fore-milk samples
+ = Luteal-phase or pregnant; − = follicular phase; ? = within ± 2 s.d. of discriminating level.

palpation per rectum 8 weeks after AI in 17 of the 18 cows: the other was sold and further data were not available.

c) Confirmation using calving data. Calving dates were obtained from a further 110 cows in 4 commercial herds for which EIA had been used for pregnancy testing at 24 days after AI. Of 62 diagnoses of pregnancy, 58 (93.5%) were shown to be compatible with subsequent calving data assuming a gestation length of 280 ± 10 days (Salisbury and Vandemark, 1961). The 46 cows diagnosed non-pregnant were shown to have calved to a subsequent service, demonstrating that the conclusion was correct.

8.4 DISCUSSION

The progesterone concentrations determined through the oestrous cycle by the homologous EIA are illustrated by results obtained from cows in the experimental group in farm B (Fig. 8.1). The luteal phase concentrations were generally higher than those measured by RIA elsewhere (Hoffmann and Hamburger, 1973; van de Wiel et al., 1978; Bulman and Lamming, 1979; Batra et al., 1980). Low levels indicative of a return to oestrus were consistently similar, however. The disparity seen at higher concentrations may
be explained in part by the different cross-reactivities exhibited when RIA and homologous EIA were compared using the same anti-progesterone γ-globulin preparation (see Sauer et al., 1982b, in reprint appendix). The higher levels may thus reflect the varying concentrations of other steroids during the oestrous cycle. This could also explain the detection of a transient peak of progesterone-like material around the time of oestrus in a number of cows (e.g. nos. 51, 247 and 251 in Fig. 8.1). The latter occurrences have been detected by others using RIA (Bulman and Lamming, 1979; Bloomfield, 1982); the nature of the substance, if not progesterone, has not been determined, however.

Although these anomalies did not affect the outcome of this particular application they raised serious questions as to specificity of the homologous assay. It was clear that this would not be acceptable where only single samples are taken (e.g. confirmation of follicular phase or pregnancy testing).

The results from the application of the homologous EIA suggested that the majority of cattle were fertile when inseminated three days after the regression of the corpus luteum had been identified by falling concentration of milk progesterone. This conclusion confirmed the early work of Ball and Jackson (1979) who similarly determined the approach of oestrus by progesterone RIA but inseminated at both two and three days after the fall. It may be implied that either most cows ovulate and are fertile at a closely defined time after natural luteolysis or that spermatozoa have a considerable life span after insemination. The results described here also suggest that the natural oestrous cycle can be monitored and successful insemination made at a time independent of behavioural observations. Under this procedure early indication may also be given of abnormalities of ovarian function that would preclude fertility.

The study employing the heterologous EIA to monitor corpus luteum
function during hormone treatment suggests that a short, seven day, treatment with PRID combined with cloprostenol injection on the sixth day can achieve effective synchronization and satisfactory fertility in dairy cows following fixed time AI. Smith et al (1984) recently observed increased synchrony of oestrus with excellent fertility (70% pregnancy rate by palpation per rectum) in heifers following a similar seven-day regimen in which prostaglandin was injected one day before PRID removal, compared with injection at the time of withdrawal. The uniformity of progesterone patterns about the time of insemination and the pregnancy rate achieved in this smaller group of cows is in full agreement with their work.

The use of the heterologous progesterone EIA enabled corpus luteum function to be clearly established throughout the treatment period. The erratic variations in progesterone concentrations observed occasionally using the homologous assay (Fig. 8.1) during the luteal phase and around oestrus in the oestrus prediction experiment were not observed for any animal in this study. This clearly indicated the greater reliability of the heterologous assays since the cyclic patterns and concentrations of progesterone in milk closely followed those established using RIA by other groups (Hoffmann and Hamburger, 1973; Bulman and Lamming, 1978; Batra et al, 1980).

The combination of precision, specificity and accuracy demonstrated for the heterologous EIA allowed a ready distinction to be made between luteal and follicular-phase concentrations of progesterone in whole milk. This was illustrated by the results obtained by assay of milk samples taken for pregnancy testing 22 - 26 days after service, although the correlations between results from RIA and EIA for the 200 samples obtained from the Milk Marketing Board were lower \( r = 0.890 \) for the MMB RIA and \( r = 0.833 \) for the RIA performed here) than those seen when 25 milk samples were assayed here \( r = 0.933; \) Fig. 7.4a) as part of the validation study. The delay in
re-assaying samples may have allowed changes in the structure of milk to occur that affected the performance of the EIA more than the RIA. Some evidence is available for this since freshly prepared milk standards reduce binding in the calibration curve perceptibly more than standards prepared 1 month previously. This is a difficult phenomenon to investigate as it would require that new standards be prepared from a fresh batch of milk whose composition may differ from the original and thus allow no absolute control for the ageing process. Since milk samples submitted for assay will usually be of recent origin, it may be a wise precaution to renew milk standards at least at monthly intervals. This apparent difficulty did not, however, prevent a high degree of correspondence between the conclusions drawn from the various assays. Such disparities as there were revolved around samples showing concentrations of progesterone near the discrimination points of the assays, so that one or other test was inconclusive. Only 5 samples (Table 8.4) produced results for which the deductions from the assays differed and 3 of these were fore-milk samples which have previously been shown to produce results that may be variable in some circumstances (Holdsworth, Booth, Sharman and Rattray, 1980). EIA of one of the two whole milk samples (No. 81) produced a value consistent with the luteal phase and was from a cow in which the occurrence of behavioural oestrus was confirmed by the veterinary surgeon. Rectal palpation indicated that the other sample (No. 108) was correctly identified by EIA and RIA as being from a cow in the luteal phase. It remains possible that the single real discrepancy (No. 81) in this study represents detection of progesterone-like material seen in the earlier study (see section 8.3.1.). Preliminary investigations using the HPLC system described in section 7.2.1f) indicated that sample No. 81 contains a cross-reacting component which is more polar than progesterone: the backflush fraction of the sample extract produced considerable binding
<table>
<thead>
<tr>
<th>APPLICATION</th>
<th>SAMPLING PROTOCOL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Establishing the onset of ovarian cyclicity prior to day 42 post-partum.</td>
<td>At 7 day intervals.</td>
</tr>
<tr>
<td>2. Estimating the period of oestrus (with observation of heat).</td>
<td>At 2 day intervals.</td>
</tr>
<tr>
<td>3. Prediction of optimum time for AI (without observation of heat)*.</td>
<td>Daily from day 16 of the oestrous cycle.</td>
</tr>
<tr>
<td>4. Confirmation of follicular phase pre- or post-AI.</td>
<td>Day of AI.</td>
</tr>
<tr>
<td>i) Assurance where oestrus behaviour is indistinct.</td>
<td></td>
</tr>
<tr>
<td>ii) Assessment of heat detection efficiency</td>
<td></td>
</tr>
<tr>
<td>5. Early detection of failure to conceive or maintain pregnancy prior to next oestrous period.</td>
<td>At day 19 post AI and, if progesterone concentrations are high, every 2 days until day 25.</td>
</tr>
<tr>
<td>6. Pregnancy testing.</td>
<td>24 – 26 days post-AI.</td>
</tr>
<tr>
<td>8. To establish precisely the stage of the oestrous cycle of donor and recipient prior to embryo transfer.</td>
<td>Daily from day 16 – 17 of cycle prior to intended transfer.</td>
</tr>
<tr>
<td>9. To estimate the efficacy of hormone treatment for superovulation.</td>
<td>At 6 – 10 days post-ovulation?</td>
</tr>
<tr>
<td>10. To enable assessment of ovarian dysfunction and objective administration of hormone therapy.</td>
<td>At 7 day intervals.</td>
</tr>
</tbody>
</table>

* day 0 = day of oestrus. + For normally cycling cows, insemination on the third day on which progesterone concentrations are low (< 5.0ng/ml whole milk) following measurement of high concentrations (> 5.0ng/ml). ? = to be determined.
inhibition when assayed by EIA.

Calving data, however, obtained from a further 110 cows in 4 commercial herds, of which 62 were determined as producing a positive test result by EIA, showed no evidence of this phenomenon. The 4 cows that did not calve to the expected insemination returned to service and were re-inseminated 31, 113, 160 and 161 days later. None of these intervals is compatible with a single cycle occurring after service and it is suggested that the luteal phase was prolonged in each of these cows. Similarly, no unexpectedly high values were seen during the follicular phase in the study (section 8.3.2.) in which oestrous cycles of 29 cows were monitored by EIA of milk samples taken each day.

These studies clearly indicate the value of the heterologous EIA in the management of reproductive performance of dairy cattle. Further applications are envisaged, many of which would be particularly appropriate if the assay was in a semi-quantitative form providing a visual end-point. Such applications are outlined in Table 8.5.

8.5 CONCLUSIONS

1. In cyclic cows, oestrus detection rates of 100 per cent may be achieved by monitoring milk progesterone concentrations. Using AI, the fertility of cows inseminated at oestrus periods predicted in this way is similar to that achieved at observed oestrus.

2. The homologous EIA was neither sensitive nor reliable enough for general application.

3. The heterologous EIA provided a fast, reliable and accurate means of pregnancy testing and monitoring corpus luteum activity.
CHAPTER 9:
GENERAL DISCUSSION, CONCLUSION AND PROSPECTS
CHAPTER 9. GENERAL DISCUSSION, CONCLUSION AND PROSPECTS

The present studies were aimed at providing a colorimetric direct EIA for progesterone in milk equivalent in performance to RIA. It was prerequisite that the assay be simple and sensitive enough to be adapted for use in a basic laboratory or in the farm office. Such a procedure would allow widespread application of the detailed knowledge of bovine reproductive physiology previously acquired through studies employing RIA.

These goals have been met as a result of the following achievements.

1. The synthesis of labels which were highly effective in EIA was made possible through the development of a new procedure enabling efficient condensation of steroid with enzyme without significant co-conjugation or denaturation of the enzyme. The procedure involved formation of an intermediate N-hydroxysuccinimide ester of the steroid using dicyclohexylcarbodiimide or disuccinimidyl carbonate. Such conjugates enabled, for the first time, development of a direct addition (homologous) EIA for progesterone in whole milk (see table 9.1).

2. The factors responsible for the poor sensitivity of the homologous EIA were thoroughly investigated. The most important of these were found to be the phenomenon of bridge recognition, the temperature at which the immuno-assay was performed and the enzyme used as label. The problem of bridge recognition was solved by use of bridge heterology. A new approach was demonstrated to be highly effective and involved substitution of a glucuronide bridge for a hemisuccinate bridge to link the steroid and enzyme while retaining the progesterone 11α-hemisuccinate antiserum: the improvements in sensitivity derived were independent of the antiserum or enzyme label used. This had not been demonstrated for any other heterologous combination. It seems probable that the same bridge combination would prove useful for the EIA of other steroid hormones.

The use of efficient labelling procedures, optimized molar ratio of steroid:
<table>
<thead>
<tr>
<th>BODY FLUID</th>
<th>SPECIES</th>
<th>DIRECT (D) OR EXTRATION (E)</th>
<th>ENZYME</th>
<th>BRIDGE</th>
<th>SEPARATION TECHNIQUE</th>
<th>DETECTION LIMIT (pg/assay)</th>
<th>ED₅₀* (pg)</th>
<th>ASSAY CV (%) WITHIN</th>
<th>ASSAY CV (%) BETWEEN</th>
<th>AUTHORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHOLE</td>
<td>Bovine</td>
<td>D</td>
<td>β-GAL</td>
<td>homol</td>
<td>S.P. mtp</td>
<td>5 300</td>
<td>4.7</td>
<td>-</td>
<td>-</td>
<td>Sevar et al, 1981</td>
</tr>
<tr>
<td>MILK</td>
<td></td>
<td>D</td>
<td>β-GAL</td>
<td>homol</td>
<td>S.P. cell</td>
<td>20 500</td>
<td>3.2</td>
<td>-</td>
<td>-</td>
<td>Sevar et al, 1981</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>PEROX</td>
<td>homol</td>
<td>S.P. paper</td>
<td>250 2500</td>
<td>10.7 - 20.0</td>
<td>21.9 - 28.5</td>
<td>Chang &amp; Estergreen, 1984</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>PEROX</td>
<td>homol</td>
<td>S.P. mtp</td>
<td>0 - 0.9 33</td>
<td>8.0 - 12.6</td>
<td>8.4 - 13.4</td>
<td>Cleere et al, 1985</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>DASP</td>
<td>20 &gt;1000</td>
<td>1.3 - 3.3</td>
<td>10.0</td>
<td>van de Wiel et al, 1982</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>PEROX</td>
<td>heterol</td>
<td>S.P. mtp</td>
<td>1 - 2  7.5 - 10.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>van de Wiel &amp; Koops, 1982</td>
</tr>
<tr>
<td>SKIM</td>
<td></td>
<td>D</td>
<td>β-GAL</td>
<td>homol</td>
<td>D.A.</td>
<td>4 600</td>
<td>10.7</td>
<td>8.6</td>
<td>Morino et al, 1984</td>
<td></td>
</tr>
<tr>
<td>MILK</td>
<td></td>
<td>E</td>
<td>β-GAL</td>
<td>homol</td>
<td>D.A.</td>
<td>10 &gt;1000</td>
<td>0.8 - 4.9</td>
<td>6.7 - 13.2</td>
<td>Nakao et al, 1983</td>
<td></td>
</tr>
<tr>
<td>CREAM</td>
<td></td>
<td>D</td>
<td>β-GAL</td>
<td>homol</td>
<td>D.A.</td>
<td>12 &gt;1000</td>
<td>-</td>
<td>-</td>
<td>Nakao &amp; Kawaoka, 1980</td>
<td></td>
</tr>
<tr>
<td>MILK</td>
<td></td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>S.P. cell</td>
<td>30 280</td>
<td>4.7 - 7.7</td>
<td>7.4 - 9.2</td>
<td>Amstad &amp; Cleere, 1981</td>
<td></td>
</tr>
<tr>
<td>FAT</td>
<td></td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>S.P. cell</td>
<td>- 94</td>
<td>-</td>
<td>-</td>
<td>Amstad &amp; Cleere, 1981</td>
<td></td>
</tr>
<tr>
<td>BLOOD</td>
<td>Porcine</td>
<td>E</td>
<td>β-GAL</td>
<td>homol</td>
<td>D.A.</td>
<td>-  &gt;1000</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Johsen et al, 1980</td>
<td></td>
</tr>
<tr>
<td>SERUM</td>
<td>Bovine</td>
<td>E</td>
<td>β-GAL</td>
<td>homol</td>
<td>D.A.</td>
<td>12 600</td>
<td>6.7 - 9.4</td>
<td>4.7 - 8.7</td>
<td>Nakao, 1980</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>S.P. cell</td>
<td>- 150</td>
<td>4.3 - 8.0</td>
<td>4.9</td>
<td>Soli et al, 1982</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>S.P. mtp</td>
<td>0.5  &gt;1000</td>
<td>2.7 - 9.0</td>
<td>-</td>
<td>-</td>
<td>Wang et al, 1984</td>
<td></td>
</tr>
<tr>
<td>BLOOD</td>
<td>Bovine</td>
<td>D</td>
<td>ALK PHOS</td>
<td>heterol</td>
<td>S.P. mtp</td>
<td>8.0 50</td>
<td>6.5</td>
<td>57.0 - 12.3</td>
<td>Boland et al, 1985</td>
<td></td>
</tr>
<tr>
<td>PLASMA</td>
<td>Rat</td>
<td>E</td>
<td>β-GAL</td>
<td>homol</td>
<td>D.A.</td>
<td>15 70</td>
<td>8.7</td>
<td>9.7</td>
<td>Dray et al, 1975</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>D.A.</td>
<td>10 100</td>
<td>8.7</td>
<td>9.7</td>
<td>Freyman et al, 1982</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swamp</td>
<td>Buffalo</td>
<td>E</td>
<td>β-GAL</td>
<td>homol</td>
<td>S.P. asp</td>
<td>- 90</td>
<td>1.0 - 5.0</td>
<td>3.0 - 7.0</td>
<td>Gros et al, 1978</td>
<td></td>
</tr>
<tr>
<td>Bovine/Ovine</td>
<td></td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>DASP</td>
<td>125 4000</td>
<td>7.0 - 7.7</td>
<td>11.0 - 18.0</td>
<td>Joyce et al, 1977</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>D.A.</td>
<td>10 300</td>
<td>6.1 - 9.2</td>
<td>11.2 - 15.0</td>
<td>Joyce et al, 1978a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swamp</td>
<td>Buffalo</td>
<td>E</td>
<td>PEROX</td>
<td>homol</td>
<td>DASP</td>
<td>50 &gt;700</td>
<td>13.6</td>
<td>13.5</td>
<td>Kamonpatana et al, 1979</td>
<td></td>
</tr>
<tr>
<td>SALIVA</td>
<td>Human</td>
<td>D</td>
<td>PEROX</td>
<td>homol</td>
<td>S.P. mtp</td>
<td>0.25 60</td>
<td>4.9 - 10.5</td>
<td>8.7 - 14.5</td>
<td>Munro &amp; Stabenfeldt, 1984</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Equine</td>
<td>E</td>
<td>β-GAL</td>
<td>homol</td>
<td>DASP</td>
<td>55 200</td>
<td>10.6</td>
<td>-</td>
<td>Patricot et al, 1978</td>
<td></td>
</tr>
</tbody>
</table>

PEROX = Peroxidase; β-GAL = β-Galactosidase; ALK.PHOS = Alkaline Phosphatase; S.P. = Solid Phase; Paper = Blotter Paper; mtp = Microtitre Plate; cell = Cellulose; D.A. = Double Antibody; DASP = Double antibody solid phase; seph = Sepharose; tube = Polystyrene test tube; * The Mass of progesterone (pg) giving rise to a 50% reduction in label binding; values were estimated from calibration curves. ? Details not given.
enzyme at conjugation and optimized methods of antibody adsorption to microtitre plates enabled practical and economic benefit to be derived from the increased sensitivity achieved by performing EIA at ambient temperature rather than 40°C. It was previously necessary to use a high incubation temperature (40°C) in order to ensure adequate label binding.

Alkaline phosphatase and penicillinase had not previously been used as labels in the EIA of progesterone and in these studies provided the most sensitive and suitable procedures. Of the four enzymes investigated, the assay for β-galactosidase was least sensitive. Difficulties in the efficient formation of heterologous conjugates with peroxidase, in combination with the potential toxicity associated with its assay, militated against further evaluation of its use in EIA. Alkaline phosphatase was selected for further development since, unlike penicillinase, a chromogenic substrate was available which greatly facilitated the enzyme assay. The optimum molar excess of steroid required for conjugate formation was dependent upon the enzyme used: it is suggested that this is related to the relative accessibility and reactivity of particular lysine ε-amino residues. In contrast with conventional dogma, steric considerations dictate that the optimum molar incorporation ratio of steroid in a particular enzyme might not be 1:1, but will be influenced by the location and orientation of the most reactive residues as this will influence their subsequent ability to bind with antibody.

3. Although microtitre plate wells have provided a convenient immuno-sorbent for the determination of antigen and antibody concentrations in ELISA, their application to hapten EIA had not been established at the start of these investigations and is only now becoming accepted (Sauer et al., 1981; Foulkes et al., 1982; Sauer et al., 1982b; van de Wiel and Koops, 1982; Munro and Stabenfeldt, 1984; Tallon et al., 1984; Cleere, Gosling, Morris et al., 1985; Sauer, Foulkes, Worsfold and Morris, 1986). The
particular contributions made to the use of microtitre plates has been in establishing optimum conditions for passive adsorption of antibodies to the well surface and in demonstrating that this system is precise and accurate enough to allow quantitative determination of haptens.

The optimum molarity and pH of the buffer established for antibody adsorption (0.17mM acetate buffer, pH 5.0) was far removed from those generally used elsewhere (0.05M carbonate buffer, pH 9.6) and stressed the importance of assessing optimum conditions for the adsorption of particular antibodies and antigens. At pH 4 - 5 γ-globulin would be expected to have a net positive charge facilitating coulombic (electrostatic) attraction to the well surface prior to the establishment of hydrophobic binding. Pre-washing wells with detergent significantly increased the degree and precision of adsorption to polyvinyl. These conditions promoted more economic use of antisera and made possible more rapid assays by increasing the quantity of antibody adsorbed. Cleere et al (1985) have shown that the degree and precision of adsorption of antibody can also be achieved by addition of progesterone antibody (rabbit) to wells pre-coated with anti-rabbit-IgG antibody.

4. The validity of the heterologous milk progesterone EIA has been established: precision, sensitivity, analytical recovery and linearity of response of the assay compared favourably with RIA and other procedures described for milk progesterone EIA (table 9.1).

The EIA of progesterone isolated from milk enabled the specificity of the assay to be assessed. The ODS column method used for steroid extraction offered practical advantages over solvent partition procedures. The high resolution HPLC system developed enabled isolation of progesterone from milk extract in at least one tenth the time described in other procedures of comparable resolution.

5. The majority of EIA procedures previously employed for the assay of
progesterone in milk involved prior extraction of milk or a milk fraction (see table 9.1) and with one exception (van de Wiel and Koops, 1982), are homologous procedures, lacking sensitivity and offering few practical advantages over RIA.

The direct, heterologous microtitre plate EIA described in abstract by van de Wiel and Koops (1982, see table 9.1) and the heterologous assay described here show very similar specifications. More recently, Cleere et al. (1985) have described a direct homologous microtitre plate assay of comparable performance, demonstrating that bridge recognition may not be a problem with particular antisera. The EIA described by van de Wiel and Koops (1982) provides an end-point (red/brown colour) giving a visual distinction between samples from different stages of the oestrous cycle and providing a semi-quantitative test. Although this can be achieved with the present test, the yellow end-point is not ideal and use of alternative chromogenic substrates such as described by McComb, Bowers and Posen (1979) could resolve this point.

6. The homologous EIA demonstrated the practicality of applying milk progesterone EIA on the day of sampling although unexpected results were found during the follicular phase. The heterologous assay eliminated this problem and proved to be as reliable as RIA when used for monitoring corpus luteum function in dairy cattle and for pregnancy testing. Practical applications envisaged for the assay are shown in Table 8.5. Some of these applications are relevant to the management of reproduction in sheep and pigs and preliminary studies indicate that the heterologous procedure may be readily adapted to the assay of whole blood and plasma samples from these species.
The heterologous EIA was designed for the assay of large numbers of samples (≈ 30 - 150 samples per day, assayed in duplicate) and the time taken for its performance (≈ 5 hours) reflects this. For smaller numbers of samples, shorter assay times could be accommodated largely by increasing the mass of conjugate and the volume of milk used in the assay although this would involve a compromise with assay sensitivity.

Adaptations of the heterologous assay have enabled quantitative and semi-quantitative visual tests to become commercially available for use on-farm or at the veterinary practice: practical aspects of these tests, when used by farmers, are currently being evaluated (B. Drew and P. Lane, personal communication) and trials are in progress aimed at providing an analysis of the cost benefits of the test when used regularly as part of a breeding management programme (B. Drew and J.A. Foulkes, personal communication.)

It must be recognised that problems will arise when the test is used for other than oestrus confirmation or pregnancy testing by individuals not conversant with the endocrine changes which form the basis of the particular application. In these circumstances, consultation with an informed veterinarian or livestock advisory officer would resolve the problem. In the longer term, the institution of courses within the regular curriculum at agricultural colleges would be appropriate. This would also ensure that new generations of dairy farmers are aware of how best to achieve the commercial benefits arising from the close control of fertility.

EIAs such as developed in these studies are currently the most suitable and convenient means of progesterone assay for use in non-specialized laboratories or on the farm; however, developments towards faster procedures involving fewer steps are inevitable. Alternative non-isotopic immuno-assays involving luminescence or fluorescence measurements may provide equivalent or greater sensitivity (Collins, 1984) but require sophisticated
Simplification of EIA could be achieved by development of label displacement EIA (use of pre-formed antibody-bound label) or homogeneous EIA, although existing procedures are generally of insufficient sensitivity for the present application. As hybridomas become increasingly used for antibody production, however, rapid screening techniques will improve the possibility of finding antibodies with properties which best suit these assay systems.

Other forms of immunoassay such as those based on the agglutination of gold sols (Leuvring, Thal, White and Schuurs, 1981) or release of liposome entrapped dye (O'Connel, Campbell, Fleming et al., 1985) will enable a visual end-point to be achieved without the use of enzymes but again, require further development to provide adequate sensitivity.

Ideally, however, progesterone determination would be made directly in the milk collection jar without intervention by the dairyman. The indications are that biosensor technology may eventually provide such a procedure since the principle of using changes in evanescent wave patterns generated in coated optical sensors to detect molecular interactions (e.g. between antigen and antibody) has already been established (Albery, Hagget and Snook, 1986): such a system would have no requirements for labelled analyte.


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In all mammals studied, progesterone is essential for the maintenance of early pregnancy and in most species, including the cow (Hawk, Brinsfield, Turner, Whitmore & Norcross, 1963) it is also pre-requisite for the implantation or attachment of the conceptus (Davis & Ryan, 1972).

In cattle the continued presence of circulating progesterone is achieved by cessation of cyclic activity through prolongation of the lifespan of the corpus luteum formed in the cycle of conception. The means by which this is brought about has not been established: the corpus luteum of the cow is not simply retrieved by a foeto-placental luteotrophin as appears to be the case with primates (see Perry, Heap, Burton & Gadsby, 1976). Nevertheless, luteal maintenance is dependent upon the presence of the conceptus in the uterus since the cow does not undergo pseudo-pregnancy in the absence of conception after mating. From evidence derived from sheep, it would seem that if gonadotrophins originating from the conceptus do arise around this time their role may well be secondary to that of the pituitary hormones since hypophysectomy in early pregnancy promptly leads to the demise of the corpus luteum and pregnancy failure. (Denamur & Martinet, 1961; Denamur, 1968; Heap et al, 1979).

Although the nature of the signal emanating from the conceptus remains to be determined, a pattern of events associated with the maintenance of corpus luteum activity during early pregnancy has emerged.
A significant increase in plasma progesterone concentration in pregnant compared with non-pregnant cattle has been reported from as early as day 9 after oestrus (Henricks, Lamont, Hill & Dickey, 1975; Bulman & Lamming, 1978; Lukaszowska & Hansel, 1980) and the decline in concentrations normally seen by day 17-18 of the cycle does not occur (Shemesh, Ayalon & Lindner, 1968; Hansel & Echternkamp, 1972).

It has been shown that transfer of embryos (Betteridge, Mitchel, Englesome & Randall, 1976; Betteridge, Randall, Englesome & Sugden, 1984), embryonic homogenates (Northey & French, 1980), trophoblastic vesicles (Heyman, Camous, Meziou & Martal, 1984) or trophoblast cells (Plante, Bosquet, Guay & Goff, 1984) into the uterus of cyclic cows can result in extension of the lifespan of the corpus luteum. The embryonic signal resulting in maintenance of luteal activity must, however, be present prior to day 17 as shown by the transfer of bovine embryos to cows at different stages of the cycle (Betteridge et al, 1976) and by flushing embryos from the uterine tracts of cows at different stages of pregnancy (Northey & French, 1980; Dalla Porta & Humblot, 1983; Humblot & Dalla Porta, 1984).

Current in vitro evidence indicates that the conceptus may exert a direct luteotrophic effect on the corpus luteum: conflicting data may perhaps be related to differences in sensitivity and specificity of the means of assessment. Using the rat seminal vesicle bioassay, Foster (1956) failed to show gonadotrophic activity in the blood and urine of pregnant cows. Lunnen & Foote (1967), however, showed a significant response in the rat ventral prostate bioassay with extracts of bovine maternal plasma and foetal cotyledons. Human chorionic gonadotrophin (HCG)-like activity has been detected in day 16 bovine blastocysts using a radioreceptor assay (Shemesh, 1980) and in maternal and foetal cotyledons using a radioreceptor assay or rat heydig cell and bovine granulosa cell bioassay (Ailenburg & Shemesh, 1983). Similarly, Beal, Lukaszewska & Hansel (1981) have demonstrated the ability of day 18 bovine blastocyte homogenates and
extracts to stimulate progesterone synthesis by dispersed bovine luteal cells. The latter study indicated that the luteotrophic substance(s) was heat labile and since it had a molecular weight (by dialysis) less than 12,000 daltons that it was not LH. Subsequent studies by Henricks & Poffenbarger (1984) using a highly specific mouse heydig cell bioassay confirmed that day 16-20 bovine conceptus do not contain LH activity or form it on incubation.

The corpus luteum is still capable of synthesizing progesterone in vitro in response to incubation with LH at day 18 of the cycle as it is in pregnancy, indicating that it is not increased responsiveness to LH which is directly responsible for luteal maintenance (Lukaszowska & Hansel, 1980).

No clear pattern has yet emerged to indicate whether a luteotrophic or antiluteolytic signal is operating in vivo or whether such a signal operates directly on the corpus luteum or via the uterus or any combination of these routes. It is apparent, though, that at least part of the effect of the conceptus is expressed locally since the corpus luteum is more readily maintained on the side ipsilateral rather than contralateral to an isolated gravid horn (Del Campo, Rowe, French & Ginther, 1977; Ginther, 1981). Given the localized counter current mechanism described earlier for luteal regression it is to be expected that one of the factors involved in luteal maintenance would be of low molecular weight, lipid soluble (ease of diffusion through blood vessel walls) and rapidly metabolizable (localization of effect), as is the luteolysin PGF$_2$$\alpha$.

It is thought that uterine release of PGF$_2$$\alpha$ is responsible for luteolysis during the oestrous cycle. It appears that day 18 pregnant cows show diminished endometrial secretion of PGF$_2$$\alpha$ (Lukaszowska & Hansel, 1980; Thatcher et al, 1984) but it is not clear whether a mechanism involving depression of PGF$_2$$\alpha$ concentrations reaching the ipsilateral corpus luteum via the ovarian vein occurs and is operating
in the prevention of luteolysis. Although Lukaszowska and Hansel (1980) found no differences between ovarian, arterial and jugular vein concentrations of PGF$_2\alpha$, Thatcher et al (1984) found evidence for a small net reduction in ovarian arterial concentration in pregnant compared with non-pregnant cows.

Recent evidence has indicated a possible role for prostaglandins in abrogating the luteolytic influence of PGF$_2\alpha$ in early pregnancy in cattle. These findings are in keeping with previous observations in various tissues that the different classes of prostaglandin such as the F and E series appear to have opposing effects in the same tissues (Kuehl, Cirillo, Hams & Humens, 1973). Work in sheep initially indicated that PGE$_2$ may antagonize the luteolytic action of PGF$_2\alpha$ in vivo and thus potentially act as an antiluteolysin (Henderson, Scaramuzzi & Baird, 1977; Pratt, Butcher & Inskeep, 1977) and that higher concentrations of PGE$_2$ are present in the utero-ovarian venous plasma of pregnant rather than non-pregnant sheep at the time of maternal recognition of pregnancy (Silvia, Ottobre & Inskeep, 1984).

A similar function is plausible in cattle since secretion of progesterone by bovine luteal tissue in vitro is stimulated by PGE$_2$ (Marsh, 1970) and intra-uterine infusion of PGE$_2$ between days 9-21 of the cycle results in extension of corpus luteum function and in delayed onset of oestrus (Chenault, 1983; Gimenez & Henricks, 1983). The demonstration that bovine embryos possess a considerable capacity for the synthesis and secretion of PGE$_2$ (Shemesh, Milaguir, Agalon & Hansel, 1979; Lewis, Thatcher, Bazer & Curl, 1982) introduces the attractive theory that such secretions may be involved in a local pathway resulting in the maintenance of the function of the ipsilateral corpus luteum. Reports by Kimball and Lauderdale (1975) and Milvaeh and Hansel (1980) on the ability of PGE$_1$ and PGI$_2$ respectively to increase plasma progesterone concentrations when administered in vivo, however, serve as a caution as to the validity of such a theory given the lack of specificity of the prostaglandins in this effect.
In the pig, it is thought that oestrogens produced and secreted by the blastocyst play a major role in corpus luteum maintenance. Evidence has been compiled suggesting that blastocyst oestrogens may operate at the level of the uterus, the ovary and the hypothalamus or pituitary. Oestrogens have been shown to produce an antiluteolytic influence at the level of the uterus involving inhibition of secretion of PGF\(_{2\alpha}\) into the uterine vein. Additionally, luteotrophic affects have been demonstrated which may involve direct antagonism of the luteolytic influence of PGF\(_{2\alpha}\) in the corpus luteum, stimulation of LH release from the pituitary or both (see Heap, Flint & Gadsby, 1979).

It seems that blastocyst oestrogens are unlikely to play a similar role in pregnancy recognition in cattle, however. Although the bovine conceptus is capable of metabolizing steroids such as progesterone and androstenedione (Gadsby, 1982), conversion into oestrogens in vitro appears to be minimal when comparisons are made with porcine blastocysts (Shemesh et al., 1979; Gadsby, 1982; Eley, Thatcher, Bazer & Fields, 1983). A direct luteotrophic effect of conceptus oestrogens seems improbable, since, in contrast with the sow, administration of oestrogens during the bovine luteal phase results in shortening of the lifespan of the corpus luteum (Wiltbank, Ingalls & Rowden, 1961; Eley, Thatcher & Bazer, 1979).

Oestradiol levels have, however, been shown to be elevated in the uterine lumenal fluid and in uterine venous blood at the time of pregnancy recognition (Ford, Chenault, Christenson, Echternkamp & Ford, 1981). They may be responsible for the transient increase in uterine blood flow occurring at this time and which is associated only with the uterine horn containing the conceptus (Ford, Chenault & Echternkamp, 1979). It has been suggested that this effect may function to enhance transport of luteotrophin to the ovary bearing the corpus luteum.

Although numerous proteins or macromolecules have been isolated which are secreted by the bovine conceptus or temporarily associated with this critical early stage of pregnancy (Roberts & Parker, 1976; Laster, 1977;
Butler, Hamilton, Sasser, Ruder, Hass & Williams, 1982; Masters, Roberts, Lewis, Thatcher, Bazer & Godkin, 1982) they have not been demonstrated to influence ovarian function, nor has any other particular function been ascribed to them.
APPENDIX 2. BASIC PROGRAMMES FOR COMMODORE PET 2001 SERIES MICROPROCESSOR.

Calculation of weighted linear regression with errors in X and Y.

i) Linear regression analysis: calculation of regression of X on Y then Y on X.

```plaintext
10 OPEN3,4
12 PRINT"INPUT NO. OF PAIRS":INPUTS
15 DIMA(S):DIMB(S):DIMC(S):DIMD(S)
16 DIME(S):DIMF(S):DIMG(S):DIMH(S):DIMR(S)
50 FORN=1 TO S:PRINT "INPUT X , Y":INPUTA(N),RCN)
100 B=B+A(N), T=T+R(N), C(N)=A(N)*R(N), D=D+C(N)
150 E(N)=A(N)^2; F=F+E(N); G(N)=R(N)^2; H=H+G(N)
200 NEXTN
250 I=(B*T)/S; J=(B^2)/S
300 K=H-(T^2)/S; L=(D-I)/(J*K)^0.5
350 M=(D-I)/(F-(B^2)/S); N=(T-(M*B))/S
361 FORAC=1 TO 3
362 PRINT£3:NEXTAC
400 PRINT"CORR. COEFF."; L; PRINT£3,
410 PRINT"SLOPE Y ON X ": M; PRINT£3,
420 PRINT"INTERCEPT": N; PRINT£3,
430 PRINT£3,
440 PRINT£3,
1120 PRINT
READY.
```

ii) Weighted linear regression: calculation of corrected slope b) and intercept a).

```plaintext
2 OPEN3,4
5 PRINT"REGRESSION WITH ERROR IN X & Y"
7 INPUT"COMPARISON":T$,
10 INPUT"S.D.Y":SY;VY=SY^2
20 INPUT"S.D.X":SX;VX=SX^2
30 L=VY/VX;PRINT"L="L
40 PRINT"SLOPE Y ON X":B1
50 PRINT"SLOPE X ON Y":B3;B2=1/B3
60 N1=(B2-(L/B1))
70 N2=(N1+((4*L)+(N1^2))^0.5)/2
80 PRINT"CORRECTED SLOPE=N2"
90 INPUT"MEAN X":X
100 INPUT"MEAN Y":Y
110 A=Y-(N2*X)
120 PRINT"INTERCEPT=A"
130 PRINTA="Y-"("N2*X")"
132 PRINT£3,T$
133 PRINT£3,"CORR SLOPE=N2,"CORR INT="A
140 GOTO5
READY.
```
iii) Compatibility of weighted regression of methods with the line of identity (slope = 1, intercept = 0).

READY.

4 OPEN3, 4
5 PRINT"F TEST FOR LINEARITY"
6 PRINT3, "F-TEST FOR LINEARITY"
7 INPUT"COMPARISON"; T$
10 INPUT"CORRECTED SLOPE"; BL
20 INPUT"CORRECTED INTERCEPT"; A
22 INPUT"S.D. X"; SX
25 INPUT"S.D. Y"; SY
30 INPUT"NO. OF POINTS"; N1
35 T=0; SS=0; P=0; RR=0
40 FORN=1 TON1
50 INPUT"X,Y"; X, Y
65 S=((SY^2)+((BL^2)*(SX^2)))
70 SS=(((Y-(X*BL)-A)^2)/S)
95 R=(((SY^2)+((1^2)*(SX^2)))
100 RR=(((Y-(X*1)-0)^2)/R)
104 T=T+SS
105 P=P+RR
110 NEXT
120 F1=((P-T)/2)
130 F2=(P/(N1-2))
135 F=F1/F2
140 PRINT"F="F,"DF=2&N1-2
150 INPUT"SIGNIFICANCE="; S$
160 PRINT3, T$
170 PRINT3, "F="F,"DF=2&N-2,S$
180 GOTO7

READY.
Calculation of progesterone concentrations in samples using a four parameter logistic function (Rodbard, 1978).

```
APPENDIX 3. BASIC PROGRAMME FOR COMMODORE PET 2001 SERIES MICROPROCESSOR.

Calculation of progesterone concentrations in samples using a four parameter logistic function (Rodbard, 1978).

READY.

2 PRINT" DATA STORE
3 PRINT" 7777777777
5 DIMA(12,9):DIMZ(10,10):OPEN2,6:PRINT"DATE"
6 INPUTD$:PRINT"FILE NO.";INPUTF
7 PRINT"BLANK ON ZERO AND RUN PLATE READER
10 DIMD9(12)
20 GET€2,A$:IFA="M"THEN50
40 GOTO20
50 PRINTA$:FORD=6700TD8175
60 GET€2,A$:A=ASC(A$):POKEN,A
80 IFST<>2THENPRINTA$:NEXTN:GOTO100
85 GOTO60
100 REM REMOVE CR
180 K=0:FORN=6700TD8160:A=PEEK(N):IFA=13THEN300
190 POKE(15200+K),A:K=K+1
300 NEXTN
305 REM REMOVE LETTERS
310 FORM=152007016555s A=PEEK(NTsIFA=64THENA=A-16
320 POKEN,NT:NEXTN
330 FORM=15210TD81540STEP14
360 A=PEEK(N):IFA=32THEN1=0:GOTO380
370 D1=A-48
380 D2=PEEK(N+1)-48:D3=PEEK(N+3)-48
400 A=PEEK(N+8):IFA=32THENB=2:GOTO470
410 D4=PEEK(N+B)-48:D5=(PEEK(N+10)-48)/10
420 D6=(PEEK(N+11)-48)/100:D7=(PEEK(N+12)-48)/1000
460 D8=D4+D5+D6+D7
470 D9=D1+D2
475 IFD9>THENPRINTD9""D3" OVER";GOTO485
480 PRINTD9""D3""DB
485 A(D9,D3)=DB
490 NEXTN
500 PRINT"CONNECT UP PRINTER THEN TYPE CONT";STOP
505 D=0:D1=0:D2=0:D3=0:D4=0:D5=0
507 OPEN3,4
510 A=(A(1,2)+A(1,3))/2
520 Z(1,2)=(A(1,4)+A(1,5))/2
530 Z(2,2)=(A(1,6)+A(1,7))/2
540 Z(3,2)=(A(2,1)+A(2,2))/2
550 Z(4,2)=(A(2,3)+A(2,4))/2
560 Z(5,2)=(A(2,5)+A(2,6))/2
570 Z(6,2)=(A(2,7)+A(2,8))/2
575 FORM=1TD6:READ Z(N,1)
580 Z(N,3)=Z(N,2)-D
590 Z(N,4)=Z(N,3)/A
600 Z(N,5)=LOG(Z(N,4)/(1-Z(N,4)))
610 Z(N,6)=LOG(Z(N,1))
620 Z(N,7)=Z(N,5)^2
630 Z(N,8)=Z(N,6)^2
640 Z(N,9)=Z(N,5)*Z(N,6)
650 D1=Z(N,6)+D1
660 D2=Z(N,5)+D2
670 D3=Z(N,8)+D3
680 D4=Z(N,7)+D4
690 D5=Z(N,9)+D5
700 NEXTN
780 E1=D3-((D1^2)/6)
790 E2=D4-((D2^2)/6)
```
APPENDIX 3. (cont.).

800 E3=D5-((D1*D2)/6)
820 PRINTE3,"&&&&&&"FILE="F"$&&&&&&&&&&"
830 PRINTE3,"D1="D1,"D2="D2,"D3="D3,"D4="D4
840 PRINTE3,"D5="D5,"D6="D6,"D7="D7
860 PRINTE3,"A="A
880 PRINT"Y=|X*+C1:PRINTE3,"Y ="B1,"X +"C1
900 C4=E3/((E1*E2)^0.5):PRINTE3,"CORR.COEFF="C4
910 PRINT"X","EXPT-Y","OBS-Y":PRINTE3,"X","EXPT-Y","OBS-Y"
915 DEF FNA(V)=(A-D)/(1+(V/C3)^B2)+D
920 FORN=iTO7:Y(N)=FNA(2(N-1))
930 PRINTE3,PRINTE3,Z(N,1),Y(N),Z(N,2)
940 NEXTN
950 DEF FNB(V)=((((A-D)/(V-D))-1)^(1/B2))*C3
960 PRINTE3,"D1&D2&M.O.D&DOSE&&&&& (NG/ML)"
970 K=-1
980 FORN1=1TO12;FORN2=1TO7STEP2
990 K=K+1
1000 P1=A(N1,N2);P2=A(N1,N2+1);M1=(P1+P2)/2
1010 IFM1<0.001THEN2000
1015 IFM1>A THEN2000
1017 IFM1>A THEN2000
1020 M2=FNB(M1);IFM2>2540 THENM2=2550;IFM2<0 THENM2=0
1021 PRINTE3,P1,"P2","M1","M2","M2/10
1023 POKE(17000+K),M2/10
1025 NEXTN2:NEXTN1
1090 PRINT"LOAD""COW FILE""AND ENTER COW NUMBERS"
1100 STOP
2000 GOSUB3000:GOT01025
2100 GOSUB3100:GOT01025
2200 GOSUB3200:GOT01025
2300 GOSUB3300:GOT01030
2400 GOSUB3400:GOT01080
2500 GOSUB3500:GOT01080
3000 PRINTE3,P1,"P2","M1","DOSE=0":RETURN
3100 PRINTE3,P1,"P2","M1","DOSE<0":RETURN
3200 PRINTE3,P1,"P2","M1","DOSE<0":K=K+1:RETURN
5020 DATA 10,20,50,100,300,800
READY*
APPENDIX 4. PUBLICATIONS.


DIRECT ENZYMEOIMMUNOASSAY OF PROGестERONE IN BOVINE MILK

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ABSTRACT

A sensitive enzymeimmunoassay has been developed for measuring progesterone in unextracted bovine milk. An N-hydroxysuccinimide ester of 11-α-hydroxyprogesterone 11-hemisuccinate has been synthesised and used to form conjugates with β-galactosidase in buffer at pH 7.0. The degree of incorporation of progesterone into the enzyme was demonstrated using (14C)-labelled steroid and by radioimmunoassay binding inhibition. Standard curves of comparable range and sensitivity to radioimmunoassay were obtained in the presence of whole milk taken from a cow at oestrus. These advances have allowed the development of a simple micro-titre plate enzymeimmunoassay of progesterone in whole milk and will be of particular value in determination of pregnancy, prediction of the day of oestrus and diagnosis of reproductive disorders.

INTRODUCTION

Radioimmunoassay (RIA) is widely used for determination of picogram quantities of steroid hormones in blood and milk but has remained confined to specialised laboratories. The concentration of progesterone in bovine milk has been used for prediction of the oestrus period (1) and in commercial diagnosis of pregnancy (2). Progesterone determination by enzymeimmunoassay (EIA) provides a simple procedure for examination of reproductive status requiring only a colorimeter for quantitation and presents the possibility of widespread application by eliminating radioisotopic hazards. If full advantage is to be taken of such techniques and for large numbers of assays to be practicable, measurements must be possible without the need for prior extraction of steroids. EIA procedures previously employed for steroid determination have not convincingly demonstrated their suitability for use in direct assays (3-5). Synthesis of an N-hydroxysuccinimide ester of 11-α-hydroxyprogesterone 11-hemisuccinate (11-α-hydroxy-4-pregnene-3, 20 dione 11-hemisuccinate) has enabled
ready formation of well characterized progesterone-β-galactosidase conjugates at pH 7.0 and eliminated side reactions associated with some conjugation techniques (6, 7). Conjugates produced by this mild and efficient procedure have allowed the development of an EIA for progesterone in the presence of whole milk with standard curves of a sensitivity and range comparable with those obtained using RIA. This advance has enabled the technique to be further adapted to provide a simple microtitre plate EIA for determination of progesterone in unextracted bovine milk.

MATERIALS AND METHODS

Preparation of 11α-hydroxyprogesterone 11-(14C)-hemisuccinoyl N-hydroxysuccinimide ester.

11α-Hydroxyprogesterone (330mg), succinic anhydride (100mg), (14C)-succinic anhydride (50μCi; 80-120μCi mmol⁻¹; Radiochemical Centre, Amersham) and dried pyridine (300μl) were heated in a loosely-capped conical glass test-tube on a sandbath at 90°C for 24 hours. The tarry residue was taken up in methanol (2ml) and evaporated at 90°C under a stream of nitrogen for 1 hour before extraction and crystallization of 11α-hydroxyprogesterone (14C)-11-hemisuccinate (“progesterone (14C)-hemisuccinate”) from ethanol: water (70:30 v/v) (8). Following recrystallization, the "progesterone (14C)-hemisuccinate" (43mg) was dissolved in dioxane (1ml) and N-hydroxysuccinimide (12.5mg) and dicyclohexylcarbodiimide (23.8mg) added. The mixture was stirred at 25°C for 30 min and the by-product dicyclohexylurea removed by washing through a sintered glass funnel with 3 x 3ml dioxane. The clear filtrate was dried under a stream of nitrogen and the residue dissolved in a minimum volume of dichloromethane at 35°C. Addition of cold diethyl ether allowed crystallization of the 11α-hydroxyprogesterone 11-(14C)-hemisuccinoyl N-hydroxysuccinimide ester. A melting point of 195 to 197°C was found after recrystallization and a molecular weight of 528.27 determined for the M + 1 ion by mass spectrometry using NH₃ chemical ionization. The formula was confirmed at C₂₉, H₃₇, N, O₉ by elemental analysis (Dr F.B. Strauss, Microanalytical Laboratory, Oxford).

Preparation of progesterone-β-galactosidase conjugates

β-Galactosidase (EC 3.2.1.23; Sigma type VI) was dialysed against 1,000 vols 0.1M phosphate-buffered saline (pH 7.0) at 4°C to remove ammonium sulphate. Aliquots (100μl) containing 2.8mg protein ml⁻¹ were placed in glass test-tubes and the N-hydroxysuccinimide ester of "progesterone (14C)-hemisuccinate" in dimethylformamide added (10μl: concentration range 60mg ml⁻¹ to 60μg ml⁻¹). This provided molar ratios of progesterone to enzyme varying from 2,000 to 1 to 2 to 1. The reaction was allowed to proceed
with stirring at room temperature for 1 hour. Conjugates were exhaustively dialysed against three changes of three litres of 0.1M phosphate-buffered saline containing sodium azide (0.12 w/v) at pH 7.0 (PAS buffer) at 4°C before elution from 10 x 1cm columns of G25 Sephadex with PAS buffer to ensure removal of any remaining traces of unconjugated progesterone. The passage of conjugate at an elution volume of approximately 4ml was detected by absorbance at 280nm using a Cecil Spectrophotometer with a flow cell fitted.

Determination of incorporation of progesterone into β-galactosidase

The protein content of the conjugates was measured (9) and their enzyme activities determined as described below for EIA. Specific activities were expressed as µmol g-nitrophenol produced mg protein⁻¹ min⁻¹ at 45°C and recorded as a percentage of the original enzyme activity. Samples of the N-hydroxysuccinimide ester of progesterone (¹⁴C)-hemisuccinate were weighed and dissolved in dimethylformamide. Aliquots (5µl) were placed in scintillation vials, Toluene Scintillator (7ml; Packard) added and radioactivity determined as 73.2 x 10³ dpm mmol⁻¹. Soluene (1ml; Packard) was added to aliquots of purified conjugates and the radioactivity similarly measured. The molar concentrations of enzyme present were calculated assuming a molecular weight of 540,000 (10) and the molar ratio of progesterone to enzyme determined. The progesterone contents of suitable dilutions of the conjugates were further measured by RIA binding inhibition and the molar ratio of immunologically apparent progesterone to enzyme calculated.

Test-tube EIA and RIA of progesterone

Progesterone-enzyme conjugate prepared using a 200 fold molar excess of steroid was used for the EIA. A range of progesterone standards in PAS buffer with gelatin (1.0g l⁻¹; 100µl) and 20µl whole milk taken from a cow at oestrus (containing a minimum level of progesterone) were added to polystyrene test-tubes and allowed to equilibrate for 1 hour at room temperature. Enzyme-progesterone conjugate (25ng in 100µl PAS-gelatin buffer; equivalent to 5pg progesterone by RIA) and 100µl of bovine serum albumin-absorbed (11), cellulose-linked (12) goat anti-progesterone antiserum (1:1600 dilution in PAS-gelatin buffer) were then added and the tubes mixed. The antiserum (G711/12) was raised against an ¹¹⁸-hydroxyprogesterone 11-hemisuccinate-BSA conjugate (8). "Non-immune" goat serum linked to cellulose (1:1600 dilution) was used in place of anti-progesterone antiserum in the blank. The assay tubes were incubated at room temperature for 1 hour with shaking every 10 min and left for 16 hours at 4°C. PAS-gelatin buffer (3.5ml) was added at 4°C and the antibody-bound label separated by centrifugation (1600g for 10 min). Unbound progesterone and progesterone-enzyme conjugate were poured off and the tubes drained by inversion over absorptive paper. The activity of enzyme bound to antibody was determined by addition of freshly prepared o-nitrophenyl β-D-galactopyranoside (3mg ml⁻¹) in 200µl assay buffer (PAS-gelatin buffer containing 0.01M magnesium chloride and 0.1M mercaptoethanol) at 4°C. The tubes were mixed and incubated at 45°C for 1 hour, cooled to 4°C for 2 min and the reaction stopped by addition of 1M sodium carbonate (2ml) before centrifugation (1600g for 10 min). The absorption of the supernatant at 450nm was determined with a Cecil CE 404-2 spectrophotometer and corrected for the non-immune serum blank.
The radioimmunoassay was conducted by essentially the same procedure as the enzyme immunoassay, but with 100μl (1, 2, 6, 7-3H) progesterone (Radiochemical Centre, Amersham; 4000 dpm, 5-6pg) in PAS-gelatin buffer in place of enzyme-steroid conjugate. After overnight incubation in the presence of antibody, PAS-gelatin buffer was added (700μl) and the tubes mixed and centrifuged as before. Unbound progesterone was determined by pouring the supernatant into scintillation vials, extracting into Toluene Scintillator (7ml) for 1 hour and counting in a Packard B2450 scintillation spectrometer.

Micro-titre plate EIA and RIA of progesterone

Progesterone-enzyme conjugate was prepared using a 20 fold molar excess of steroid. BSA-absorbed rivanol-purified (11) goat anti-progesterone gammaglobulin in 0.1M carbonate buffer, pH 9.6 (200μl; 10μg protein ml⁻¹) was added to each well of a polystyrene micro-titre plate (Dynatech M129A) and incubated at 37°C for 3 hours. The wells were emptied by inversion, washed twice with PAS-gelatin buffer, drained and stored at -20°C until used. Whole milk taken from a cow at oestrus (10μl) and PAS-gelatin buffer containing progesterone standards (180μl) were added to the wells and incubated at 37°C for 1 hour. Enzyme-progesterone conjugate (150ng in 10μl; equivalent to 13pg progesterone by radioimmunoassay) was added and the plate incubated for a further 2 hours. The wells were emptied, washed three times with PAS-gelatin buffer and inverted on absorptive paper. o-Nitrophenyl-β-D-galactopyranoside in assay buffer (200μl; 3mg ml⁻¹) was added to each well and the plate incubated at 45°C for 1 hour. The reaction was stopped by dispensing the contents of each well into 1M sodium carbonate (2ml) and the absorbance determined at 405nm in a Cecil 404-2 spectrophotometer.

The micro-titre plate RIA was conducted by essentially the same procedure but with 4000 dpm (3H) progesterone per well in place of enzyme-steroid conjugate. After overnight incubation at 4°C free progesterone was determined by transferring well contents into scintillation vials and extracting and counting as described above.

RESULTS AND DISCUSSION

N-Hydroxysuccinimide esters of acyl-amino acids have previously been employed in the synthesis of peptides (13). The ability of the N-hydroxy-succinimide ester of "progesterone 11-hemisuccinate" to combine with β-galactosidase under mild conditions was demonstrated by both direct measurement of incorporated radioactivity and RIA binding inhibition (Table 1). Low molar ratios of progesterone to enzyme in the reaction mixture produced substantial incorporation. There was little increase when a 2000 rather than a 500 fold molar excess of steroid was employed.
### TABLE 1
Molar Incorporation of Progesterone into β-galactosidase and Enzyme Activity of the Resulting Conjugates

<table>
<thead>
<tr>
<th>Molar ratio of progesterone: enzyme in conjugation reaction mixture</th>
<th>Enzyme activity of conjugates (% of original activity)</th>
<th>Molar ratio of progesterone to enzyme: Radioactivity</th>
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<td>103.4</td>
<td>0</td>
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<td>2</td>
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<td>500</td>
<td>62.6</td>
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<tr>
<td>2000</td>
<td>22.6</td>
<td>107.0</td>
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Molar incorporation calculated from the radioactivity data approached the theoretical maximum of 116 molecules of progesterone per enzyme molecule that could be attached to lysine residues in the enzyme (10). Enzyme specific activity was not reduced by the conditions of the conjugation procedure but a reduction was seen when near maximal numbers of progesterone molecules were incorporated (Table 1). This could be explained either by alterations occurring at the active site of the enzyme when progesterone was added near this position or by allosteric effects induced when high levels of incorporation occurred. The only comparable data available (3) indicated that the mixed anhydride technique (14) was an order of magnitude less efficient at conjugating a steroid (cortisol) to β-galactosidase than the N-hydroxysuccinimide ester method reported here. The carbodiimide reaction used by other groups to link steroid derivatives directly to β-galactosidase (6, 7) has been shown to be inefficient: only 30% of β-galactosidase was reported to be conjugated with estradiol-17β when a 20 - 50 fold excess of the steroid was employed (15). Furthermore, the procedure inevitably leads to considerable enzyme to enzyme conjugation.
Radioimmunoassay of conjugates formed using the N-hydroxysuccinimide ester of "progesterone hemisuccinate" detected substantially lower molar incorporation ratios than did direct measurement of incorporated radioactivity (Table 1): clearly, the only progesterone molecules detected by immunoassay are those situated at such positions in the enzyme molecule that antibody attachment is not sterically hindered. This is in keeping with earlier findings (3) that only 1 in 10 of the cortisol molecules incorporated into β-galactosidase were detected by immunoassay. Claims that optimum sensitivity in EIA is in part dependant on achieving a low (around 1:1) molar ratio of progesterone to enzyme (4, 7) would only be true where all conjugated steroid was immunologically apparent as well as physically detectable. Immunological assessment of progesterone incorporation is, therefore, clearly of the greater relevance to the performance of an EIA.

The quantity of each conjugate used in the EIA procedure was based on its immunoreactivity and the amount of label used for both EIA and RIA was adjusted to be approximately equivalent. Quantities of conjugate equivalent to 5 - 15 pg progesterone resulted in sufficient enzyme activity binding to the antibody for standard curves to be produced by EIA. Their range and sensitivity was comparable with those seen following RIA using the same antibody dilution. The addition of whole milk from a cow at oestrus to the EIA system did not reduce the resolution of the standard curves and the results obtained were comparable with those from RIA performed under the same conditions (Figs. 1 and 2). The test-tube system (Fig. 1) had a within assay coefficient of variation of 3.2% for the EIA and 6.7% for the RIA. The limits of sensitivity at the 95% confidence level were 20 pg and 21 pg respectively. The micro-titre plate system (Fig. 2) showed a within assay coefficient of variation of
Fig. 1. Comparison of test-tube EIA (●-●) and RIA (○-○) standard curves in the presence of whole milk from a cow at oestrus. Values are mean of 6 determinations ± 2 SEM.

Fig. 2. Comparison of micro-titre plate EIA (●-●) and RIA (○-○) standard curves in the presence of whole milk from a cow at oestrus. Values are mean of 6 determinations ± 2 SEM.
4.7% for EIA and 2.7% for RIA, with limits of sensitivity of less than 5 pg and 7.8 pg respectively at the 95% confidence level.

The particular solid phase antibody system employed in the EIA procedure determined which steroid-enzyme conjugate provided optimal sensitivity. Using the microgranular cellulose-linked antibody system in the test-tube EIA, optimal sensitivity was achieved with a conjugate prepared using a 200 fold molar excess of steroid. This system required a quantity of conjugate (25 ng) immunologically equivalent to 5 pg progesterone per assay tube. It was found, however, that in the micro-titre plate EIA optimal sensitivity was obtained with a conjugate prepared using a 20 fold molar excess of steroid. The quantity of label required per well (130 ng) was equivalent to 13 pg progesterone. The variation seen may be partly explained by the different antibody concentration used in the two systems. In the micro-titre plate assay the quantity of antibody which passively adsorbs to the wells has a finite and comparatively low limit. This in turn limits the total amount of label which may be bound.

The techniques described in this study will enable the widespread use of progesterone assays for determination of reproductive status and the diagnosis of reproductive disorders. Such methods will be invaluable to small laboratories or in developing countries where radioimmunoassay would involve levels of capital expenditure too high for economic consideration. The arrangements necessary for the safe handling and disposal of isotopes are eliminated and equipment requirements greatly reduced. The micro-titre plate method in particular offers a sensitive and convenient assay which may be readily automated at comparatively low cost. The successful assay of progesterone in whole milk without prior extraction of steroid will be of particular value as an aid to farmers and veterinary practitioners for pregnancy diagnosis in cattle and in the prediction of the
day of ovulation, perhaps eventually allowing insemination without
recourse to observation of oestrous behaviour.

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THE USE OF ENZYME IMMUNOASSAY FOR THE MEASUREMENT OF HORMONES WITH
PARTICULAR REFERENCE TO THE DETERMINATION OF PROGESTERONE IN
UNEXTRACTED WHOLE MILK

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ABSTRACT
Prior to the development of enzymeimmunoassay (EIA) techniques, radioimmunoassays or radioreceptor assays were the most practical means by which hormone concentrations could be measured in large numbers of samples. EIA requires only simple colorimetry for quantitation and avoids the use of radioisotopes and associated counting equipment.

EIA's have been developed for various trophic, thyroid and steroid hormones during the past six year. These developments should enable veterinary laboratories to monitor abnormal hormone secretion in various disorders. Periodic assessment of progesterone levels can provide considerable information regarding the reproductive status of an animal. Milk would be the sample of choice in many domestic species.

The use of EIA for the determination of hormones in biological fluids and the methods by which hapten-enzyme conjugate may be formed are reviewed and factors influencing EIA sensitivity discussed. The development of a simple microtitre plate EIA for progesterone in unextracted whole milk is described.

INTRODUCTION
Enzyme immunoassay (EIA) or enzyme linked immunosorbent assay (ELISA) techniques have been the subject of continuous intensive development since their use was first reported (Engvall and Perlmann, 1971; Van Weeman and Schuurs, 1971). In the veterinary field, however, the application of EIA has remained almost totally restricted to the investigation and detection of infectious diseases. This is reflected in the scope of topics reported at this meeting. Other areas of application should surely include residue determinations in tissues and body fluids (anabolic steroids, antibiotics etc.) in order to police legislation relating to meat inspection and testing for drugs in racing.

The measurement of hormones associated with growth, development and reproductive function have obvious value in the livestock industry and the use of EIA procedures for their determination would enable their routine assessment to become a practical proposition.

The hormone EIA's which are listed in Table 1 and the procedures which we shall later describe are quantitative, competitive immunoassay procedures analogous with radioimmunoassay (RIA). This paper will not
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<th>Hetero/ Homology</th>
<th>Body Fluid Assayed</th>
<th>Direct Addition or Extraction</th>
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<tr>
<td>Thyroxine</td>
<td>β-Gluc AP Glut hetero (b) serum D</td>
<td>DA</td>
<td>400</td>
<td>Albert et al, 1978</td>
<td></td>
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<tr>
<td>HRP</td>
<td>β-Gal MHSae homo serum D</td>
<td>DA</td>
<td>1000</td>
<td>Monji et al, 1979b</td>
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<td>HCG</td>
<td>β-Gal MCAE - urine D</td>
<td>SPbc</td>
<td>2.5mIU/ml</td>
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<td>β-Gal MBS - plasma D</td>
<td>DA</td>
<td>0.4mIU/ml</td>
<td>Kitagawa et al, 1979</td>
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<td>Tomoda et al, 1978</td>
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<td>HRP</td>
<td>Glut - - D</td>
<td>DASpc</td>
<td>0.4mIU/ml</td>
<td>Van Weeman &amp; Schuurs, 1971</td>
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<td>Insulin</td>
<td>GA Glut - - -</td>
<td>SPs</td>
<td>1μl/tube</td>
<td>Ishikawa, 1973</td>
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<td>β-Gal PDM - DA</td>
<td>Sp</td>
<td>1μl/tube</td>
<td>Kato et al, 1975b</td>
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<tr>
<td>β-Gal MBS - serum D</td>
<td>SPsr</td>
<td>5μl/tube</td>
<td>Kato et al, 1979</td>
<td></td>
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<tr>
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<td>DA</td>
<td>0.5μl</td>
<td>Kitagawa &amp; Aikawa, 1975</td>
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<tr>
<td>LH</td>
<td>AP PAC - urine serum D</td>
<td>SPgb</td>
<td>100mIU/ml</td>
<td>Saxena et al, 1979</td>
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<tr>
<td>FMSG</td>
<td>AP Glut* - - -</td>
<td>SPme</td>
<td>0.011IU/ml</td>
<td>Marion et al, 1978</td>
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<td>HPL</td>
<td>AP + HRP Periodate - plasma D</td>
<td>DA</td>
<td>0.1μg/ml</td>
<td>Williams, D.G., 1978</td>
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<td>Thyrotropin</td>
<td>AP Glut - - -</td>
<td>DA</td>
<td>1μg/ml</td>
<td>Miyai et al, 1976</td>
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<tr>
<td>CO</td>
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<td>DA</td>
<td>1.5μg/ml</td>
<td>Albert et al, 1978</td>
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**ABBREVIATIONS**

Hormone - hCG human chorionic gonadotrophin; LH luteinizing hormone; FMSG, pregnant mare serum gonadotrophin; hPL, human placenta lactogen. Enzyme - β-Gal, β-galactosidase; AP, alkaline phosphate; HRP horse radish peroxidase; GA, glucoamylase; CO, glucose oxidase; β-Gluc, β-glucosidase; *enzyme conjugated to antibody ('sandwich' technique or *) immunoenzymometric assay. Con., Meth. - MCAE & MBS, N-hydroxysuccinimide esters of N-(4-carboxyphenylmethyl)-maleimide & maleimido benzoic acid respectively (heterobifunctional reagents). Glut, glutaraldehyde; PDM, N,N'-o-phenylemaleimide (homobifunctional reagent); PAC, photo affinity coupling; MA, mixed anhydride; CDI, water soluble carbodiimide; MBSae, maleimido benzoate active ester; MHSae, N-hydroxysuccinimide active ester. Procedure - Homo, homologous assay (hapten linkage to immunogen is same as linkage to enzyme); hetero, heterologous assay (linkage differs, either in bridge (b) or site (s) of conjugation). Body Fluid - Species is human except where indicated; bovine (b), equine (e) or porcine (p). Direct Addition or Extraction - D, direct addition; E, hapten extracted prior to assay. Separation Technique - SP, solid phase antibody; DA, double antibody; DASP, double antibody solid phase; PEG, polyethylene glycol precipitation; rbc, red blood cells; sr, silicone rods; c, cellulose; s, sephadex; gb, glass balls; mt, microtiter plate; ct, coated tube; so, sepharose.
consider the relative merits of the different methods by which enzymes can be employed as labels in EIA nor the merits of various enzymes used since these areas have been adequately reviewed by others (O'Sullivan et al., 1979; Oellerich, 1980; Scharpe et al., 1976; Schuurs and Van Weerman, 1977). Attention will, however, be paid to the methods by which enzyme-immunogen conjugates may be made.

Table 1 illustrates hormones for which EIA's have been developed. It can be seen that interest in this procedure has remained largely confined to the human clinical sphere. Progesterone is an exception and is the only hormone to have been assayed in a number of species as well as in several body fluids. This is perhaps because the patterns of progesterone secretion provide a wealth of information on aspects of reproductive performance such as the occurrence of cyclical activity or ovulation, differential diagnosis of luteal or ovarian cysts and pregnancy diagnosis.

The sensitivity of EIA is in many cases comparable with that achieved using RIA although many of the hapten EIA procedures have involved extraction of the analyte before assay (Table 1). Where cross-reacting substances are likely to be present in the test sample, purification by means of extraction or even chromatography may be necessary: this obviously applies equally to RIA. If high specificity is not required, however, prior extraction of sample may not be essential for the EIA provided the conjugates used are of suitable purity and do not contain a substantial proportion of unconjugated enzyme or steroid, or conjugated but denatured enzyme since this may affect the sensitivity of the assay. We believe, therefore, that particularly where quantitative, direct-addition assays are to be performed the mode of preparation of the conjugate is of the utmost importance.

PREPARATION OF PROTEIN-ENZYME CONJUGATES

A number of conventional procedures are available for the conjugation of enzymes to proteins and these include the one-step (Avrameas, 1969) and two-step (Avrameas and Ternynck, 1971) gluteraldehyde methods, the periodate method (Nakane and Kawaol, 1974) and the use of homobifunctional reagents such as N,N'-o-phenylenedimaleimide (Kato et al., 1975a). These methods have been reviewed by Kennedy et al. (1976) and others at this symposium (Doel and Collen, 1981).

The nature of these reactions makes co-conjugation of either enzyme to enzyme or protein to protein almost inevitable. For this reason, the
development of heterobifunctional reagents represents a major advance for production of enzyme-protein conjugates since they eliminate the possibility of co-conjugation of enzyme or protein.

Protein-enzyme conjugation using heterobifunctional reagents

A number of heterobifunctional reagents have recently been developed which, as the name implies, contain two differing reactive groups. The reagents reported up to now have reactivity towards sulphydryl groups at one end and towards amino groups at the other (fig. 1). Thus, in the first instance, if the reagent is reacted with a protein (or enzyme) which does not contain available sulphydryl groups no polymerization can occur: if excess reagent is then removed (i.e. by gel filtration or dialysis) the other protein may then be added in order that conjugation may proceed. If the second protein does not normally contain available sulphydryl residues it may be possible either to introduce them by chemical means (mercaptoimidation or mercapto-succinylation (Traut et al., 1973; Kato et al., 1975b) or by reduction of existing disulphide bridges (Kato et al., 1975a).

Bifunctional compounds which have been employed in the preparation of protein-enzyme conjugates include m-maleimidobenzoyl-N-hydroxysuccinimide (MBS) for conjugation of enzymes with insulin (Kitagawa and Aikawa, 1976) and donkey anti-sheep IgG Fc fragment (O'Sullivan et al., 1978a), N-hydroxysuccinimide esters of N-(4-carboxycyclohexylmethyl)-maleimide and N-(4-carboxyphenylmethyl)-Maleimide for conjugation with protein hormones (Hamada et al., 1978; Ishikawa et al., 1978) and N-succinimidyl 3-(2-pyridyl
dithio) propionate (SPDP) for conjugation with protein A (Pain, D. and Surolia, A.).

PREPARATION OF HAPTEN-ENZYME CONJUGATES

The preparation of enzyme-hapten conjugates for EIA has generally involved use of the standard mixed anhydride or carbodiimide procedures originally developed for linking haptens to protein carriers such as BSA for use in raising hapten antisera (fig. 2). In essence, the procedures give rise to the formation of peptide bonds between an acid group on the hapten or hapten derivative and lysine ε-amino groups on the enzyme.

a) Carbodiimide reaction

\[
\text{Hapten-}O_2 + R'N=C=NR^2 \xrightarrow{\text{(Carbodiimide)}} \text{Hapten-C-N-Enzyme}
\]

(eg. progesterone hemisuccinate)

\[\text{Enzyme-NH}_2\]

b) Mixed anhydride reaction.

\[
\text{Hapten-}O_2 + (\text{CH}_3)_2\text{CHCH}_2-O-C\xrightarrow{\text{DMF, Anhydride condition}} \text{Hapten-C-}R^\prime\]

\[
\xrightarrow{\text{tertiary amine, HCl}} \text{Hapten-C-}R^\prime\text{O-C}_2\xrightarrow{\text{Enz-NH}_2, \text{aq}} \text{Hapten-C-N-Enzyme}
\]

\[+ R^\prime \text{O-C}_2\]

iso-butyl chloroformate

Fig. 2 Summary of carbodiimide and mixed anhydride reactions.

The carbodiimide method

The basic water-soluble carbodiimide procedure has been use by a number of workers for producing enzyme-hapten conjugates (Table 1) but is losing favour since it inevitably results in co-conjugation of enzyme molecules. This is because it is not possible with currently available carbodiimide reagents to conjugate enzyme to hapten without the continued presence of excess carbodiimides since the intermediate o-acylurea is unstable (Kurzer and Douraghi-Zadeh, 1967).
The mixed anhydride procedure

This procedure eliminates the possibility of co-conjugation of enzyme since the generation of the intermediate mixed anhydride is only possible under anhydrous conditions (fig. 2). When aqueous enzyme solution is added for conjugation enzyme carboxylic acid groups cannot themselves form anhydrides and the enzyme-mixed anhydride reaction prevails.

Although a within laboratory comparison of the different methods of conjugation has not been reported, it has been argued that the mixed anhydride procedure may not be so efficient as procedures utilising hapten "active esters" described later.

Homobifunctional reagents

Homobifunctional reagents such as dimethyl adipinate (fig. 3) may also be used for coupling haptens to enzymes (Al-Bassam et al., 1978; O'Sullivan et al., 1978b) but reaction conditions must be carefully selected to minimize the inevitable co-conjugation of either enzyme or hapten.

dimethyl adipinate

\[
\begin{align*}
\text{H}_2\text{C}-\text{O-} & \text{C}-(\text{CH}_2)_4\text{-O-CH}_3 \\
\text{Enz.NH}_2 & \quad \text{H}_2\text{O-Hapten}
\end{align*}
\]

Specific for amino groups

bisoxirane

\[
\begin{align*}
\text{H}_2\text{O-Hapten} & \\
\text{CH}_2-\text{CHCH}_2\text{O(CH}_2)_4\text{OCH}_2\text{CH-CH}_2
\end{align*}
\]

hydroxyl and amino groups

\[
\begin{align*}
\text{N,N'-O-phenylenedimaleimide}
\end{align*}
\]

Fig. 3 Conjugation using homobifunctional reagents. \( R' \) and \( R^2 \) can be protein or hapten moieties.

Heterobifunctional reagents

Heterobifunctional reagents may similarly be used for conjugating haptens essentially without the hazards of co-conjugation (fig. 1). They have not found general application in the hapten hormone field largely because derivatives containing amino or sulphydryl groups are not generally
available or easily synthesised.

Heterobifunctional reagents have, however, found application in coupling certain drugs to enzymes: MBS has been used for coupling β-galactosidase to Viomycin (Kitagawa et al., 1976) and N-(3-maleimidopropionyl glycoloxy) succinimide for coupling Viomycin, Gentamycin (Kitagawa et al., 1978a) and Ampicillin (Kitagawa et al., 1978b) to β-galactosidase.

"Active ester" derivatives of haptens

The synthesis of reactive esters of haptens or hapten derivatives provides the basis for what may be the most convenient and efficient means of producing enzyme-hapten conjugates (fig. 4). These esters may be isolated and purified and thus used directly for conjugation with the enzyme under aqueous conditions and neutral pH. This eliminates the possibility of co-conjugation and enzyme denaturation (fig. 4). N-hydroxysuccinimide and p-nitrophenyl esters will readily react with primary, unhindered amino groups such as the ε-amino groups of lysine to form a peptide bond: such reactions have been used for conjugating progesterone

1) N-hydroxy succinimide esters.

\[
\begin{align*}
R'\text{C-OH} + \text{dicyclohexyl carbodiimide} & \rightarrow R'-\text{O-N} - \text{H}_2\text{O} \\
\text{Enz.}\text{-NH}_2
\end{align*}
\]

2) p-Nitrophenyl esters.

\[
\begin{align*}
R'\text{C-OH} + \text{dicyclohexyl carbodiimide} & \rightarrow R'-\text{O-} - \text{H}_2\text{O} \\
\text{Enz.}\text{-NH}_2
\end{align*}
\]

3) m-Maleimidobenzoate esters.

\[
\begin{align*}
R'\text{CH}_2\text{OH} + \text{ maleimidobenzoyl chloride} & \rightarrow R'\text{CH}_2\text{-O-} - \text{HCl} \\
\text{Enz SH}
\end{align*}
\]

Fig. 4 Active ester derivatives of haptens. R' represents the hapten portion.
11-hemisuccinate (Sauer et al., 1981) and testosterone derivatives (Hosada et al., 1979; 1980) to β-galactosidase and for conjugating oestrone sulphate 6-hemisuccinate to BSA (Nambara et al., 1980). Gros et al., (1978) have also used N-hydroxysuccinimide esters to link progesterone 11-hemisuccinate and progesterone 11-hemimaleate to β-galactosidase but without prior purification or isolation of the active ester (carbodiimide may therefore have been present when the enzyme was added). Monji and Castro (1979a) have isolated and purified m-Maleimidobenzoate esters of cortisol and cortisol derivatives which they subsequently used to enable direct conjugation with the free sulphhydryl residues of β-galactosidase in buffer at pH 7.0. These reports indicate that these active esters react with enzyme or protein in a near stoichiometric manner and result in only minimal loss of enzyme activity.

Typical procedure for coupling haptens to enzymes using N-hydroxysuccinimide esters

The formation of the N-hydroxysuccinimide ester of 11-α-hydroxyprogesterone 11-hemisuccinate (Sauer et al., 1981) provides a typical example of the means by which N-hydroxysuccinimide esters of carboxylated haptens or hapten derivatives may generally be made (fig. 5).

A slight molar excess (10–20%) of N-hydroxysuccinimide and dicyclohexylcarbodiimide was added to progesterone 11-hemisuccinate (43mg) and dissolved in a minimum volume of dry dioxane. The mixture was stirred at 25°C for 30 mins and the insoluble by-product dicyclohexylurea removed by washing through a sintered glass funnel. The clear filtrate was dried under a stream of nitrogen and the residue dissolved in a minimum volume of dichloromethane at 35°C. Addition of cold diethyl ether allowed crystallization of the N-hydroxysuccinimide ester.

Progesterone-β-galactosidase conjugates were subsequently produced as described (Sauer et al., 1981) by addition of a suitable molar excess of the active ester (in a minimum volume of dry dimethylformamide) to β-galactosidase in 0.1M phosphate buffered saline, pH 7.0. The reaction was performed at room temperature with stirring for one hour before "exhaustive" dialysis against three changes of 3 litres of 0.1M phosphate-buffered saline containing sodium azide (0.1% w/v) at pH 7.0. Conjugates were then subjected to gel filtration (Sephadex G25) prior to assessment and subsequent use for EIA. The importance of extensive dialysis and gel filtration for

* see appendix
1) SYNTHESIS OF $^{14}C$ LABELLED 11-$\text{O}$-HYDROXYPROGESTERONE 11-HEMISUCCINATE.

2) SYNTHESIS OF PROGESTERONE "ACTIVE ESTER".

3) SYNTHESIS OF ENZYME-PROGESTERONE CONJUGATE.

Fig. 5 Formation of β-galactosidase-progesterone conjugates by the N-hydroxysuccinimide ester method.

The removal of unconjugated progesterone cannot be over-stressed; it is essential if a high degree of assay sensitivity is to be achieved. Even after dialysis against four changes of buffer (over 5 days), and gel filtration followed by a final dialysis, we have still been able to detect immunoreactive material in the dialysate. By this stage, however, the quantity present would not be sufficient to interfere with the EIA at the conjugate dilution employed.

Several methods have been employed to assess the number of hapten molecules conjugated per enzyme and these include spectrophotometric methods (Erlanger et al, 1957), or analysis of free amino groups on the enzyme subsequent to conjugation (Fields, 1971). The most direct and perhaps least misleading approach is through the use of radioactively-labelled hapten (Abuknesha and Exley, 1978; Comoglio and Celada, 1976; Sauer et al, 1981).
Using the latter method of assessment the N-hydroxysuccinimide ester method of conjugation was shown to be highly efficient, particularly at lower molar ratios (Table 2). Incorporation approaching the theoretical maximum of 116 progesterone molecules per enzyme can be achieved (there being 116 lysine residues per β-galactosidase molecule; Craven et al., 1965) although high molar incorporation was found to result in loss of enzyme activity (Sauer et al., 1981). Determination of steroid incorporation by binding inhibition tests detected substantially lower molar incorporation (see Table 2), indicating that the actual molar steroid incorporation may have little bearing on the sensitivity of an assay except where it can be shown that all conjugated steroid is immunologically apparent as well as physically detectable.

METHODS OF SEPARATION OF FREE FROM BOUND ENZYME CONJUGATE

The EIA's described in this paper (termed heterogenous EIA's) require separation of free from bound enzyme label following the immunoreaction in order to quantitate the unlabelled hapten or protein. Separation techniques which have been employed are generally methods adapted from RIA procedures. These include polyethylene glycol precipitation, double antibody precipitation, double antibody solid phase and solid phase primary antibody techniques (Table 1). The use of dextran-coated charcoal for separation is probably the most widely used technique for hapten RIA but since absorption is based on the large difference in molecular weight (of the order of 200 fold) between free label and that bound to antibody it cannot be applied to EIA.

Where possible the use of solid phase antibodies (preferably primary
antibodies) gives rise to the most convenient form of separation. Where particulate solid phases are used it has been necessary to employ a centrifugation stage. To avoid a centrifugation step, methods have been developed enabling magnetic solid phases to be prepared: materials used include polyacrylamide-agarose beads (Guesdon and Avrameas, 1977), ferric oxide particles (Hersch and Yaverbaum, 1975; Nye et al, 1976), cellulose (Anderson, 1978) and plastic coated steel balls (Smith and Gehle, 1977).

Using polystyrene test-tubes or polyvinyl or polystyrene microtitre plates coated with primary antibody, separation of free from bound enzyme-conjugate is achieved simply by pouring off the free fraction. The quantity of enzyme label bound may be simply determined by addition of substrate following an initial washing to remove residual free enzyme conjugate.

In this laboratory we have largely used solid phase separation techniques, including primary antibody linked to microgranular cellulose. We now use microtitre plates in a progesterone enzymeimmunoassay (Sauer et al, 1981) in what we believe is the first reported use of such plates in a hapten EIA. This procedure combines a convenient, simple and less tedious method with rapid assay and end-point determination - particularly when used in conjunction with an automatic plate reader such as the "twin beam" Dynatech MR 580.

MICROTITRE PLATE ENZYMEIMMUNOASSAY FOR PROGESTERONE

For the majority of quantitative coated test-tube or microtitre plate immunoassays it is essential to isolate the \( Y \)-globulin fraction of the antiserum prior to coating of tubes or microtitre plate wells. This enables significant antibody binding to be achieved at reasonable antibody dilution.

Antibody purification

We have used BSA-absorbed (Thorneycroft et al, 1970) goat antiprogestrone serum (G711/12) raised against 11\( \alpha \)-hydroxyprogesterone 11-hemisuccinate-BSA for our assay. Adequate purification was achieved by treating the antiserum with 4 volumes of 0.4% Rivanol; this precipitates the majority of serum proteins leaving globulins in solution (Horejsi and Smetana, 1956). Thorneycroft et al, (1970) indicated the importance of pH in this purification procedure and our results underline the point. The indications are that it is essential to establish not only the pH for the precipitation but also the volume of 0.4% Rivanol to be added, since these optima may vary from species to species. Goat antiserum was treated with 4 volumes of
The effect of pH on the precipitation of serum proteins by Rivanol.

Fig. 7  Effect of pH on recovery of anti-progesterone γ-globulin from serum treated with Rivanol (pH 6.0, - - ; pH 7.0, Δ-Δ; pH 8.0, Δ-Δ; pH 9.0, ■■■; pH 10.0, · · ·; untreated serum, ○-○).
buffered 0.4% Rivanol (0.1M phosphate or tris buffer, pH 6-10) for 15 minutes at room temperature and then centrifuged. Rivanol was removed by Sephadex G-25 column chromatography (eluting with 0.005M phosphate buffer, pH 7.0) and the protein fraction freeze-dried and re-dissolved in deionized water to give a volume of 10 x that of the original serum. Polyacrylamide gel electrophoresis (fig. 6) and subsequent assessment of antibody titre (fig. 7) indicated that pH 9.0 was optimal and resulted in removal of the majority of other serum proteins. Antiserum treated in this way was subsequently used for binding to microtitre plates. When dilution curves obtained with the original antiserum were compared with those following treatment at pH 6-9, a small but similar drop in binding was seen consistent with experimental losses. At pH 10, however, a further more substantial drop in binding was found, indicating a loss of γ-globulins by treatment at this pH (fig. 7).

Adsorption of antibodies to microtitre plate wells

We have used Dynatech 96-well flat-bottomed polyvinyl plates (M29A) and found that these gave more consistent binding than did the use of their polystyrene equivalent reported previously (Sauer et al, 1981). Binding of 3H progesterone to the wells was used to assess the extent to which the anti-progesterone γ-globulin was adsorbed. At temperatures of 25, 30, 35 and 40°C binding of γ-globulin was virtually complete by one hour (fig. 8A). We eventually chose a 3hr incubation at 40°C to ensure good standardization.

![Graph](image)

Fig. 8 Effect of A, incubation duration and temperature (25°C, O-0; 30°C, △-△; 35°C, ■-■; 40°C, E-E); B, pH and C, buffer molarity on adsorption of antibody onto microtitre plate wells. Values are mean of 4 determinations (A) or 8 determinations (B and C) ± 2 SEM.
The pH of the coating buffer had a considerable influence on binding of \( \gamma \)-globulin to the wells, maximum binding being achieved at pH 5.0 (Fig. 8B). The influence of acetate buffer concentrations at pH 5.0 on binding of antibody to the wells is shown in Fig. 8C. Buffer solutions were prepared in water which had undergone a primary deionization, glass distillation and further deionization prior to use. The highest binding levels were achieved using 1mM buffer.

Later studies therefore used standard adsorption conditions: \( \gamma \)-globulin diluted in 1mM acetate buffer pH 5.0 (0.2ml), was added to each well and incubated at 40°C for 3 hrs. The plates were then inverted and sharply tapped to eject the residual antibody. The wells were washed once with 0.1M phosphate buffer (pH 7.0) containing sodium chloride (0.9% w/v), sodium azide (0.1% w/v) and 0.1% (w/v) gelatin (PAS-gelatin buffer) and then incubated overnight at 4°C with the same buffer (250\( \mu \)l). Plates were stored in this condition at 4°C and further washed with PAS-gelatin buffer immediately prior to use for EIA.

**Enzymeimmunoassay procedure**

This assay procedure was developed to enable the determination of progesterone in milk by direct addition of whole milk to the assay system. Experiments to determine optimum conditions with regards antibody and enzyme conjugate dilutions were therefore carried out in the presence of whole milk. Progesterone-\( \beta \)-galactosidase conjugate prepared using a 20 fold molar excess of steroid was used in subsequent EIA procedures unless otherwise indicated.

Standards were prepared by the dissolution of progesterone in whole milk (containing a minimum of endogenous progesterone) from a cow at oestrus. These standards (10\( \mu \)l), or whole milk from test animals (10\( \mu \)l) were added to each well of an antibody-coated microtitre plate followed by 200\( \mu \)l of progesterone-enzyme conjugate suitably diluted in PAS-gelatin buffer. After a three hour incubation period at 40°C the wells were emptied and washed three times as above. The amount of enzyme conjugate bound was determined by addition of freshly prepared substrate, o-nitrophenyl \( \beta \)-D-galactopyranoside (3mg ml\(^{-1} \)), in PAS-gelatin buffer containing 0.01M magnesium chloride and 0.1M mercaptoethanol. Following an incubation period of 1 hr at 45°C
the optical density (OD) at 405nm was measured directly through the wells using a Dynatech MR580 automatic plate reader.

Selection of antibody and enzyme conjugate dilutions

A 1 hr period was chosen for incubating enzyme and substrate to minimise time differences associated with substrate addition to the first and last well. This also enabled economical use to be made of antibody and conjugate. The antibody dilution at which wells were coated and the conjugate dilution used in the EIA procedure were determined on an empirical basis; account was taken of the optical density achieved following binding in the absence of competing progesterone (Bo value) since this dictates the OD range measured in the standard curve.

Conjugate and antibody dilutions giving rise to acceptable standard curves were compared in order to assess their influence on the limits of detection. It can be seen from figs. 9A and 9B that neither parameter had

![Graph A](image1.png)

![Graph B](image2.png)

*Fig. 9 Effect of A, antibody dilution (at 1/400 conjugate dilution) and B, conjugate dilution (at 1/200 antibody dilution) on the sensitivity of EIA standard curves. Values are mean of 4 determinations ± 2 SEM.*
a dramatic effect over the ranges tried. In some respects this may not be surprising. The range of antibody dilutions indicated in fig. 9A would result in a quantity of antibody bound to the wells, which, if used in RIA would bind a maximum of 1pg of 3H progesterone in the absence of competing steroid. Further reduction in the quantity of antibody bound to the wells would be unlikely to result in a substantial increase in sensitivity under the immunoassay conditions adopted here; the theoretical arguments applied to RIA by Ekins (1974) would suggest that by using such small quantities of antibody the system may already be operating around the limits of sensitivity for the assay. At a 1/2000 antibody dilution (fig. 9A) a significant difference in binding of conjugate was shown between 0 and 10pg (p<0.05). No significant difference in the limit of detection was found between standard curves using the different conjugate dilutions.

In subsequent assays of progesterone in test milk samples each well was coated with anti-progesterone γ-globulin (1/2000 dilution ≡ 0.6µg/well) and conjugate (1/400 dilution; 135ng in 200µl PAS-gelatin buffer ≡ 6pg of immunoreactive progesterone) added to 10µl of milk.

**Comparative RIA systems**

A microtitre plate RIA and a liquid phase RIA were developed to compare whole milk progesterone concentrations determined by RIA and EIA.

The microtitre plate RIA was carried out in essentially the same manner as the EIA procedure except that the plates were coated with γ-globulin at 1/200 dilution. Following removal of the free steroid by washing as before, bound label was measured by cutting up the plates and counting individual wells in Toluene Scintillator (7ml, Packard).

The liquid phase RIA was carried out as follows:

Whole milk standard (10µl; 0-300pg progesterone) was pipetted into polystyrene test tubes and 3H progesterone in PAS-gelatin buffer (100µl, 4,000 dpm ≡ 6pg progesterone) and progesterone antiserum (200µl G711/12 at 1/30,000 dilution in PAS-gelatin buffer) added. The tubes were mixed and incubated for 1 hr at room temperature and then overnight at 4°C. Free 3H progesterone was separated from bound by the addition of dextran-coated charcoal suspension (500µl, 2.5mg charcoal; "Separex", Steranti, UK) in PAS-gelatin buffer at 4°C. After 6 mins at 4°C tubes were centrifuged at 4,000g (10 min) and the supernatant poured off into scintillation vials and counted following extraction into Toluene Scintillator (7ml).
Fig. 10 Comparison of EIA standard curves using standards prepared in buffer (•-•) or milk (▲-▲). Values are mean of 4 determinations ± 2 SEM. For ease of comparison results are expressed in terms of optical density (A) and B/Bo (B).

Fig. 11 Comparison of liquid phase (A) and microtitre plate (B and C) RIA standard curves using standards prepared in buffer (•-•) or milk (▲-▲). Values are mean of 4 determinations ± 2 SEM. For ease of comparison microtitre plate results are expressed in terms of B/Bo (B) and % bound (C).
The application of EIA to the determination of milk progesterone

Fig. 10 shows a typical comparison between EIA standard curves prepared in the presence and absence of milk. It is apparent that milk caused a considerable drop in enzyme conjugate binding (21%), but even so the sensitivity of the curve was still 10 pg/well at the 95% confidence limit. A similar drop in binding was noted for the microtitre plate RIA (fig. 11B). These EIA standard curves were not as sensitive as we have reported in a previous publication (Sauer et al., 1981); this may be due to the presence of slightly higher levels of endogenous progesterone in the "oestrus milk" sample used to prepare the standards. The milk used for these standards had been stored for about 5 months at 4°C in the presence of 0.1% sodium azide. Storage of the milk in this way had a minimal effect on progesterone concentrations when assayed by liquid phase RIA but may influence the stability of certain milk proteins over a period of several months: this may in turn influence conjugate binding to the antibody.

During November and December 1980, representative milk samples were

Fig. 12 Comparison of progesterone concentrations in milk samples collected around the oestrus period. Samples were assayed by microtitre plate EIA (A-A) and RIA (O-O) and by liquid phase RIA (#-#). Values are mean of 4 determinations.
taken from the milk collection jars of two cows at the morning milking. Aliquots (1ml) were stored at 4°C in the presence of 0.1% sodium azide. The samples were assayed within three months by liquid phase RIA and subsequently by liquid phase RIA, microtitre plate RIA and microtitre plate EIA in August 1981. Results from samples collected around the oestrus period at which artificial insemination (AI) was carried out are illustrated in fig. 12.

A correspondence can be seen when comparing profiles obtained by the three methods. The long period of storage may have influenced the EIA results since protein precipitation occurred in some of the samples. Such changes in milk constituents may conceivably have a greater influence on the binding of enzyme conjugate in the EIA system than the RIA system. A comparison of milk samples assayed after three months with those assayed after eight months revealed only minimal changes in progesterone concentration when examined by liquid phase RIA. A study of progesterone concentrations in fresh whole milk is currently being undertaken to resolve the differences observed between the EIA and RIA systems.

Clinical value of milk progesterone determinations in fertility control

Progesterone concentrations in milk have been shown to correlate closely with the growth and secretary function of the corpus luteum (Hoffmann et al, 1976; Laing and Heap, 1971; Pope et al, 1976): luteal function in cattle may therefore be conveniently monitored through determination of progesterone concentrations in milk rather than plasma, allowing sampling by lay personnel.

Analysis of progesterone in milk samples has been used for the investigation of a number of physiological conditions. These include pregnancy diagnosis, enabling early re-submission of non-pregnant cows for service (Laing and Heap, 1971), confirmation of cyclic activity (Günzler et al, 1979; Lamming and Bulman, 1976), accurate differential diagnosis of ovarian cysts (Hoffmann et al, 1976) and the confirmation and prediction of oestrus (Ball and Jackson, 1979; Günzler et al, 1979). The latter application is of particular interest to the artificial insemination industry since it is now well established that in large herds the detection of oestrus can be a major problem (McCaughey and Cooper, 1980; Oltner and Edqvist, 1981) and can result in a considerable extension of the calving to conception interval. The economic losses involved can be considerable since a missed oestrus period will result in a delay of 21 days before subsequent AI and each day lost has been
estimated to cost of the order of £1.50 in lost production.

Failure to detect oestrus can be a particular problem during the early post-partum period when "silent" oestrus periods may occur. Whether failure to detect oestrus is due to "silent" heat or management problems, a quick and simple progesterone test could circumvent the necessity for oestrus observation. Ball and Jackson (1979) used progesterone levels (measured by RIA) as the basis for determining when cattle should be inseminated. Subsequent conception rates of animals in which oestrus was not observed demonstrated the value of this approach. The potential of a suitable on-farm milk progesterone test in this respect is obvious. The microtitre plate EIA currently offers the quickest and most convenient means for determining progesterone concentrations directly in whole milk. The use of enzyme rather than isotopic labels offers the opportunity for further development of "dip-stick" techniques for use by stockmen.

PRELIMINARY APPLICATION OF DIRECT EIA OF SERUM SAMPLES

In heifers and non-lactating cows it is obviously not possible to obtain a milk sample. Similarly, in other species such as the pig, sheep and horse, progesterone estimations would need to be carried out on blood samples under most circumstances, although saliva might be suitable. Preliminary findings suggest that the microtitre plate procedure may be equally applicable to the assay of serum samples. Fig. 13 shows standard curves obtained using essentially the same conditions described for the direct assay of milk. Serum samples (10μl) were taken from animals in which endogenous levels of progesterone should have been minimal. RIA standard curves are also shown for comparison (fig. 14). The results appear particularly promising in the pig since plasma progesterone concentrations during the oestrus cycle cover a similar range to those in bovine milk. The large depression in binding observed with the RIA standard curve in the presence of prepubertal pig serum (fig. 13) could have arisen from the presence of endogenous progesterone or steroid binding globulins.

FACTORS INFLUENCING EIA SENSITIVITY

Discussions regarding the limits of sensitivity that may be achieved by hapten EIA have largely evolved from the development of steroid enzyme immunosays. Two main factors have been considered important (given that the conjugative procedure is efficient and does not result in enzyme denaturation) namely,

1) the number of steroid molecules incorporated per molecule of enzyme and
Fig. 13 Comparison of EIA standard curves in the presence of serum (10μL) from pre-pubertal gilt (●-●), pre-pubertal heifer (▲-▲), castrate ram (△-△) and gelding (□-□). Values are mean of 4 determinations. For ease of comparison results are expressed in terms of optical density (A) and B/B₀ (B).

Fig. 14 Comparison of liquid phase (A) and microtitre plate (B and C) RIA standard curves without serum (●-●) and in the presence of serum (10μL) from pre-pubertal gilt (●-●), pre-pubertal heifer (▲-▲), castrate ram (○-○) and gelding (□-□). Values are mean of 4 determinations. For ease of comparison results are expressed in terms of % bound (A and C) and B/B₀ (B).
ii) the affinity of the antibody for the hapten relative to its affinity for the conjugate.

1) The steroid: enzyme incorporation ratio aspect has been stressed by a number of workers (Exley and Abukneshaa, 1977; Joyce et al., 1977b). It has been suggested, either on theoretical grounds (Exley and Abukneshaa, 1977) or on the basis of experimental findings (Joyce et al., 1977a; Hosada et al., 1980), that the ratio of steroid : enzyme should be of the order of 1:1 for optimal sensitivity to be achieved. The findings of Joyce et al (1977b) indicated that with oestradiol - horse radish peroxidase conjugate, reducing the incorporation ratio from 11:1 to 2.6:1 gave rise to a 30% increase in sensitivity. No explanation was given as to how, by reducing the ratio by a further small degree (from 2.6:1 to 2.1:1) they achieved an increase in sensitivity of 70%. Their results indicate rather that the assay system was not optimised for particular conjugates. This is reinforced by their further observations that using an optimised double antibody separation technique rather than solid phase primary antibody, a 50 fold increase in sensitivity was achieved, considerably more than that realised by alteration in steroid incorporation ratios. More recent publications by this group (Joyce et al., 1978; Turkes et al., 1979) better reflect the high degree of sensitivity which can be attained by using a heterologous EIA under optimum conditions. The work of Hosada et al (1980) does not enable conclusions to be drawn regarding the limits of detection achievable using conjugates with different steroid : enzyme ratios, particularly since they have not determined the degree of steroid incorporation in their enzyme conjugates.

The actual number of hapten molecules conjugated to an enzyme may be at least a magnitude higher than those which are accessible to antibody binding sites (Cosoglio and Celada, 1975; Hayashi et al., 1981; Komimami et al., 1980; Sauer et al., 1981). Since only conjugated hapten molecules which are not sterically hindered are likely to be of immunological significance it is our view that for hapten EIA systems the total number of haptens incorporated may have little bearing on the absolute sensitivity of the system (the sensitivity will be appreciably reduced, however, when, as a result of very high steroid incorporation, a large proportion of the conjugated steroids are immunoreactive). Despite their arguments to the contrary, this view is supported by the data of the Riad-Fahmy group. For their progesterone and testosterone EIA systems they have indicated that 20-50ng of steroid-horse radish peroxidase conjugate (1.2:1 steroid : enzyme
incorporation ratio) proved optimal (Turkes et al., 1979 and 1980). Theoretical considerations argue against the possibility of realising the high degree of sensitivity which they have achieved (Joyce et al., 1978; Turkes et al., 1979) were all the steroid in this quantity of conjugate immunologically apparent: 20 - 50ng of conjugate is approximately equivalent to 200 - 500pg of steroid, assuming a molecular weight for HRP of 40,000. Further, it has not been our experience that optimal sensitivity can be achieved using β-galactosidase-progesterone conjugates which contain of the order of one steroid molecule per molecule of β-galactosidase. It can be seen by comparison of figs. 15A and 15B that such low incorporation requires the use of considerably more conjugate per assay in order to achieve an acceptable degree of conjugate binding in the absence of competing steroid. Note that in fig. 15B the 5, 10 and 20pg standards cannot be distinguished from the B0 value. In practise, where steric hinderance of conjugated hapten occurs it will be necessary to employ a conjugate with appreciably more hapten incorporated since only a fraction of these will be immunoreactive (Table 2).

ii) It is widely accepted that in an homologous hapten EIA system the affinity of binding of enzyme conjugate to antibody is higher than that for native hapten since the same hapten derivative is used in forming the immunogen as is used for conjugating hapten to enzyme. This phenomenon was originally investigated for oestrogens by Van Weeman and Schuurs (1975) who argued that by employing 'site' or 'bridge' heterology more sensitive assays could be achieved. It can now be seen that similar improvements in sensitivity can be achieved by the use of more refined conjugation and separation techniques. In a practical sense, however, antibody avidity dictates that, for an homologous hapten-enzyme conjugate, very high concentrations of hapten are required in order to reduce enzyme label binding to blank values (10 - 100 times those required to reduce ¹¹⁵I label binding in a comparable RIA system to blank levels) even though the sensitivity achieved may still be very high (compare figs. 10 and 11).

A number of workers have investigated aspects of bridge and site heterology in order to increase the sensitivity of steroid EIA's but with varying degrees of success (Van Weeman and Schuurs, 1975; Joyce et al., 1977b; Exley and Abuknesha, 1977; Dawson et al., 1979; Hosada et al., 1980). Different methods of assessing sensitivity have, however, made it difficult to compare their findings. Although increases in sensitivity have been achieved in some instances (Dawson et al., 1979; Van Weeman and Schuurs, 1975) they may be accompanied by loss of specificity (Hosada et al., 1980)

The data of Dray and co-workers (Dray et al., 1975; Gros et al., 1978)
appear conflicting. They indicate in one instance (Gros et al., 1978) that it is necessary to use bridge heterology (hemimaleate rather than hemisuccinate) to achieve similar dose response curves when comparing binding of tritium and enzyme-labelled progesterone whereas in a previous publication (Dray et al., 1975) an almost identical response was achieved using an homologous system. We have found that such comparisons can be misleading since

![Graph](image_url)

**Fig. 15** Comparison of EIA standard curves (in the absence of milk) using progesterone hemisuccinate (●-●) or progesterone hemimaleate (○-○) conjugates prepared with a molar ratio of progesterone derivative: β-galactosidase of 25:1 (A) or 2.5:1 (B). Results are mean of 4 determinations ± 2 SEM. For A, $B_0$ values ± 2 SEM for the hemisuccinate (23ng conjugate/well) and hemimaleate (21ng/well) conjugates were 0.930 ± 0.055 OD and 0.784 ± 0.010 OD respectively. For B, $B_0$ values ± 2 SEM for the hemisuccinate (54ng conjugate/well) and hemimaleate (57ng conjugate/well) conjugates were 0.460 ± 0.015 OD and 0.331 ± 0.011 OD respectively.

it cannot be assumed that assay conditions which are optimal for one conjugate are also optimal for the other or that conjugation efficiency is the same when using different hapten derivatives. We have compared β-galactosidase conjugates of progesterone hemimaleate (heterologous) and progesterone hemisuccinate (homologous); under the same conditions it was apparent, using conjugates prepared with a 25:1 molar steroid excess that greater sensitiv-
ity was achieved using the hemimaleate conjugate (fig. 15A). Standard curves obtained using conjugates formed with a 2.5:1 steroid excess, however, showed no significant difference in sensitivity (fig. 15B).

**SUMMARY**

The fragmentary nature of the information which has been derived from investigations of heterology and the extent of hapten conjugation on the performance of EIA's is such that it is impossible to draw hard and fast conclusions about their influence on assay sensitivity. In general, where heterology has been introduced to effect increases in sensitivity little attention has been given to the degree of hapten incorporation achieved under these conditions (Van Weeman and Schuurs, 1975; Hosada et al, 1980). Increases or decreases in sensitivity under these circumstances may partly be attributed to differing degrees of steroid incorporation. Further, it has proved possible to achieve equally sensitive EIA's using homologous systems (Sauer et al, 1981; Turkes et al, 1979; Dray et al, 1975) as was previously thought possible only by using heterology (Joyce et al, 1977; Van Weeman and Schuurs, 1975). Closer attention to the elimination of free hapten following conjugation may have contributed to these improvements.

To our knowledge no studies have been performed under suitably comparable conditions to furnish evidence that for hapten assays an actual incorporation ratio of approximately 1 hapten per enzyme molecule does indeed result in standard curves of the greatest sensitivity. This argument has a theoretical attraction which cannot be ignored but it does not take into account the extent to which incorporated haptens are sterically available for antibody binding. In future it would seem pertinent, when evaluating the influence of heterology on the sensitivity of EIA standard curves to consider concurrently the relationship between the total number of haptens incorporated into the enzyme and the number of haptens which are immunoreactive.

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**Appendix**

It is only recently that the Sigma Chemical Company have indicated in their catalogue that the grade IV E. Coli β-galactosidase used in these studies is standardized by the addition of BSA. Since this would clearly be conjugated by the progesterone active ester and subsequently interfere with the assay, we now use the grade VIII product.
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USE OF MICROTITRE PLATE EIA FOR DIRECT DETERMINATION OF PROGESTERONE IN WHOLE MILK: APPLICATION OF HETEROLOGOUS SYSTEMS FOR IMPROVED SENSITIVITY

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SUMMARY

An homologous microtitre plate enzymeimmunoassay (EIA) for the direct determination of progesterone in whole milk is described. Aspects of the assay are discussed in relation to progesterone profiles obtained from several cows over an oestrous cycle. The β-galactosidase conjugate used was prepared from the 11α-hemisuccinate derivative of 11α-hydroxyprogesterone and was subsequently compared with heterologous conjugates prepared in a similar way using 11α-hemimaleate, 11α-glucuronide and 3-(0-carboxymethyl)oxime derivatives. The progesterone 11α-glucuronide-β-galactosidase conjugate provided standard curves with the steepest slope. Approximately 1/10 the mass of progesterone (56 pg) was required to reduce conjugate binding by 50% when compared with the homologous hemisuccinate conjugate. The results suggest that the use of a glucuronide bridge for linking progesterone to enzyme may reduce 'bridge-recognition' phenomena sufficiently for a visual distinction to be made between luteal and follicular phase concentrations of progesterone.

INTRODUCTION

The analysis of progesterone concentrations in milk is invaluable for the investigation of a number of reproductive parameters (Laing & Heap, 1971; Hoffman et al., 1976; Lamming & Bulman, 1976; Ball & Jackson, 1979; Günzler et al., 1979). Until recently, however, such analyses were confined to specialized laboratories since radio-immunooassay (RIA) was the only practicable means of progesterone determination. EIA procedures, developed from the early work of Engvall & Perlmann (1971) and Van Weeman & Schuurs (1971), now provide an alternative to RIA and have raised the prospect of a progesterone analysis which could be performed in any laboratory or even on-farm. In both cases it is desirable for the assay procedure to be simple and involve no sample preparation, i.e. an assay carried out on whole milk without prior extraction of steroid. Such a system involving the use of microtitre plates or
microgranular cellulose-linked antiserum has been described previously (Sauer, Foulkes & Cookson, 1981; Sauer et al., 1982). The microtitre plate method, however, offers much in terms of speed and simplicity of operation and so has been used for all our subsequent studies. Although microtitre plate readers are available which will rapidly quantitate enzyme activity in situ, a similar but more sensitive plate system would allow a visual distinction to be made between luteal and follicular-phase levels of progesterone.

The EIA procedure described first in this paper is termed homologous since the progesterone derivative coupled to the enzyme is identical to that used for preparation of the immunogen used in raising the anti-progesterone serum. Generally, when homologous externally-labelled steroids are used in immunoassays an overall increase in the binding affinity of the antibody for them is observed which is associated with additional recognition of the bridge linking steroid to label (Scarisbrick & Cameron, 1975). It has long been appreciated that the maximum sensitivity attainable decreases when antibody affinity for the label is greatly dissimilar to that for the unlabelled antigen (Ekins, Newman & O’Riordan, 1968). This was found to hold true for $^{125}$I-based steroid RIA procedures (Allen & Redshaw, 1978; Corrie, Hunter & MacPherson, 1981a; Stanezyk & Goebelsmann, 1981) and steroid EIA (Van Weemen & Schuurs, 1975; Exley & Abuknesha, 1977; Hosada et al., 1980) when both systems were compared with RIA using $^{3}$H-labelled steroids. The effect of bridge recognition can be considerably reduced by judicious use of heterologous systems in which site of attachment of the enzyme on the steroid, the bridge used or both are altered. Although the heterologous system finally adopted is often determined on an empirical basis, the work of Corrie, Ratcliffe & MacPherson (1981b), using $^{125}$I steroid RIA systems, suggests that where antisera have been raised against hemisuccinate derivatives the use of a glucuronide linkage at the same site on the steroid will generally provide conjugates with the desired characteristics without the need for subsequent selection of exceptional antisera. A comparison of four different conjugate systems is described here which suggests that their findings may also be applicable to EIA procedures.

Using the non-equilibrium homologous microtitre plate EIA for whole milk as described below, it is apparent that although milk progesterone profiles of cows correspond well with those determined by RIA, luteal phase concentrations are invariably over-estimated and occasional transient (less than one day) progesterone peaks may appear around oestrus which are not apparent using RIA. In a preliminary attempt to explain these differences we have investigated the cross-reactivity of various steroids in both the EIA and RIA systems. Since it has been shown that the degree of cross-reactivity may be significantly altered in immunoassays in which equilibrium has not been attained (Vining, Compton & McGinley, 1981), the extent of non-equilibrium in this system has also been investigated.

MATERIALS AND METHODS

Antiserum

The same antiserum (G711/12) was used for all EIA and RIA procedures and was prepared by the method of Furr (1973) using progesterone 11α-hemisuccinate-BSA as immunogen.
Preparation of steroid derivatives

11α-hydroxyprogesterone 11-hemisuccinate (P11-HS) and 11α-hydroxyprogesterone 11-hemimaleate (P11-HM) were prepared by essentially the same means from 11α-hydroxyprogesterone (Steraloids, Croydon, UK) and the respective acid anhydrides (Sauer et al., 1981). 11α-hydroxyprogesterone 11-glucuronide (P11-G) was formed by the method of Corrie et al. (1981). Progesterone 3-(0-carboxymethyl)oxime (P3-CMO) was purchased from Steraloids.

The N-hydroxysuccinimide esters of the carboxylated steroids were prepared in dry dioxane using a dicyclohexylcarbodiimide (Sigma Chemical Co., Poole, UK)-mediated condensation reaction as described by Sauer et al. (1981). The esters of P11-HS and P11-HM were prepared and isolated as crystalline products and stored dessicated until used for conjugation with β-galactosidase. The esters of P3-CMO and P11-G were used directly without purification following addition of acetic acid in dioxane (molar ratio steroid: acetic acid, 1:0.5) to eliminate unreacted carbodiimide and filtration to remove the by-product dicyclohexylurea.

Preparation of steroid-enzyme conjugates

The β-galactosidase preparation was dialysed against 0.1M phosphate-saline buffer pH 7.0 before use. Solutions of the N-hydroxysuccinimide esters in dry dioxane were conjugated directly with β-galactosidase (grade VIII, Sigma Chemical Co.) in phosphate-saline buffer by dropwise addition as described by Sauer et al. (1981): a 25:1 or 250:1 molar excess of steroid was used. One hundred percent conversion of P3-CMO and P11-G to the esters was assumed. The volume ratio of β-galactosidase in buffer to steroid in dioxane was maintained at or above 10:1.

Following conjugation of steroid with enzyme, unconjugated steroid was eliminated by exhaustive dialysis and gel filtration (Sephadex G25) as previously described (Sauer et al., 1981) and the conjugates stored at 4°C in 0.1M phosphate-buffered saline (pH 7.0) containing 0.1% (w/v) sodium azide and 0.1% (w/v) gelatin (PAS-gelatin buffer).

Microtitre plate EIA procedure

The wells of flat-bottomed polyvinyl plates (Dynatech M29) were coated with the y-globulin fraction of BSA-absorbed anti-progesterone serum as described by Sauer et al. (1982) after washing the plate wells with Tween 20 (Sigma Chemical Co. 0.05% v/v) and rinsing twice with distilled water. The microtitre plate EIA procedure was carried out essentially as reported by Sauer et al. (1982) using progesterone standards prepared in whole milk from a cow at oestrus or from an ovariectomized cow. Addition of immunoassay reagents (standards, 10 µl; conjugate, 200 µl) was performed at 4°C for standardization purposes, either simultaneously using a pipettor/diluter (IMV, L'Aigle, France) or in rapid succession using solid interface pipettes (Socorex, Renens, Switzerland or SMI, Berkeley, USA). Both the immunoassay incubation (3 h) and enzyme incubations (1 h) were carried out at 40°C. The substrate (o-nitrophenyl β-D-galactopyranoside, 3 mg/ml; Sigma Chemical Co.) solution was cooled to 4°C before addition. The colour produced on incubation was measured directly through the wells using an automatic plate reader (Dynatech MR 580).
Radioimmunoassay procedures

Both the liquid-phase and microtitre plate RIA procedures were carried out at 4°C essentially as described by Sauer et al. (1982).

Cross-reactivity studies

Steroid standards were prepared in PAS-gelatin buffer and assays performed as described. The cross-reactivity data was assessed by the criteria of Abraham (1969).

Collection of milk samples

Representative morning milk samples from Friesian cows were taken from the individual bulk milk jars by stockmen following agitation of the milk. Sodium azide solution (20% w/v) was added to the samples (5 μl/ml) and the milk stored at 4°C until assayed.

Study of conjugate association time

Standards prepared in whole milk were used to assess the time taken for maximum binding of label to the antibody-coated plates with or without competing steroid present. A comparison was made between the binding of enzyme conjugate at both 4°C and 40°C with the binding of 3H-progesterone at 4°C.

RESULTS AND DISCUSSION

Standard curves typical of those obtained in the presence and absence of milk using homologous EIA are shown in Fig. 1. The presence of milk caused a considerable drop (26%) in conjugate binding, necessitating the use of standards prepared in whole milk when assaying unknown milk samples. A drop in label binding (65%) in the

![Absorbance graphs](image)

Fig. 1. Comparison of EIA standard curves using standards prepared in buffer (○ - ○) or milk (△ - △). Values were corrected for substrate blanks and are mean of four determinations ± 2 SEM. For ease of comparison results are expressed in terms of absorbance and B/B₀.
comparable microtitre plate RIA (Fig. 2C) but not in liquid phase RIA (Fig. 2A) indicated that binding inhibition is a feature of the microtitre plate system. The quantity of antibody adsorbed on the microtitre plate wells is far less than that present in the liquid phase system: approximately 50% in the case of the microtitre plate RIA and 20% in the case of the EIA as determined by binding of $^3$H-progesterone to the wells in the presence of milk.

![](image)

Fig. 2. Comparison of liquid phase (A) and microtitre plate (B and C) RIA standard curves using standards prepared in buffer (●●●) or whole milk (▲▲▲). Values are mean of four determinations ± 2 SEM.

Although luteal phase concentrations of milk progesterone were over-estimated using the homologous EIA system (Fig. 3) profiles corresponded well with those determined by liquid phase RIA and have been successfully used in predicting the time of artificial insemination in cattle (Foulkes, Cookson & Sauer, 1982). Progesterone peaks may occur around oestrus in some animals (e.g. Pro 39, Fig. 3). Such transient peaks have also been noted using RIA (E. D. Watson, unpublished data) although none were detected here. These anomalies could be explained in part by the increased cross-reactivity observed for certain steroids using EIA when compared with liquid-phase microtitre plate RIA (Table I). In particular, 5α-pregnanedione and pregnenolone show cross-reactivities an order of magnitude higher than those seen with liquid phase RIA. The presence of 5α-pregnanedione in milk has been demonstrated previously (Darling & Harkness, 1973; Purdy et al., 1980) and could therefore be responsible for the 'spikes' seen around oestrus; it is less likely that levels would be high enough (Purdy et al., 1980) to completely explain the large over-estimation observed here in the luteal phase or that oestrogens (Mønk, Erb & Mollet, 1975; Heap & Hammon, 1979; Pandey et al., 1981) or corticosteroids (Schwalm & Tucker, 1978) are present in sufficient quantities in whole milk to cross-react significantly in the assay. It is improbable that use of the γ-globulin fraction of the progesterone antiserum for the EIA rather than whole antiserum as used in the liquid
Fig. 3. Comparison of progesterone concentration in whole milk assayed by microtitre plate EIA (---) and liquid phase RIA (----). Samples were collected over a 30-day period around oestrus.

Fig. 4. Dynamics of enzyme-progesterone conjugate (A and B) or \(^{3}H\)-progesterone (C) binding to antibody-coated microtitre plate wells at 4°C or 40°C in the presence of 0 (\(\triangleleft\)), 10 (\(\triangleright\)), 20 (\(\blacktriangleleft\)), 50 (\(\bullet\)), 100 (\(\bigcirc\)), 200 (\(\bigtriangleup\)), 800 (\(\square\)) or 5000 (\(\bigcirc\)) pg progesterone. Whole milk standards were used. Values are mean of two determinations or four determinations ± 2 SEM.
phase RIA was responsible for the differences in cross-reactivity since such differences were not apparent when comparing the liquid phase RIA with the microtitre plate RIA (Fig. 4).

The work of Vining et al. (1981) indicated that equilibrium must be achieved in an immunoassay in order that maximum specificity and sensitivity are attained. Equilibrium was not achieved in the EIA system even after incubation for 8 h at 40°C or 72 h at 4°C whereas in the RIA system equilibrium was attained after 4 to 6 h at 4°C (Fig. 4). The changed cross-reactivities seen with the microtitre plate EIA could therefore be a result of the non-equilibrium nature of this procedure.

If non-specific influences resulted in the conjugate binding inhibition seen in the standard curves (Fig. 1) and were present in greater quantities in the test milk samples a further depression of binding would occur, with a consequent over-estimation of progesterone concentration. Since no attempt has been made to eliminate such non-specific binding inhibition or to correct progesterone estimations by use of individual sample blanks, such an occurrence could also contribute to the over-estimation of luteal progesterone concentrations.

The progressive increase in sensitivity achieved when a series of heterologous bridge and site systems were compared with the homologous assay is shown in Fig. 5. The effect of using conjugates prepared using a 25 or 250-fold molar excess of steroid derivative is given in Figs 5A and 5B respectively. Where the 25 molar excess of steroid was used the slopes of the curves increased in the order P11-HS, P11-HM, P3-CMO, P11-G. A similar picture was seen using a 250 molar steroid excess but the slopes of the P3-CMO and P11-HM curves were almost identical. The P11-G conjugate prepared

### TABLE I

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Cross-reaction at 50% label* displacement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liquid-phase RIA</td>
</tr>
<tr>
<td>Progesterone</td>
<td>100</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>5α-Pregnane-3, 20-dione</td>
<td>6.8</td>
</tr>
<tr>
<td>5α-Pregnane-3, 20-dione</td>
<td>1.3</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>3.6</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>0.05</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>11α-Hydroxyprogesterone</td>
<td>75.8</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1.6</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0.03</td>
</tr>
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</table>

*RIA [3H]-progesterone label, EIA progesterone 11α-hemisuccinate-β-galactosidase label (conjugate 98, 11 ng/well) prepared using 25:1 molar steroid excess at conjugation.
using a 25 molar excess clearly gave rise to the most sensitive standard curve. Since the hemisuccinate and hemimaleate bridges differ only in respect of one double bond (Fig. 6) it is not surprising that use of the latter gave rise to the smallest increase in sensitivity. This contrasts with the findings of Gros, Flecheux & Dray (1978) who reported that the use of hemimaleate conjugates resulted in binding inhibition curves markedly steeper than those seen using the hemisuccinate conjugates and essentially identical in slope to those obtained in RIA using $^3$H-progesterone as the label.

Fig. 5. Comparison of EIA standard curves using progesterone 11α-hemisuccinate (● – ●), progesterone 11α-hemimaleate (○ – ○), progesterone 3-(0-carboxymethylene)oxime (□ – □) or progesterone 11α-glucuronide (■ – ■) conjugates prepared using a 25 molar (A) or 250 molar (B) excess of steroid. Values are mean of four determinations ± 2 SEM.

Fig. 6. Comparison of structures of enzyme-progesterone conjugates formed using: A 11α-hydroxyprogesterone 11-hemisuccinate, B 11α-hydroxyprogesterone 11-hemimaleate, C progesterone 3-(0-carboxymethylene)oxime and D 11α-hydroxyprogesterone 11-glucuronide.
TABLE II
THE INFLUENCE OF SITE AND BRIDGE HETEROLOGY AND THE DEGREE OF STEROID INCORPORATION ON THE BINDING OF PROGESTERONE-β-GALACTOSIDASE CONJUGATES TO ANTI-PROGESTERONE 11αHEMISUCCINATE γ-GLOBULIN

<table>
<thead>
<tr>
<th>Conjugate no.</th>
<th>Bridge position and type</th>
<th>Molar ratio steroid: enzyme at conjugation</th>
<th>Mass conjugate used in EIA ng/well</th>
<th>B_0 value absorbance units*</th>
<th>Mass progesterone required for 50% conjugate displacement pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>104</td>
<td>11α-hemisuccinate</td>
<td>25:1</td>
<td>12</td>
<td>0.320</td>
<td>875</td>
</tr>
<tr>
<td>105</td>
<td>11α-hemisuccinate</td>
<td>250:1</td>
<td>10</td>
<td>0.487</td>
<td>1220</td>
</tr>
<tr>
<td>101</td>
<td>11α-hemimaleate</td>
<td>25:1</td>
<td>21</td>
<td>0.389</td>
<td>380</td>
</tr>
<tr>
<td>90</td>
<td>11α-hemimaleate</td>
<td>200:1</td>
<td>40</td>
<td>0.412</td>
<td>216</td>
</tr>
<tr>
<td>110</td>
<td>3-(0-carboxymethyl)-oxime</td>
<td>25:1</td>
<td>136</td>
<td>0.217</td>
<td>100</td>
</tr>
<tr>
<td>111</td>
<td>3-(0-carboxymethyl)-oxime</td>
<td>250:1</td>
<td>11</td>
<td>0.247</td>
<td>230</td>
</tr>
<tr>
<td>114</td>
<td>11α-glucuronide</td>
<td>25:1</td>
<td>102</td>
<td>0.109</td>
<td>56</td>
</tr>
<tr>
<td>115</td>
<td>11α-glucuronide</td>
<td>250:1</td>
<td>10</td>
<td>0.156</td>
<td>87</td>
</tr>
</tbody>
</table>

* Corrected for substrate blank.

Table II summarizes the sensitivity of each standard curve in terms of the mass of progesterone required to displace 50% of each conjugate. It was previously demonstrated that higher steroid ratios at conjugation resulted in a higher degree of steroid incorporation (Sauer et al., 1981). It was apparent, therefore, that lower levels of steroid incorporation resulted in greater sensitivity in all conjugates except those using the hemimaleate bridge. Using the P11-G and P3-CMO conjugates, approximately ten times more conjugate was required to attain a similar degree of binding in the absence of free progesterone (B_0 value) when conjugates with the lower degree of steroid incorporation were employed. With P11-HS and P11-HM conjugates, however, this difference was less marked possibly due to a higher degree of bridge recognition. The efficiency of the conjugation procedure using the P11-HM, P3-CMO and P11-G derivatives has not been investigated and therefore the effect of variation in the degree of steroid incorporation cannot be excluded.

It can be argued that increasing the assay sensitivity would enable less milk to be used for the progesterone determination and, as a consequence, that this would reduce the non-specific influence of milk components on conjugate binding. It has been suggested by others (Van Weeman & Schuurs, 1975; Hosada et al., 1980) that such an increase in sensitivity might be attained by the use of heterologous systems although evidence derived from 125I-RIA systems indicates that previous success has been largely as a result of the chance availability of exceptional antisera (Cameron et al., 1974; Pratt, 1978; Corrie et al., 1981a). The work of Corrie et al. (1981b) suggests that a more systematic approach might be adopted for selection of bridge-heterologous
systems. In particular, where a hemisuccinate bridge had been used for immunogen production (the most common in the case of anti-progesterone sera), selection of a glucuronide bridge for the conjugate would be indicated. This approach resulted in an 125I-RIA for progesterone in which bridge recognition was apparently reduced to a minimum and which as a result was effective using a number of antisera (Corrie et al., 1981b). One antiserum was examined in this laboratory but since it was not selected for particular use in EIA the improved sensitivity achieved (Fig. 5) when P11-G-β-galactosidase conjugates were employed supports the argument of Corrie et al. (1981b).

It is hoped that a sensitive heterologous system will provide a sound basis for the development of an on-farm test for progesterone enabling a visual distinction to be made between milk samples from cows at follicular or luteal phases of the oestrous cycle. This will allow ready investigation of reproductive disorders, improved timing of AI and convenient pregnancy diagnosis.

ACKNOWLEDGEMENTS

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REFERENCES


3

Principles of Enzyme Immunoassay

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INTRODUCTION

The development of radioimmunoassay (RIA) 25 years ago (Berson & Yalow, 1959) marked the beginning of a new era in biochemical analysis, offering the possibility of assaying a wide variety of proteins and polypeptides. Almost 10 years passed before the potential of this method for the determination of haptens was realised (Oliver et al., 1968). With this came the very real possibility of assaying, with unprecedented sensitivity and specificity, any biochemical substance against which an antibody could be raised. Disadvantages, such as there are, revolve around the use of radioisotopes for quantification. Some isotopes have short half-lives, their determination requires sophisticated and expensive equipment and their toxic nature necessitates the application of strict regulatory controls. As a result, RIA has been excluded from many small laboratories, especially in the food manufacturing industry.

Although many alternative forms of labelling have been considered (Schall & Tenoso, 1981), the use of enzyme-labelled reagents for immunoassays (Engvall & Perlmann, 1971; van Weemen & Schuurs, 1971) has provided the most convenient means by which the sensitivity and specificity of RIA can be more generally applied. The reagents used are
those commonly employed in general laboratories and are thus not associated with special hazards: the enzymes used for labelling are cheap and may be stored for in excess of one year at 4°C, or at room temperature when freeze-dried. Similarly, the equipment required for enzyme immunoassay (EIA) is common to the majority of the laboratories and requires only the availability of accurate pipettes and dispensers, and a colorimeter or spectrophotometer. End-point determination, i.e. the quantification of colour or fluorescence, need not be rate limiting. Microtitre plate readers enable automatic optical density (OD) determination at approximately one per second and microprocessor interfacing is possible for data reduction. Since EIA can be easy to perform and colorimetric end-points visually assessed, it has been adopted for simple screening procedures for use in the field (Voller et al., 1976a).

ASSAY PRINCIPLES

Two basic types of immunoassay employ enzyme-labelled reagents: the enzyme immunoassay (EIA) and the sandwich assay, which are broadly analogous with RIA and immunoradiometric assay, respectively. They rely on the inherent characteristic of antigen and antibody to bind with high avidity and specificity.

Enzyme immunoassay

EIA encompasses immunoassays based on the 'saturation assay' principle in which the analyte is enzyme-labelled (Fig. 1). The assay comprises three components: (i) a limited and constant quantity of antiserum specific for the analyte, (ii) a limited and constant quantity of enzyme-labelled analyte, and (iii) standard quantities of analyte for calibration purposes (or unknown quantities of analyte in the test sample). When the

- **Ag** = antigen or hapten
- **Enz** = enzyme label
- **P** = solid phase
- **Ab** = specific antibody

![Fig. 1](image-url)  
**Fig. 1.** Principle of enzyme immunoassay; **Ag** = antigen or hapten, **Enz** = enzyme label, **P** = solid phase, **Ab** = specific antibody.
three components of the system are mixed, labelled and unlabelled analyte compete for the limited number of antibody binding sites (Fig. 1). Since each test contains the same amount of antibody and labelled analyte, the greater the quantity of unlabelled analyte present, the smaller will be the quantity of labelled analyte binding to antibody. Separation of free labelled analyte from that bound to antibody, and subsequent addition of substrate to the bound fraction, allows enzyme activity to be measured and the degree of competition to be ascertained. The greater the quantity of analyte present, the fainter will be the colour produced. The use of standard amounts of analyte enables calibration curves to be constructed and quantification of analyte in the unknown sample by interpolation (Fig. 2). A typical EIA protocol is given in Table 1.

This type of EIA requires separation of free analyte from antibody-bound analyte for quantification and is termed 'heterogeneous'. The homogeneous EIA or enzyme-mediated immunoassay technique (EMIT) is a special case of EIA which does not depend on free and bound label separation to enable assessment of the degree of competition (Boguslaski & Li, 1982). Until recently, this method could only be applied to the determination of haptens. It relies on the use of enzyme–hapten conjugates in which enzyme activity is altered on binding with the antibody. Figure 3 illustrates the principle behind the most common form of EMIT in which enzyme activity is inhibited by specific interaction with the antibody: enzyme activity is proportional to the concentration of hapten since the degree of inhibition will be reduced by its presence. Although these assays can provide very rapid tests (2–3 min) since separation is not required, these procedures are far less sensitive than their heterogeneous counterparts (100–1000 fold) and their application has largely been limited to the monitoring of drugs during therapy.

Sandwich Assays
'Enzyme-linked immunosorbent assay' (ELISA) encompasses all solid-phase immunoassays using enzyme-labelled reagents, but is most commonly used to describe non-competitive solid-phase sandwich assays: it will, therefore, be used here in the more restricted latter context. These heterogeneous methods are analogous with immunoradiometric assays (Miles & Hales, 1968; Addison & Hales, 1971) and are based on the principle of 'reagent excess'. In contrast to EIA, there is no element of competition and the amount of enzyme label bound is directly proportional to the concentration of analyte. Solid-phase reagents are used
Fig. 2. Typical calibration curves for enzyme immunoassay.

Table 1
Typical Assay Protocol for Microtitre Plate Enzyme Immunoassay

1. Adsorb specific antibody onto wells of microtitre plate (Voller et al., 1976a). This involves addition of diluted, purified antibody (200–300μl, dependent on plate type) and incubation for 3 h at room temperature or overnight at 4°C. Store until required. Wells are emptied and washed prior to use.

2. Add sample or standard, then enzyme-labelled antigen (or hapten) to the wells and incubate for 2–3 h at room temperature.

3. Empty and wash wells with buffer (× 3).

4. Add substrate solution and incubate at a temperature suited to enzyme (20°C for peroxidase, 20–40°C for alkaline phosphatase or β-galactosidase). Stop reaction by addition of sodium hydroxide and determine absorbance. The reaction need not be stopped if an automatic microtitre plate reader is used since all 96 wells may be read in 1–1½ min.

5. Plot calibration curve and interpolate results.

for separation of free label from bound label which also facilitates the removal of excess reagents after each stage.

Although based on the same principle, there are numerous variants of the ELISA procedure (Schuurs & van Weemen, 1980), the majority of which use enzyme-labelled antibody as tracer: Table 2(a) illustrates the
Fig. 3. Principle of homogeneous enzyme immunoassay. In this type of homogeneous assay, enzyme activity is inhibited when antibody is bound to the enzyme conjugate (a). The presence of hapten reduces the degree of antibody binding to conjugate and thus the degree of enzyme inhibition (b).

principle of these procedures and Table 2(b) the end product of some of these variants. Solid-phase-linked antigen or antigen-specific antibody is required for the binding and subsequent detection of antibody or antigen, respectively. In the latter case, an assay is performed by sequential addition of antigen and enzyme-labelled antibody to the solid-phase-linked antibody system with a washing step being performed after each addition. Addition of substrate enables quantification of the bound label.

Whichever procedure is used, the solid-phase reagent should ideally be present in sufficient excess to ensure complete immunoadsorption of the analyte in the standard or test sample. Similarly, an excess of all other reagents ensures complete sandwich formation for each analyte molecule. The process of sandwich formation obviously requires that the antigen be multivalent. The solid-phase antibody and labelled antibody must both be specific for the antigen but should be directed against different and spatially separated antigenic determinants. A typical calibration curve and assay protocol are shown in Fig. 4 and Table 3, respectively.

The enzyme-labelled species-specific anti-immunoglobulin is one of the more commonly used labels and forms the basis of the indirect ELISA (Table 2(b)). Although this procedure may involve an additional step in the assay, it obviates the need to label the analyte-specific antiserum or the analyte which may be in limited supply. Furthermore, the same label may be used in assays of a number of analytes depending upon the species of the specific antiserum. In order to avoid non-specific binding of label, it is important that the two analyte-specific antisera are raised in phylogenetically distant species, the labelled species-specific anti-
Table 2
Principles of the Sandwich Assay

(a) Sandwich assay for detection of antigen

\[ \text{3*-Ab} + \text{Ag} \rightarrow \text{3*-Ab}...\text{Ag} \rightarrow \text{3*-Ab}...\text{Ag}...\text{Ab}_1\text{-Enz} \]

(Kato et al., 1975; Maiolini & Masseloff, 1975; Ishikawa & Kato, 1978; Ishikawa et al., 1982)

(b) Final products of commonly employed types of sandwich assay

(i) Detection of antibody.
(Schmitz et al., 1977)

(ii) Detection of antibody.
(Schuurs & van Weemen, 1980)

Indirect sandwich assay:
(iii) Detection of antibody.
(Engvall & Perlmann, 1972)

(iv) Detection of antigen.
(Voller et al., 1978)

\[ \text{3*} = \text{Solid phase; Ab} = \text{Antigen-specific antibody; Ab}_1 = \text{antigen-specific antibody raised against different antigenic determinants to those of Ab; Ab}_2 = \text{antibody against immunoglobulin of the species in which Ab}_1 \text{ was produced; in (iv) Ab}_1 \text{ should therefore be raised in a different species than Ab; Protein A = a protein isolated from S. aureus which can interact specifically with the Fc region of most mammalian immunoglobulins.} \]

immunoglobulin being raised against immunoglobulin of the species in which the second anti-analyte antibody (Ab}_1 \text{ in Table 2(b)) was raised.}

ENZYME-LABELLED REAGENTS

Choice of Enzyme
The choice of enzyme and its specific activity are primary considerations in relation to assay sensitivity since it will determine the size of signal at the assay end-point. Table 4 indicates important criteria for the selection of enzymes for use as labels. Few enzymes fulfill all these criteria but
Fig. 4. Typical calibration curves for a sandwich assay.

Table 3
Typical Assay Protocol for a Microtitre Plate Sandwich Assay for the Determination of Antigen

1. Adsorb specific antibody onto wells of the microtitre plates (Voller et al., 1976α), as Table 1. Store plates (containing buffer and a bacteriostat) at 4 °C until required. Plastic film may be used to seal the plates.

2. Empty and wash wells with buffer prior to use.

3. Add sample or standard at a suitable dilution and incubate for 2 h at room temperature. Empty and wash the wells with buffer (× 3).

4. Add enzyme-labelled specific antibody and incubate for 2 h at room temperature. Empty and wash wells (× 3).

5. Add substrate solution, incubate for a suitable period and, if necessary (see Table 1), stop the reaction. Determine the absorbance.

6. Plot calibration curve and interpolate results.

Other types of sandwich assay (Table 2) are performed in a similar way. In the indirect assay for antigen a further incubation stage is required prior to addition of substrate.

enzymes such as β-galactosidase, alkaline phosphatase and peroxidase are most commonly used. All produce assays of approximately equivalent sensitivity and are available commercially in a suitably pure form and at reasonable cost. Given this, the prudent operator would perhaps
Table 4
Considerations for Selection of Enzyme

1. Purity of enzyme preparation.
2. Specific activity of enzyme.
4. Absence of interfering factors or enzyme-like activity in test fluid.
5. Ease and speed of estimation of enzyme activity and the safety of the reagents used.
6. Availability and cost of enzyme.
7. Stability of the enzyme and its conjugate.

base selection on the ease of enzyme activity determination and the safety of the chemicals used. Peroxidase, for instance, is one of the least expensive enzymes and has many of the properties required for EIA, but the majority of chromogens used in its determination are mutagenic or carcinogenic; the formation of carcinogenic products is also likely whichever chromogen is used (Saunders et al., 1964).

Although colorimetric end-points are preferred for most applications, β-galactosidase, alkaline phosphatase and peroxidase may also be assayed using substrates that form fluorescent products. This can increase the sensitivity of detection of enzyme by at least an order of magnitude (van der Waart & Schuurs, 1977; Ishikawa & Kato, 1978; Arakawa et al., 1979; Ishikawa et al., 1982).

Formation of Enzyme-Conjugates
Many potential users of EIA or ELISA are apprehensive of the chemistry involved in production of enzyme-labelled reagents. In ELISA, however, it is invariably possible to adopt procedures such as the indirect sandwich assay (Table 2(b)) in which commercially-available labels such as species-specific enzyme-labelled anti-immunoglobulin are used.

In general, enzyme-labelled forms of particular analytes or primary antibodies however, are not available commercially, and careful consideration should be given to the method of conjugate formation. Conjugates are required in which both the catalytic activity of the enzyme and immunoreactivity of the analyte or binding characteristics of the antibody are retained.

Conjugation of analyte or antibody with enzyme is generally achieved by covalent bonding through the lysine ε-amino groups of the enzyme or,
where glycoprotein enzymes such as peroxidase are used, through carbohydrate residues. Methods of conjugation and their relative merits have been discussed by a number of authors (Schuurs & van Weemen, 1977; O'Sullivan et al., 1979; Sauer et al., 1982a) and will be described only briefly here.

**Protein-Enzyme Conjugates**

Since enzymes are themselves proteins containing reactive groups, the main problems in the production of enzyme–protein conjugates are related to the prevention of co-conjugation (polymerisation) of the enzyme or protein. Coupling reagents such as glutaraldehyde (Avrameas, 1969; Avrameas & Ternynck, 1971; Voller et al., 1976a) and periodate oxidation (Nakane & Kawaoi, 1974) are still widely used and reported to provide conjugates of useful quality despite the probability of some degree of polymerisation and denaturation. It has been reported that formation of these undesirable by-products can be avoided using heterobifunctional reagents (Kitagawa & Aikawa, 1976; O'Sullivan et al., 1978; Tae, 1983). Normally, incorporation of 1–2 enzyme molecules per antigen or antibody will be optimum since more extensive coupling may result in reduced enzyme activity or immunoreactivity.

**Hapten Enzyme Conjugates**

Haptens may, in some instances, be coupled directly with enzymes, but usually it is necessary to form a derivative to provide a reactive group at a suitable location (Robinson et al., 1975). Hapten–enzyme conjugates are commonly produced through formation of a peptide bond between carboxylic acid groups of the hapten and amino-groups on the enzyme or vice versa. Since enzymes contain both of these groups, methods which avoid polymerisation of the enzyme are preferred: mixed anhydride (Erlanger et al., 1957) and N-hydroxysuccinimide ester (Anderson et al., 1964) procedures have been used to covalently bind haptens or their derivatives to enzymes with minimal enzyme denaturation (Comoglio & Celada, 1976; Joyce et al., 1978; Hosoda et al., 1980; Sauer et al., 1981). Direct carbodiimide condensation, on the other hand, will inevitably cause some degree of denaturation since the condensation reagent will necessarily come into contact with the enzyme with the result that the conjugate may need to be purified by fractionation or affinity chromatography (Dray et al., 1975; Exley & Abuknesh, 1977). In theory, the coupling of one hapten per enzyme should be optimal, but in practice the location and orientation of the hapten on the enzyme may be important since the possibility of steric hindrance of antibody binding arises (Sauer
et al., 1981). It may, therefore, be necessary to attach several hapten molecules to each enzyme to ensure immunoreactivity: the optimum degree of incorporation may thus need to be determined experimentally and will depend on the enzyme and hapten employed.

Purification of Conjugates
It is essential that enzyme-labelled reagent is free from unlabelled analyte or antibody following conjugation to prevent a reduction in the sensitivity of the immunoassay. For both enzyme–hapten and enzyme–protein conjugates, this is most commonly achieved by gel filtration on the basis of molecular-weight difference. The presence of unconjugated enzyme is rarely problematic since this is washed away with the unbound label during the immunoassay.

Conjugates can often be stored at 4°C in buffer containing preservatives such as sodium azide (β-galactosidase and alkaline phosphatase) or merthiolate (peroxidase). The preservative used should be carefully considered since some may inhibit enzyme activity.

SEPARATION OF FREE FROM ANTIBODY-BOUND LABEL

Numerous methods are employed for separation of free label from antibody-bound label (Table 5) although the most convenient means is through the attachment of one of the immunoreagents to a solid support. The antigen or antibody may be attached covalently to particulate material such as cellulose or agarose (Wide, 1981) or by passive adsorption to the inner walls of plastic tubes or microtitre plate wells (Engvall & Perlmann, 1971; Voller et al., 1974). Separation of free and bound label during the immunoassay can be achieved by pouring-off or aspirating the free label: a particulate solid phase would obviously require to be sedimented before separation.

Immunoprecipitation (by the double antibody technique) has often been used in EIA, particularly in the performance of steroid hormone assays (Dray et al., 1975; Comoglio & Celada, 1976; Joyce et al., 1978). It offers the advantage of better availability of primary antibody to the other reagents, since all reagents remain in solution until the precipitating second antibody is added. It has been suggested that this may result in assays of increased sensitivity (Joyce et al., 1978) although this has not been a general finding (Arakawa et al., 1979; van Weemen et al., 1979). This method does, however, require an extra incubation stage fol-
Table 5
Methods of Separating Bound from Free Label

1. Polyethylene glycol precipitation (Österman et al., 1979).
2. Double antibody precipitation (Comoglio & Celada, 1976; Joyce et al., 1978).
3. Double antibody solid-phase (Comoglio & Celada, 1976; Bosch et al., 1978).
4. Solid-phase primary antibody:
   - Polycarbonate-coated, polystyrene or nylon balls (covalent or passive binding: Miranda et al., 1977; Smith & Gehle, 1977; Hendry & Herrman, 1980).
   - Polystyrene test tubes (passive binding: Engvall & Perlmann, 1972; Stimson & Sinclair, 1974; Voller et al., 1975).
   - Polystyrene or polyvinyl microtitre plates (passive binding: Voller et al., 1976a, 1976b; Sauer et al., 1982a).

Following addition of second antibody and so may be less convenient than solid phase EIA procedures. Again, separation is achieved following centrifugation.

EIA and ELISA procedures were considerably simplified with the adoption of disposable plastic microtitre plate systems (Voller et al., 1976b) and these are worth particular consideration. The 125 mm x 85 mm plates are manufactured in polystyrene or polyvinyl and comprise 96 wells in a 8 x 12 format; dependent upon type, each well may contain a volume of up to 400μl. Antigen or antibody is passively adsorbed onto the inside of the wells and the plates stored until required. They may be sealed and kept at 4°C with buffer/preservative in the wells or dried and stored at room temperature in sealed dehydrated sachets, depending on the stability of the adsorbed material. During immunoassay, separation of free from bound label is performed simultaneously in all 96 wells by inversion and tapping dry over absorbent material. Over the past few years numerous automatic (through-the-well) colorimeters with microprocessor interface facilities have become available. These enable optical density determinations to be carried out without transfer of the samples into cuvettes. Since all 96 wells may be read in 1–1½ minutes it is not generally necessary to stop the enzyme reaction.
FACTORS INFLUENCING SENSITIVITY

The factors that influence sensitivity of EIA and ELISA parallel those in their radio-labelled analogues, although additional consideration must be given to factors which may modify enzyme activity and have been the subject of recent reviews (van der Waart & Schuurs, 1977; Sauer et al., 1985).

The sensitivity of immunoassays is commonly defined as the smallest amount of analyte which gives rise to a response significantly different from zero at the 95% confidence level (limit of detection). This would be determined from the assay calibration curve and is defined as the mean value of the zero standard minus two standard deviations for EIA and for ELISA is the mean of the zero standard plus two standard deviations. When optimising conditions in order to improve sensitivity, the slope of the standard curve and the precision of determinations must be taken into account. A calibration curve of shallow slope may, for instance, be more capable of discriminating between similar concentrations of analyte than a steeper slope if precision of the former is greater (Ekins, 1983).

Influence of the Antiserum

The limit of detection of EIA is essentially determined, in accordance with the Law of Mass Action, by the avidity of binding of analyte to antibody: the more avid the antiserum the more sensitive will be the assay. In ELISA, the influence of avidity of binding is not so apparent since excess reagents are used and sensitivity will depend on the signal to noise ratio and, by implication, on the specific activity of the label (Ekins, 1978; Ishikawa & Kato, 1978).

The mass of antibody used in EIA or ELISA is determined by experiment through comparison of a range of antibody dilutions and conjugate concentrations in the presence or absence of clinically significant quantities of analyte (antibody dilution curves). In this way, the mass of reagents required to provide calibration curves over the range of clinical concentrations and with the required sensitivity can be ascertained. Objective assessment of the effect of alterations in method on assay performance may be aided by plotting precision profiles (Ekins, 1983).

The quantity of antigen required for detection of antibodies in ELISA would be similarly assessed by the preparation of dilution curves. For serological diagnosis of infectious diseases, e.g. Trichinella spiralis infection in pigs (see Bibliography index p. 20), however, only crude
antigens are normally available. In these circumstances coatingconcentrations of antigen for ELISA are normally determined by doing chequer-board titrations against positive and negative sera and subsequent tests performed using the antigen dilution which gave the greatest discrimination (Voller et al., 1976b).

**TIME AND TEMPERATURE OF INCUBATION**

There are no hard and fast rules regarding optimum time and temperature for incubation, and where sensitivity is not critical, conditions are often adopted on the grounds of convenience. Short incubations may not be appropriate where large numbers of samples are involved since this can result in overlap of assays: 2–3h immunoreaction time is normally suitable. In ELISA, room temperature or 37°C incubations are common and can enable binding to approach completion within this period. EIA procedures are often carried out at 4°C however, since elevated temperature can drastically reduce assay sensitivity: where the antigen–antibody reaction involves a large enthalpy component, binding affinity will decrease with increased incubation temperature (Keane et al., 1976). Enthalpy plays only a small part in the binding reaction of some antisera, however, and in these cases sensitivity may be relatively independent of incubation temperature (Malvano & Rolleri, 1975; Keane et al., 1976). Such antisera should be identified at an early stage since they may enable incubations to be carried out at higher temperatures (e.g. 37°C or 40°C) and therefore for shorter periods without loss of sensitivity.

**THE NATURE OF THE LINK BETWEEN HAPTEN AND ENZYME**

RIAs of haptens are often performed using tritium-labelled hapten and the label is considered to be immunologically identical to the unlabelled hapten. In the case of enzyme-labelled hapten, however, the label may be bound with greater avidity than the native hapten because structurally it is very similar to the immunogen against which the antibodies were raised — a phenomenon referred to as 'bridge recognition'. This may result in a considerable loss in sensitivity (van Weemen & Schuurs, 1975) typified by a reduction in slope of the standard curve compared with
tritium-based RIA using the same antiserum. Where such a loss in sensitivity is problematic, it has been found that changing the bridge linking the hapten to the enzyme and/or the site of attachment on the hapten can result in recovery of sensitivity (van Weemen & Schuurs, 1975; Exley & Abuknesha, 1977; Arakawa et al., 1979; Hosoda et al., 1980; Sauer et al., 1982b).

NON-SPECIFIC EFFECTS

Non-specific adsorption of enzyme label, interference with specific binding and factors interfering with determination of enzyme activity have been reported to reduce assay performance, especially where relatively large volumes of undiluted or unextracted sample are assayed. Biological samples may, for instance, contain high concentrations of endogenous enzyme or substances which inhibit or enhance enzyme activity. In general, however, adequate wash procedures between assay stages can prevent such interference.

Non-specific adsorption of enzyme-label to the solid phase has been reported with solid-phase systems using passive adsorption of reagent to the solid phase (plastic tube and microtitre plate methods). This can normally be avoided by procedures which are now standard in most protocols: excess adsorption sites unoccupied by antibody may be blocked by addition of a high concentration of unrelated protein after antigen or antibody has been adsorbed. Alternatively, the inclusion of detergent or high molarity salt at the immuno-incubation stage can be used to inhibit non-specific binding (Engvall & Perlmann, 1971; Engvall et al., 1971; Engvall & Ruoslahti, 1979; Saunders, 1979).

In steroid hormone EIA, as in RIA, the presence of steroid binding globulin in the plasma or serum of some species has been reported to interfere with the specific interaction of steroids and antibody. Specific displacement of steroid from the binding globulin has been achieved by reducing the pH at which the immunoreaction is performed (Riad-Fahmy et al., 1981) or by prior heating of the sample (Ogihara et al., 1977).

CONCLUSIONS

EIA and ELISA have been extended to many disciplines in the biological sciences and assays have been developed for a broad range of biochemi-
cal substances. These include serum proteins (Schuurs & van Weemen, 1977), hormones (Sauer et al., 1982a), antibodies against infectious diseases (Voller et al., 1978), viral antigens in infected plants (Voller et al., 1976c; Clark & Adams, 1977), food constituents (Morgan et al., 1983; chapters in this book and others listed in the Bibliography), antibiotics (Standefer & Saunders, 1978; Miura et al., 1981) and herbicides (Niewola et al., 1983). Heterogeneous EIA and ELISA are in limited use on a routine basis outside the sphere of infectious disease diagnosis although an increasing number of assays have been demonstrated to compare favourably with long established techniques (Oellerich, 1980; Schuurs & van Weemen, 1980).

The increasing demands made for more, and more varied, tests to be performed while staff numbers are reduced means that either tests will need to be circumvented by a greater degree of selectivity on the part of the person requesting the analysis or that they should be met by the use of simpler, more flexible and more rapid procedures. Microtitre plate and related tests hold much promise since they can be readily performed in a semi-automated form, using dispensing and washing devices and quantified using automatic plate readers. Under less favourable circumstances, qualitative and semi-quantitative results can be obtained using pipettes and wash bottles and the colour simply read by eye, comparisons being made with calibrators.

REFERENCES


Principles of Enzyme Immunoassay


Use of progesterone 11-glucuronide–alkaline phosphatase conjugate in a sensitive microtitre-plate enzymeimmunoassay of progesterone in milk and its application to pregnancy testing in dairy cattle

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Summary. A simple direct-addition microtitre plate enzymeimmunoassay (EIA) for progesterone in whole milk is described. The assay used antiserum raised against 11α-hydroxyprogesterone 11-hemisuccinate (progesterone 11-hemisuccinate) and a heterologous label prepared by conjugation of 11α-hydroxyprogesterone 11-glucuronide (progesterone 11-glucuronide) with alkaline phosphatase using an active ester procedure. The sensitivity, analytical recovery, linearity of response and precision of the assay compared favourably with radioimmunoassay (RIA). Results from EIA of milk samples were compared with determinations made after isolation of progesterone by HPLC (r = 0.910). Milk samples (200) were assayed by RIA at both the Milk Marketing Board and the Cattle Breeding Centre and the results were correlated with EIA performed at the Cattle Breeding Centre (r = 0.890 and r = 0.833 respectively). Calving data were obtained from a further 110 cows for which the milk progesterone EIA had provided a pregnancy test 24 days after AI; 46 cows were correctly identified as non-pregnant and 58 as pregnant and there were 4 false positive and 2 inconclusive results.

Introduction

In 1971, Laing & Heap demonstrated that concentrations of progesterone in the milk of cows reflected the stage of the oestrous cycle as assessed by palpation of the ovary per rectum. Progesterone concentrations in milk were later shown to parallel those in plasma (Heap, Gwyn, Laing & Walters, 1973; Hoffmann & Hamburger, 1973) and to provide a means of determining whether or not pregnancy had been established after insemination. Such measurements have enabled the functional status of the corpus luteum of dairy cows to be monitored and the causes of reproductive failure to be identified (Hoffmann, Günzler, Hamburger & Schmidt, 1976; Lamming & Bulman, 1976; Foote et al., 1979; Ball & Jackson, 1979). However, the enforced restriction of commercial RIA services to licenced specialized laboratories has meant that the only routine practical application of this approach has been a postal pregnancy-testing service (Booth & Holdsworth, 1976). The potential advantages of enzymeimmunoassay (EIA) over RIA have been discussed previously in terms of general accessibility, low cost and the innocuous nature of the reagents employed (Landon, Crookall & McGregor, 1975; Schuurs & Van Weeman, 1977; Blake & Gould, 1984).

EIAs have been described for the determination of progesterone in extracts of serum or plasma from animals of several species (Dray, Andrieu & Renaud, 1975; Joyce, Read & Fahmy, 1977;
M. J. Sauer et al.

Patricot, Poggi & Revol, 1978; Seeger, Thurow, Haede & Knappe, 1979; Kamonpatana et al., 1979; Johnen, Nakao, Tsunoda & Kawata, 1980) and in extracts of whole milk of cows (van de Wiel, Kamonpatana, Ngramsurijaroj, Koops & Singhajan, 1982), defatted milk (Nakao, Sugihashi, Saga, Tsunoda & Kawata, 1983) and milk fat (Arnstadt & Cleere, 1981). Direct EIA procedures requiring no extraction of progesterone have been described for bovine whole milk (Sauer, Foulkes & Cookson, 1981; Chang & Estergreen, 1984) and cream (Nakao & Kawata, 1980). The development of a microtitre-plate progesterone EIA (Sauer et al., 1981; Sauer, Foulkes & O'Neil, 1982a; van de Wiel & Koops, 1982; Munro & Stabenfeldt, 1984; Tallon et al., 1984) has, in particular, removed many of the constraints imposed by RIA. Progesterone measurements made directly on whole milk samples by an homologous microtitre-plate EIA enabled assessment of luteal function to be used for management of fertility in commercial herds (Foulkes, Cookson & Sauer, 1982). This successful application, however, emphasized that a more robust and sensitive technique was required to ensure greater reliability in general use.

The comparative novelty of direct EIA requires that special attention be paid to validation procedures and stresses the need for full discussion of its essential characteristics, particularly since the labelled and unlabelled analyte are not physically identical. There have been no reports of direct EIA or RIA of progesterone in milk in which such procedures have included comparisons with results after chromatographic isolation of progesterone from the same samples. Corrie, Ratcliffe & MacPherson (1982) have demonstrated the benefit of the glucuronide bridge of 125I-labelled progesterone 11-glucuronide-tyramine conjugate in reducing bridge recognition and improving RIA sensitivity. In the present study a heterologous microtitre-plate EIA using progesterone 11-glucuronide label was investigated.

**Materials and Methods**

**Materials**

**Reagents.** N,N'-dicyclohexylcarbodiimide, N-hydroxysuccinimide, p-nitrophenylphosphate disodium salt ("Sigma 104", pNPP), alkaline phosphatase (EC 3.1.3.1) type VII-T (from bovine intestinal mucosa), sodium azide, activated charcoal (untreated powder) and diethanolamine were obtained from Sigma Chemical Company Limited (Poole, U.K.). Toluene Scintillator was obtained from Packard Instruments Limited (Caversham, U.K.) and Lactab Mk III potassium dichromate tablets from Thomson and Capper Limited (Runcorn, U.K.). Progesterone 11α-glucuronide (Corrie, Hunter & MacPherson, 1981) was a gift from Dr J. E. T. Corrie of the MRC Immunoassay Team, Edinburgh, U.K. [1,2,6,7-3H]Progesterone (sp. act. 80 Ci/mmol) was purchased from Amersham International PLC (Amersham, U.K.). Progesterone was obtained from Koch Light Laboratories (Colnbrook, U.K.) and all other steroids from Steraloids Limited (Croydon, U.K.). All solvents used in the conjugation procedure were dried over molecular sieves (type 3A, BDH Chemicals Limited, Poole, U.K.). All other reagents were of Analar quality and supplied by BDH Chemicals Limited. Goat anti-progesterone 11-hemisuccinate (anti-progesterone) serum (G711/12) was a gift from Dr G. S. Pope, NIRD, Shinfield, and was used for all EIA and RIA studies at the Cattle Breeding Centre.

**Buffers.** Phosphate buffers (0·1 M, pH 7·0) were prepared with disodium hydrogen orthophosphate (12 H₂O) and sodium dihydrogen orthophosphate (2 H₂O) with sodium chloride (0·9 % w/v; PS buffer), sodium azide (0·1 % w/v; PAS buffer) and gelatin (0·1 % w/v; PAS-gelatin buffer) added. DEM buffer (pH 9·8) contained diethanolamine (1 M), magnesium chloride (0·5 mM) and sodium azide (0·1 % w/v).

**Treatment of milk samples.** Milk samples (20 ml) were taken from the collection jar after agitation of the complete milking, preserved by addition of one Lactab Mk III tablet and stored at 4°C until assayed.

**Progesterone standards.** Progesterone (100 µg/ml methanol) was added (0·1 ml/20 ml) to whole
Milk progesterone EIA and pregnancy testing in cattle

Milk collected from an ovariectomized cow and preserved by addition of one Lactab Mk III tablet/20 ml. This solution was further diluted with the same milk to provide standards (milk standards) containing 0–80 ng progesterone/ml. Buffer standards were prepared in PAS-gelatin buffer in the same manner.

Antibody preparation. The anti-progesterone serum (G711/12) was raised in a castrated male goat after immunization against progesterone 11-hemisuccinate-bovine serum albumin (Furr, 1973). The γ-globulin fraction of the bovine serum albumin (BSA)-absorbed antisera was prepared by treatment with Rivanol pH 9.0 (Sauer, Cookson, MacDonald & Foulkes, 1982b).

Radioimmunoassay at the Cattle Breeding Centre

Standards (10 μl, in quadruplet) or samples (10 μl, in duplicate) were pipetted into polystyrene test tubes. [3H]Progesterone in PAS-gelatin buffer (200 μl, = 7 pg progesterone) and anti-progesterone γ-globulin (100 μl, equivalent to 1:15 000 dilution of serum) in PAS-gelatin buffer were then added. The tubes were mixed and incubated overnight at 4°C. Ice-cold PAS-gelatin buffer (500 μl) was added to each tube and free [3H]progesterone was separated from bound by the addition of dextran-coated charcoal suspension (200 μl, containing 6.25 mg charcoal/ml and 0.625 mg dextran/ml) in PAS-gelatin buffer at 4°C. After 10 min at 4°C the tubes were centrifuged at 1600 g (5 min), the supernatant was poured into scintillation vials and the radioactivity determined following extraction into Toluene Scintillator (7 ml).

Preparation of antibody-coated microtitre plates

The anti-progesterone γ-globulin was diluted (equivalent to 1:8000 dilution of serum) in 0.17 mM-sodium acetate buffer, pH 5.0, and an aliquant (200 μl) was added to each well of the microtitre plates (NUNC-immunoplate IF, GIBCO, Uxbridge, U.K.). The plates were covered with a lid and even adsorption of antibody was ensured by maintenance in a draught-free environment away from point-sources of heat for 3 h at room temperature (20–22°C). The microtitre plates were emptied and PAS-gelatin buffer (350 μl) was added to each well before tightly covering with plastic film (Payne Scientific Apparatus, Croydon, U.K.) and storing at 4°C. Binding activity was retained for at least 6 months under these conditions.

Preparation of alkaline phosphatase–progesterone conjugates

Alkaline phosphatase (2.5 mg/0.3 ml; 25 nmol) was dialysed against PS buffer (1 litre), for 24 h at 4°C and adjusted to a concentration of 5 mg/ml with PS buffer before use. N-hydroxysuccinimide (110 nmol in 20 μl dry dioxan) and dicyclohexylcarbodiimide (110 nmol in 20 μl dry dioxan) were added to progesterone 11-glucuronide (100 nmol in 20 μl dry dioxan) and stirred for 1 h at room temperature (20–22°C). Unreacted dicyclohexylcarbodiimide was inactivated by addition of acetic acid (50 nmol in 20 μl dry dioxan). After a further 15 min the insoluble by-product diclohexylhexyurea was removed by centrifugation (2000 g for 10 min). A 100% conversion of progesterone to ‘active ester’ was assumed. Steroid solution (40 μl) was slowly added to alkaline phosphatase in PS buffer (500 μl) to provide a molar ratio of 2:1. The mixture was stirred for 2 h at room temperature before dialysing at 4°C against 4 changes of 3 litres of PAS buffer over 4 days. The conjugate solution was finally passed down a Sephadex G25 column to remove any remaining free steroid. Gelatin solution (10% w/v in PAS buffer) was then added to a final concentration of 0.1% (w/v). The conjugate was stored at 4°C and under these conditions has been shown to be stable for at least 1 year.

Enzymeimmunoassay

Microtitre plates previously coated with antibody (above) were emptied, shaken dry and
blotted. Whole milk standards and samples were allowed to come to room temperature (20–22°C) and thoroughly mixed before assay. Standards (10 μl, containing 0–800 pg progesterone) or samples (10 μl) were added to duplicate wells. Conjugate diluted in PAS-gelatin buffer (200 μl; 20 ng/well) was then added at ambient temperature. Blank values were determined by addition of buffer alone to duplicate wells containing 10 μl of the zero progesterone standard. The plates were covered with a plate lid and incubated at ambient temperature for 3 h in a covered plastic box lined with damp paper before emptying and washing 3 times with PAS-gelatin buffer (350 μl/well) at 40°C delivered from an 8-channel peristaltic dispenser (Micro Compu-Pet Multi-Dilutor, General Diagnostics, Abingdon, U.K.). Substrate (p-nitrophenylphosphate, 15 mM in DEM buffer) was added to each well (200 μl) and the plates were incubated for 1 h at 40°C in a waterbath on a grid at water level. The absorbance (405 nm) of each well was recorded directly using a Dynatech MR580 automatic plate reader. Since the whole plate was read in 1–2 min the enzyme reaction was not stopped. The progesterone concentration in each sample was estimated by interpolation from the calibration curve on each plate (Fig. 1). For large numbers of samples this procedure has been performed automatically by interfacing with a micro-computer and applying a four-parameter logistic curve fit (Rodbard, 1978).

![Calibration curves for EIA (a) and RIA (b) using standards prepared in milk (a) or buffer (b). For EIA, mean B/B0 values ± s.d. and CV (—) for 8 (a) or 6 (b) consecutive assays are shown. Values for a single RIA calibration curve are included for comparison. Mean absorbance values in the absence of competing progesterone (B0) fell within the ranges 0.659–0.894 (a) and 0.624–1.017 (b).](image)

**Assay validation**

*Extraction of progesterone from milk samples.* Whole milk samples (n = 25) were selected, using a random numbers table, from samples collected on a routine basis. [3H]Progesterone (0.9 ng in 50 μl methanol; 5 × 10⁵ d.p.m.) was added to aliquants (15 ml) of each sample and allowed to equilibrate at 4°C overnight. Subsequent determination of radioactivity by liquid scintillation spectrometry allowed corrections to be made for procedural losses.

Progesterone was extracted by a method similar to that described by Axelson & Sahlberg.
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Fig. 2. Separation of steroids by HPLC using conditions described in the text. Peaks are in order of elution, (1) 5α- and 5β-pregnane-3,20-dione; (2) progesterone; (3) 3β-hydroxy-5-pregn-20-en-3-one; (4) 3β-hydroxy-5α-pregn-20-en-3-one; (5) 17α-hydroxy-4-pregnene-3,20-dione; (6) 20β-hydroxy-4-pregnene-3-one; and (7) 11β-hydroxy-4-pregnene-3,20-dione.

(1981) using 6 ml Baker-10 octadecyl silica columns (Linton Products Limited, Harlow, U.K.). The columns were conditioned by elution with methanol (2 × 5 ml) and deionized water (2 × 5 ml). Milk samples were heated in a waterbath at 64°C for a minimum of 10 min and immediately added to the columns in three aliquots of 4-66 ml using a positive displacement pipette (Transferpettor, A. R. Horwell Limited, London, U.K.). Samples were extracted under reduced pressure using a Baker-10 manifold: the flow through each column was adjusted to a maximum of 1 ml/min. Columns were washed with distilled water (64°C, 2 × 5 ml) before elution of steroids with methanol (3 × 0.5 ml) into conical glass test tubes. The tubes were left overnight at −20°C and the lipids that precipitated were separated by centrifugation (1600 g, 10 min). The extracts were aspirated into screw-capped glass vials (2 ml capacity), dried down under nitrogen, redissolved in the high-pressure liquid chromatography (HPLC) mobile phase (ethanol, 1% v/v in dichloromethane; 0-7 ml) and stored at −20°C until required.

Isolation of progesterone from milk extracts by HPLC. Progesterone was isolated from milk extracts (100 μl) by HPLC using a 125 mm × 4.6 mm stainless-steel column packed with RoSil 3 μm spherical silica (Alltech Associates, Carnforth, U.K.), an ACS 351 pump (Applied Chromatography Systems, Luton, U.K.) and a Valco 10-port injection valve (C10U, Alltech Associates) fitted with a 100-μl sample loop to permit timed collection of samples and column backflushing. An ultraviolet (280 nm) detector (Pye Unicam PU 4020, 8-μl flow cell) was used to establish optimum isocratic conditions for separating progesterone from related steroids. The mobile phase (flow rate 2 ml/min) allowed baseline resolution (Fig. 2) of progesterone from all steroids tested (5α- and 5β-pregnane-3,20-dione; 3β-hydroxy-5-pregn-20-en-one; 3β-hydroxy-5α-pregn-20-en-one; 20β-hydroxy-4-pregnene-3-one; 11β-hydroxy-4-pregnene-3,20-dione and 17-hydroxy-4-pregnene-3,20-dione). Since the occurrence and identity of progesterone metabolites
in milk is not fully established the column was backflushed for 6 min after collection of the progesterone fraction, a period three times the retention time of progesterone. This minimized any carry-over of slow running components from one sample to another. The progesterone fraction was dried down at 60°C under a stream of nitrogen and redissolved in PAS-gelatin buffer (0-7 ml) before EIA and RIA using buffer standards (in quadruplet).

Comparison of pregnancy test results using RIA and EIA

Milk samples (200) submitted to the Milk Marketing Board by commercial farms for pregnancy testing 22–26 days after service or by veterinary surgeons investigating herd fertility were assayed by the RIA in routine use at the MMB Veterinary Laboratory (Holdsworth, Chaplin & Booth, 1979) before transport to the Reading Cattle Breeding Centre for further determination by RIA and EIA. The majority (168) were whole milk samples taken after agitation of the milk collecting jar but 32 were fore-milk samples collected by hand.

Confirmation of pregnancy test results obtained by EIA

A further 110 whole milk samples were obtained from cows in four herds 24 days after service and progesterone concentrations were determined by EIA. The conclusions were subsequently verified from calving data.

Statistical analysis

When assay techniques were compared, weighted linear regressions were calculated assuming error in both procedures: slopes and intercepts were corrected after estimating the variance in each technique from their coefficients of variation and their compatibility with a line of identity (slope = 1, intercept = 0) tested using an F-ratio based on the ‘extra sum-of-squares’ principle (Munson & Rodbard, 1982).

Results

Assay sensitivity

The sensitivity of each assay is defined in Table 1 in terms of both limit of detection (Abraham, 1969) and the mid-point of the calibration curve (Feldman & Rodbard, 1971). Values shown were from calibration curves using standards prepared in milk or PAS-gelatin buffer (Figs 1a & 1b).

<table>
<thead>
<tr>
<th>Standards</th>
<th>EIA Limit of detection* (confidence limit)</th>
<th>EIA Calibration curve mid-point†</th>
<th>RIA Limit of detection* (confidence limit)</th>
<th>RIA Calibration curve mid-point†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk</td>
<td>5 pg (95%)</td>
<td>24 pg</td>
<td>5 pg (99%)</td>
<td>40 pg</td>
</tr>
<tr>
<td>PAS-gelatin</td>
<td>2 pg (99%)</td>
<td>10 pg</td>
<td>5 pg (99%)</td>
<td>20 pg</td>
</tr>
</tbody>
</table>

*Smallest quantity of progesterone measured which gives rise to a response significantly different from zero (Abraham, 1969). Confidence limits were derived from the appropriate t statistic.
†The mass of progesterone required to produce a 50% reduction in label binding (Feldman & Rodbard, 1971).
Precision of EIA

Precision of adsorption of antibody to microtitre-plate wells. The enzyme-labelled steroid conjugate, diluted in PAS-gelatin buffer (20 ng/200 µl), was added to 88 wells on each of 6 plates previously coated with anti-progesterone γ-globulin: 8 wells in the centre of the plates received buffer alone to provide blank values. The plates were treated as described for EIA and the standard deviation (s.d.) of the absorbances of the 88 wells was calculated for each plate. The mean within-assay coefficient of variation (CV) for the 6 plates was 5.1% (range 4.1-7.9%). The mean absorbance calculated for the wells at the outside of each of the 6 plates did not differ significantly from the mean value for the remaining wells.

Blank values. The binding of enzyme-progesterone conjugate to nonimmune γ-globulin and enzyme to anti-progesterone γ-globulin was tested to investigate non-specific binding in the microtitre-plate system. Each of 2 plates was coated with both anti-progesterone γ-globulin (2 x 4 rows of 8 wells) and nonimmune γ-globulin (4 rows of 8 wells). Milk samples (10 µl; n = 8) containing high (> 15 ng/ml) concentrations of progesterone were added in quadruplicate to each group of 4 rows on one plate and milk samples (10 µl; n = 8) containing low (< 5 ng/ml) concentrations of progesterone to the other plate. Alkaline phosphatase in PAS-gelatin buffer (40 ng/200 µl) was added to one group of wells containing anti-progesterone γ-globulin on each plate and conjugate in PAS-gelatin buffer (40 ng/200 µl) was added to the remaining groups containing anti-progesterone or nonimmune γ-globulin. The plates were treated as described for EIA and the non-specific binding of conjugate or enzyme was expressed as a percentage (± s.d.) of the specific binding seen in the presence of the high or low concentrations of progesterone in milk. Conjugate binding to the nonimmune γ-globulin was 4.4 ± 1.2% and 2.15 ± 0.61% in the high and low samples respectively and enzyme binding to the specific antibody was 6.1 ± 1.2% and 2.5 ± 0.8%.

EIA drift. Repeated duplicate estimations (1-12) were made of the progesterone concentration in each of 3 milk samples arranged in sequence across a microtitre plate. The slopes and intercepts obtained by least squares regression analysis of the progesterone concentrations determined (Y) for each sample against order of application to the plates (x = 1 to 12) were compared with 0 slope and intercept at the mean sample concentrations. At means ± s.d. of 2.6 ± 0.13 ng/ml, \( Y = 0.015x + 2.505 \) (F = 0.619); at 7.8 ± 0.4 ng/ml, \( Y = 0.021x + 7.970 \) (F = 0.150); and at 28.4 ± 3.1 ng/ml, \( Y = -0.360x + 30.758 \) (F = 1.026). There was no significant difference for any sample.

Within- and between-assay precision. Milk samples were assayed by EIA and 9 samples, containing 15-30 ng progesterone/ml milk, were combined to form a 'high' quality control. Nine samples containing 5 ng/ml were similarly combined ('low' quality control). Equal volumes of the 2 pools were mixed to provide a 'medium' quality control. The 3 quality controls were assayed in duplicate at 5 different positions on each of 8 microtitre plates during the course of a routine assay and the mean and standard deviation were calculated for each to allow the within-assay coefficient of variation to be determined (Table 2). A mean duplicate determination from each quality control on each plate was selected using random numbers tables and the mean between-assay coefficients of variation were calculated by repeating the exercise a further 4 times (Table 2). The between-assay precision of calibration curves obtained using either milk or PAS-gelatin standards are shown in Figs 1(a) and 1(b), respectively.

Precision of RIA

Cattle Breeding Centre. The assay was performed in batches of 56 or 64 tubes to allow synchronization of charcoal addition and centrifugation. Replicate estimates of quality control samples assayed in duplicate within each batch (n = 5) or between batches (n = 8) allowed within- and between-batch precision to be calculated (Table 2).

Milk Marketing Board. Milk samples submitted for pregnancy testing in a 1-week period and
Table 2. Within- and between-assay precision for EIA and RIA at the Cattle Breeding Centre (CBC) and for RIA at the Milk Marketing Board (MMB) at low (L), medium (M) and high (H) concentrations of progesterone in milk

<table>
<thead>
<tr>
<th>Quality control</th>
<th>Mean within-assay precision</th>
<th>Mean between-assay precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EIA (n=40)</td>
<td>RIA (CBC) (n=5)</td>
</tr>
<tr>
<td></td>
<td>ng/ml</td>
<td>CV (%)</td>
</tr>
<tr>
<td>L</td>
<td>3.2</td>
<td>11.0</td>
</tr>
<tr>
<td>M</td>
<td>11.2</td>
<td>16.7</td>
</tr>
<tr>
<td>H</td>
<td>17.7</td>
<td>9.7</td>
</tr>
<tr>
<td>Overall mean CV</td>
<td>12.5</td>
<td>11.1</td>
</tr>
</tbody>
</table>
found to contain >9 ng progesterone/ml were pooled to provide a quality control sample. Eight assays, each including 3 quality control determinations, were selected using random numbers tables from 34 assays conducted in 1 week and the mean within-assay CV was calculated (Table 2). The between-assay CV was calculated from 40 values selected at random from assays performed during the same period (Table 2).

Specificity of EIA and RIA at the Cattle Breeding Centre

Cross-reactivity. The cross-reactivities of various steroids (dissolved in PAS-gelatin buffer) with the antiserum (G711/12) were determined by the criteria of Abraham (1969) and are presented in Table 3.

<table>
<thead>
<tr>
<th>Steroid*</th>
<th>Cross-reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RIA† at CBC</td>
</tr>
<tr>
<td>4-Pregnen-3,20-dione</td>
<td>100</td>
</tr>
<tr>
<td>4-Androstene-3,20-dione</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>5α-Pregnen-3,20-dione</td>
<td>1.30</td>
</tr>
<tr>
<td>5β-Pregnan-3,20-dione</td>
<td>6.80</td>
</tr>
<tr>
<td>17-Hydroxy-4-pregnen-3,20-dione</td>
<td>3.60</td>
</tr>
<tr>
<td>3β-Hydroxy-5-pregnen-20-one</td>
<td>0.05</td>
</tr>
<tr>
<td>11β,17α,21-Trihydroxy-4-pregnen-3,20-dione</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>11β,21-Dihydroxy-4-pregnen-3,20-dione</td>
<td>1.60</td>
</tr>
<tr>
<td>3β-Hydroxy-5-androsten-17-one</td>
<td>0.03</td>
</tr>
<tr>
<td>11α-Hydroxy-4-pregnen-3,20-dione</td>
<td>75.80</td>
</tr>
<tr>
<td>21-Hydroxy-4-pregnen-3,20-dione</td>
<td>—§</td>
</tr>
<tr>
<td>17β-Hydroxy-4-androsten-3-one</td>
<td>—§</td>
</tr>
<tr>
<td>20α-Hydroxy-4-pregnen-3-one</td>
<td>—§</td>
</tr>
</tbody>
</table>

Cross-reactivity is defined as the molar quantity of steroid, relative to progesterone (mol. progesterone/mol steroid) required to reduce label binding by 50%.

* IUPAC-IUB (1967) revised tentative rules for steroid nomenclature.
† Antiserum G711/12.
‡ Antiserum AF18/3 (data of Sheldrick, Mitchell & Flint, 1980).
§ Not tested.

Comparison of results obtained by direct assay or after chromatographic isolation of progesterone. The mean ± s.d. recovery of the progesterone tracer after C18 column extraction of the 25 milk samples examined was 78.8 ± 4.5%. The recovery after HPLC and redissolving the progesterone fraction in PAS-gelatin buffer was 52.8 ± 10.4%.

Progesterone concentrations were determined both before and after extraction and purification and were corrected for procedural losses. Comparisons of results from EIA with those from RIA before (r = 0.933) and after (r = 0.969) extraction were highly correlated and the slopes and intercepts did not differ significantly from 1 and 0 respectively (Figs 3a & 3b). Linear regressions for EIA before and after extraction (r = 0.910) and RIA before and after extraction (r = 0.952) were also not significantly different from a line of identity (Figs 3c & 3d).

Linearity of response. Ten milk samples shown to contain progesterone concentrations consistent with the luteal phase (>10 ng/ml) by EIA were taken at random and serially diluted with milk from an ovariectomized cow. The diluted samples were assayed by both EIA and RIA and linear regressions were calculated for each set of results against dilution. Correlation coefficients
fell within the range \( r = 0.984-0.999 \) for the RIA and \( r = 0.946-0.997 \) for the EIA. Correlation coefficients after linear regression of the results from EIA on those obtained by RIA were in the range \( r = 0.946-0.998 \). For 6 of the dilution series, analysis of the F-ratio showed no significant deviation from slope = 1 and intercept = 0. Significant differences were found in 4 of the series (Fig. 4) although good correlations were still exhibited \( (r = 0.967-0.997) \).

**Analytical recovery of progesterone added to milk samples.** Progesterone was added to milk samples taken from 6 different cows during the oestrous period (endogenous progesterone <1.0 ng/ml by EIA), in the manner described for preparing progesterone standards, to provide concentrations of 20 ng/ml. The samples were diluted in milk from an ovariectomized cow to provide a range of concentrations (20, 16, 12, 8, 4 and 2 ng/ml). The samples were assayed by EIA and RIA and the mean concentrations and percentage recoveries of progesterone are shown in Table 4. Linear regression of mean concentrations measured \( (y) \) on the values expected \( (x) \) gave \( y = 1.010x - 0.784 \) and \( y = 1.026x - 0.718 \) for EIA \( (r = 0.993) \) and RIA \( (r = 0.995) \) respectively.

**Pregnancy testing**

**Comparison of RIA results at the Milk Marketing Board and RIA and EIA results at the Cattle Breeding Centre.** Progesterone concentrations determined in 200 milk samples by the three procedures showed some scattering, particularly when high values were recorded. This was true when
results from the RIA or EIA at the Cattle Breeding Centre were compared with those from the Milk Marketing Board RIA ($r = 0.890$ and 0.833 respectively; Table 6). The Milk Marketing Board used 4.5 ng/ml and 1.0 ng/ml in whole milk and fore-milk respectively to discriminate between samples from pregnant and non-pregnant animals at 22–26 days after insemination. Using linear regression equations calculated and corrected for error in both techniques as described, equivalent discrimination points were determined for the assays at the Cattle Breeding Centre and standard deviations were established from their coefficients of variation (Table 5). Results falling within two standard deviations of the discriminating point were considered inconclusive. Excluding these, 129 samples were classified by the Milk Marketing Board RIA as indicating pregnancy and 63 non-pregnancy. Conclusions from the EIA results coincided for 122 and 52 samples respectively, with 7 values in each case falling within two standard deviations of the discrimination value. The figures from the RIA at the Cattle Breeding Centre were 126 and 56, with 2 and 5 results inconclusive. Both the EIA and the RIA at the Cattle Breeding Centre produced 4 results that were at
Table 5. Regression of results from EIA and RIA of 200 milk samples

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Regression</th>
<th>Discrimination point ± 2 s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$y = ax + b$</td>
<td></td>
</tr>
<tr>
<td>Whole milk ($n = 168$)</td>
<td>EIA on MMB RIA</td>
<td>0.901</td>
<td>$y = 0.832x + 1.72$</td>
</tr>
<tr>
<td></td>
<td>EIA on CBC RIA</td>
<td>0.838</td>
<td>$y = 0.699x + 3.21$</td>
</tr>
<tr>
<td></td>
<td>CBC RIA on MMB RIA</td>
<td>0.905</td>
<td>$y = 1.140x - 1.63$</td>
</tr>
<tr>
<td>Fore-milk ($n = 32$)</td>
<td>EIA on MMB RIA</td>
<td>0.662</td>
<td>$y = 1.579x - 0.555$</td>
</tr>
<tr>
<td></td>
<td>EIA on CBC RIA</td>
<td>0.768</td>
<td>$y = 1.365x + 0.815$</td>
</tr>
<tr>
<td></td>
<td>CBC RIA on MMB RIA</td>
<td>0.901</td>
<td>$y = 1.023x - 0.286$</td>
</tr>
<tr>
<td>All samples ($n = 200$)</td>
<td>EIA on MMB RIA</td>
<td>0.890</td>
<td>$y = 0.831x + 2.00$</td>
</tr>
<tr>
<td></td>
<td>EIA on CBC RIA</td>
<td>0.833</td>
<td>$y = 0.702x + 3.36$</td>
</tr>
<tr>
<td></td>
<td>CBC RIA on MMB RIA</td>
<td>0.908</td>
<td>$y = 1.133x - 1.45$</td>
</tr>
</tbody>
</table>

†Calculated for the deviation from a line of identity: *not significantly different ($P > 0.25$).
‡Calculated from 4.5 ng/ml (whole milk) and 1.0 ng/ml (fore-milk) in the MMB RIA and standard deviations from the mean overall coefficients of variation observed in the individual assays (Table 3).

Table 6. Progesterone concentration (ng/ml) in milk samples submitted to the MMB for which different conclusions were reached on reproductive status

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>MMB RIA</th>
<th>EIA</th>
<th>CBC RIA</th>
<th>Veterinary examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>81</td>
<td>1.9 (-)</td>
<td>1.0 (+)</td>
<td>4.3 (+)</td>
<td>Oestrus</td>
</tr>
<tr>
<td>108†</td>
<td>3.0 (-)</td>
<td>7.3 (+)</td>
<td>5.1 (+)</td>
<td>Luteal</td>
</tr>
<tr>
<td>145‡</td>
<td>0.7 (-)</td>
<td>2.1 (+)</td>
<td>1.3 (+)</td>
<td>—</td>
</tr>
<tr>
<td>149‡</td>
<td>0.6 (-)</td>
<td>1.6 (+)</td>
<td>1.0 (?)</td>
<td>Pregnant</td>
</tr>
<tr>
<td>154‡</td>
<td>3.6 (+)</td>
<td>1.2 (?)</td>
<td>0 (-)</td>
<td>—</td>
</tr>
</tbody>
</table>

†Fore-milk samples
+ = Luteal-phase or pregnant; — = follicular phase; ? = within ± 2 s.d. of discriminating level.

variance with conclusions drawn from the Milk Marketing Board RIA and these are summarized in Table 6: three (Nos 145, 149 and 154) were fore-milk samples. Veterinary investigation of the 5 apparent anomalies provided an indication of the correct conclusion in 3 cases (Table 6).

Confirmation of pregnancy test results obtained by EIA. Calving dates were obtained from a further 110 cows in 4 commercial herds for which EIA had been used for pregnancy testing at 24 days after AI. Of 62 diagnoses of pregnancy, 58 (93.5%) were shown to be compatible with subsequent calving data assuming a gestation length of 280 ± 10 days (Salisbury & VanDemark, 1961). All 46 cows thought to be non-pregnant calved to a subsequent service.

Discussion

The method described is the first reported use of alkaline phosphatase in a progesterone EIA: this provided a more sensitive assay than use of β-galactosidase as previously described (Sauer et al.,
Milk progesterone EIA and pregnancy testing in cattle

1982a). Peroxidase is also well suited for use as a label for progesterone EIA (Joyce, Wilson, Read & Riad-Fahmy, 1978; Arnstatt & Cleere, 1981; van de Wiel & Koops, 1982; Munro & Stabenfeldt, 1984; Tallon et al., 1984) but many of the chromogens used for its assay are known to be mutagenic or carcinogenic (Voogd, van der Stel & Jacobs, 1980) and this may limit its areas of application.

Homologous progesterone EIA's are adequate for many applications (Nakao & Kawata, 1980; Arnstatt & Cleere, 1981; Foulkes et al., 1982) but are often less sensitive than RIA. Some homologous EIA's have good sensitivity (see Riad-Fahmy, Read, Joyce & Walker, 1981); however, the preparation of enzyme conjugates using a progesterone derivative heterologous to that used for preparation of the immunogen has been shown to improve performance throughout the calibration curve (Gros, Flecheux & Dray, 1978; Sauer et al., 1982a; van de Wiel & Koops, 1982; Munro & Stabenfeldt, 1984). The use of progesterone 11-glucuronide-β-galactosidase label with antiserum raised against progesterone 11-hemisuccinate-BSA produced calibration curves an order of magnitude more sensitive than the homologous system (Sauer et al., 1982a). This particular combination of bridges effectively eliminated bridge recognition with a large proportion of antisera raised against progesterone 11-hemisuccinate-protein (M. J. Sauer, P. M. O'Neill & J. A. Foulkes, unpublished data); this reinforces the conclusions of Corrie et al. (1982) who used the same combination in a RIA with 125I-labelled progesterone 11-glucuronide-tyramine. The progesterone 11-glucuronide-alkaline phosphatase label used here provided calibration curves with limits of detection, mid-points and precision values that were at least comparable or better than those of RIA (Fig. 1; Table 1). The limits of detection and mid-point sensitivities were ~50 times better than those of the homologous EIA used by Chang & Estergreen (1984) for the direct assay of progesterone in whole milk.

The presence of milk has been shown to cause a significant depression in antibody-antigen binding and sensitivity in both RIA (Heap, Holdsworth, Gadsby, Laing & Walters, 1976) and EIA (Sauer et al., 1981; Chang & Estergreen, 1984) of progesterone and in recognition of this standard are often prepared in milk. Indeed, variations in non-specific effects between samples have been reported (Heap et al., 1976; Pennington, Spahr & Lodge, 1976; Holdsworth et al., 1979; Stevens, Long & Perry, 1981) although many descriptions of immunoassays for progesterone have made no reference to such an occurrence. This problem was illustrated in the present study when 10 samples shown to contain luteal-phase concentrations of progesterone were diluted with milk from an ovariectomized cow. All samples showed linear correlations with dilution when measured by RIA or EIA and when these results were compared. Four of the samples, however, showed significant variation from a line of identity between EIA and RIA (Fig. 4), indicating that the sample matrix had affected the concentration of progesterone perceived by one technique or the other. A similar effect was noted when progesterone was added to 6 milk samples taken from cows at oestrus. Although the mean recoveries of progesterone after further dilution with milk from an ovariectomized cow were close to those expected (Table 4), greater variation in the recovery of progesterone from individual milk samples was seen with EIA than with RIA. In comparison, Chang & Estergreen (1984) reported recovery values between 80 and 88% but a high degree of variability (s.d. 26–71%). Although comparable recovery data for RIA are scarce or limited (Heap et al., 1976), recovery ranges have been reported by some laboratories and vary considerably, e.g. 97–110% (Bulman & Lamming, 1978), 104–126% (van de Wiel, van Eldik, Koops, Postma & Oldenbroek, 1978); and 100–136% (Batra, Pahwa, Suri & Pandey, 1980).

An explanation for the variation seen in EIA in particular might lie in the composition or structure of milk since the label used could interact with particular milk samples. The investigation of non-specific binding in 16 milk samples containing high or low concentrations of progesterone showed no evidence of individual non-specific binding effects and mean values of 2.15% and 4.4% were recorded in the presence of follicular- and luteal-phase samples respectively. Equivalent studies with RIA have produced variable values as high as 20% (Holdsworth et al., 1979; Stevens et al., 1981).

Differences in cross-reactivity between RIA and EIA could produce similar discrepancies in
values measured: the occurrence of progestagens in milk has been investigated by Darling, Laing & Harkness (1974) but since the oxidative procedures employed would only allow broad classification the influence of specific steroids is hard to discern. Cross-reactivity studies were conducted using the criteria of Abraham (1969) but no large differences were found between EIA and RIA (Table 3) although Van Weeman & Schuurs (1975) and Van Weeman, Bosch, Dawson, Van Hell & Schuurs (1978) have shown that the use of heterologous assay systems can increase cross-reactivity. Steric considerations, however, suggest that this is less likely to occur when the heterology involves the bridge itself rather than the site of attachment of the bridge to the steroid. Darling et al. (1974) and Purdy, Durocher, Moore & Rao (1980) showed that 5α- and 5β-pregnanediones occur in milk but it is the suggested presence of progestagen conjugates (Heap, Henville & Linzell, 1975) that should give rise to concern since these are not defined and were not tested. Their preponderance in the aqueous phase of milk would enhance their interaction with the antibody in comparison with progesterone and their greater availability would be accentuated by the non-equilibrium nature of EIA (Sauer et al., 1982a; Morino, Nakao, Tsuoda & Kawata, 1984; Munro & Stabenfeldt, 1984); the importance of the ‘first-come, first-served’ principle (Pratt & Woldring, 1976) in non-equilibrium RIA and EIA has been demonstrated by a number of other investigators (Van Weeman et al., 1978; Vining, Compton & McGinley, 1981).

With these considerations in mind, it was necessary to demonstrate that progesterone was the milk component being measured by EIA. After correction for procedural losses, progesterone concentrations determined in 25 milk samples before and after extraction and isolation by HPLC were highly correlated for both EIA and RIA (r = 0.910 and 0.952: Figs 3c & 3d). In addition, results from the two techniques were closely correlated before (Fig. 3a; r = 0.933) and after (Fig. 3b; r = 0.969) extraction and, most importantly, none of the regressions differed significantly from a line of identity, indicating that progesterone was the major component in milk measured by both RIA and EIA.

The use of microtitre plates to provide both a solid-phase support for the antibody and a vessel to contain the assay greatly facilitates its application in unsophisticated laboratories. Previous studies (Kricka et al., 1980) have found evidence for variation in the adsorption of antibody to plates (CV = 5-2-30%). Although similar problems were originally encountered with both polyvinyl and polystyrene plates in this laboratory, the use of plates specifically manufactured for immunoassay and attention to detail in ensuring a stable environment during antibody coating and incubation resulted in acceptable precision (CV = 5-1%). In particular, no evidence was seen of preferential binding to wells at the edge of the plate (see Kricka et al., 1980). The within- and between-assay precision of EIA were 12-5 and 14-8% respectively and compared favourably with values for RIA both in this study (Table 2) and others (Heap et al., 1976; Holdsworth et al., 1979; van de Wiel, Kalis & Shah, 1979; Batra et al., 1980). Bulman & Lamming (1978), however, have given details of an RIA with greater precision (CV = 8-9% and 10-3%).

If standards and 40 samples are assayed in duplicate on a microtitre plate, about 15 min will elapse between introduction of the first sample and addition of the conjugate. Munro & Stabenfeldt (1984), using 50 μl antibody per well to coat the plate and 50 μl sample, found that this resulted in a systematic variation in the value determined for a sample depending on the time of addition to the plate ('drift'), presumably because the earlier the addition the greater the time for contact between the sample and antibody. Since the microtitre plate EIAs described do not attain equilibrium (Sauer et al., 1982a; Munro & Stabenfeldt, 1984) this presents a potential source of error. The use of 200 μl of antibody to coat the plate and only 10 μl of the sample apparently prevented drift in the present application.

The combination of precision and specificity demonstrated for this EIA allows a ready distinction to be made between luteal- and follicular-phase concentrations of progesterone in whole milk from dairy cows. This was illustrated by the results obtained by assay of milk samples taken for pregnancy testing 22–26 days after service, although the correlations between results from RIA and EIA for samples obtained from the Milk Marketing Board were lower (r = 0.890 for the MMB RIA.
and $r = 0.833$ for the CBC RIA) than those seen when 25 milk samples were assayed at the Cattle Breeding Centre alone ($r = 0.933$; Fig. 3a) as part of the validation study. The delay in re-assaying samples may have allowed changes in the structure of milk to occur that affected the performance of the EIA more than the RIA. Some evidence is available for this since freshly prepared milk standards reduce binding in the calibration curve perceptibly more than standards prepared 1 month previously (unpublished observations). This is a difficult phenomenon to investigate since it would require that new standards be prepared from a fresh batch of milk whose composition may differ from the original and thus no absolute control is available for the ageing process. Since milk samples submitted for assay will usually be of recent origin it may be a wise precaution to renew milk standards at least at monthly intervals. This apparent difficulty did not, however, prevent a high degree of correspondence between the conclusions drawn from the various assays. Such disparities as there were revolved around samples showing concentrations of progesterone near the discrimination points of the assays so that one or other test was inconclusive. Only 5 samples (Table 6) produced results for which the deductions from the assays differed and 3 of these were fore-milk samples which have previously been shown to produce results that may be variable in some circumstances (Holdsworth, Booth, Sharman & Rattray, 1980). EIA of one of the two whole milk samples (No. 81) produced a value consistent with the luteal phase and was from a cow in which the occurrence of behavioural oestrus was confirmed by the veterinary surgeon. Rectal palpation indicated that the other sample (No. 108) was correctly identified by EIA and RIA at the Cattle Breeding Centre as being from a cow in the luteal phase. An earlier homologous EIA technique recorded a proportion of results relating to the follicular phase of oestrous cycles for which progesterone concentrations were higher than expected (Foulkes et al., 1982). It remains possible that the single real discrepancy (No. 81) in this study represents a similar occurrence.

In conclusion, this EIA for progesterone has been shown to rival RIA in precision and sensitivity. Its simplicity, cheapness and safety will allow practical expression of the detailed knowledge of bovine reproductive physiology, acquired through studies employing RIA, by making a technique available to laboratories with limited equipment. The use of microtitre plates permits large numbers of samples to be examined if required and automation of both sample and data handling if necessary. The colorimetric nature of the test allows a visual endpoint, with high and low concentrations readily distinguished. Recent adaptations have provided tests that may be performed in less than 1 h. They have been successfully used by veterinary surgeons and farmers (B. Drew & J. A. Foulkes, unpublished observations) and such widespread availability promises great economic benefit.

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