ADRENERGIC CONTROL OF
HEPATIC CARBOHYDRATE METABOLISM
IN THE PERINATAL RAT

A thesis submitted to the
University of Surrey

by

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ABSTRACT

Carbohydrate metabolism in the liver of the laboratory rat immediately before and after birth has been shown to be regulated by the hepatic adrenergic system.

\[ ^{125}I \text{-iodopindolol} \] was used to characterise the \( \beta \)-adrenoceptor population of liver membranes prepared from fetal and neonatal rats. The receptors were predominantly of the \( \beta_2 \)-subtype. The dissociation constant of this radioligand (about 75pM) was not significantly different when membranes from animals of different ages were used, indicating that the type and subtype of receptors being labelled did not change with age. However, the size of the \( \beta \)-adrenoceptor population did vary with age and reached a maximum in membranes isolated from term fetal rats.

Hepatocytes were isolated from rats during the perinatal period and incubated with various adrenergic agents. Both glucose production and glycogen breakdown in freshly isolated hepatocytes were shown to be stimulated by adrenergic agonists. The use of adrenoceptor type- and subtype-specific agents confirmed that adrenergic modulation of carbohydrate metabolism in these cells was \( \beta_2 \)-mediated. When hepatocytes were isolated from term fetuses of gestationally diabetic dams, glucose production and glycogen breakdown in these cells was shown to be resistant to adrenergic action.

Adrenaline administered in vivo to newly-delivered rats was shown to increase plasma glucose and lactate concentrations and to decrease liver glycogen content at two hours after delivery. At appropriate doses, adrenaline prevented the hypoglycaemia normally seen in the hours immediately following birth in the rat. Adrenergic antagonists, when administered intraperitonely immediately after delivery, prevented the natural increase in plasma glucose concentration at three
hours post partum. The β-adrenergic antagonist propranolol was more effective than the α-adrenergic antagonist phentolamine. These findings support activation of the adrenergic system as an essential factor in the mechanism for recovery from postnatal hypoglycaemia in the laboratory rat.
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    Dissociation time course
  Radioligands for adrenoceptors
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Materials and Methods

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  Liver membrane preparation
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  $[^3\text{H}]$-Dihydroalprenolol
  $[^3\text{H}]$-Dihydroergocryptine
  $[^{125}\text{I}]$-Iodocyanopindolol
  $[^{125}\text{I}]$-Iodopindolol

Analysis of data

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  Protein dilution profile
  Association time course
  Dissociation time course
  Inhibition profile
  Saturation assay
  Investigation of high non-specific binding

$[^3\text{H}]$-Dihydroergocryptine binding assay
  Protein dilution profile
  Investigation of the effects of $\alpha$-antagonists on binding

$[^{125}\text{I}]$-Iodocyanopindolol binding assay
  Inhibition profile
  Saturation assay
  Association time course
  Dissociation time course

$[^{125}\text{I}]$-Iodopindolol binding assay
  Protein dilution profile
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<td>cyclic AMP</td>
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<tr>
<td>Adenosine 5'-triphosphate</td>
<td></td>
<td>AR</td>
</tr>
<tr>
<td>Analytical grade reagent</td>
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<td>Association rate constant of radioligand</td>
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<td>Centimetre</td>
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<td>Concentration producing 50% of maximal effect</td>
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<td>5-Hydroxy tryptamine</td>
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<td>ICYP</td>
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<td>Iodopindolol</td>
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<tr>
<td>Kilogram</td>
<td></td>
<td>kg</td>
</tr>
<tr>
<td>Litre</td>
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<tr>
<td>Micro (10⁻⁶ x)</td>
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<td>Milli (10⁻³ x)</td>
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<td>Nicotinamide adenine dinucleotide, reduced</td>
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<td>NADH</td>
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<td>Phosphoenolpyruvate carboxykinase</td>
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post coitum  p.c.
second  s
standard error of the mean  SEM
total receptor concentration  \( B_{\text{max}} \)
uridine 5'-pyrophosphate  UDP
weight by volume  w/v
CHAPTER 1

GENERAL INTRODUCTION
1.1 METABOLISM IN THE PERINATAL RAT

A number of books and reviews on the topic of metabolism in the neonate are available (Ballard, 1971a; Battaglia & Meschia, 1978; Bohme et al., 1983; DeMeyer, 1981; Hommes & Van Den Berg, 1975; Jones, 1982; Snell & Walker, 1973a).

1.1.1 OVERVIEW

Major changes occur in metabolism during the transition from fetus to adult; such changes are a consequence of the very different nutritional environment at various stages of development. At birth, and following weaning, the composition of nutrients available changes sharply; the transition from fetus to neonate, and the mechanism of adaption of glucose metabolism to this transition, is the topic of investigation of this project.

For the rat fetus in utero, it has been estimated that 50-80% of the caloric requirement is satisfied by glucose supplied transplacentally from the mother (Battaglia & Meschia, 1978). Although fetal plasma glucose levels are generally found to be lower than maternal values (Girard et al., 1973a; DiMarco et al., 1976), when the gravid rat is fasted, fetal glucose levels are maintained preferentially (Bossi & Greenberg, 1972; Goodner et al., 1969). Following parturition, milk is the sole nutritional source and hence the majority of calories taken by the neonate are in fat or protein (Luckey et al., 1954). It has been estimated that the calorie content of milk due to carbohydrate is only 8% (Dymsza et al., 1964; Hahn et al., 1961), which is insufficient to balance the glucose utilisation of the suckling animal (Snell & Walker, 1973a).

Fatty acids and ketone bodies are the major substrates of oxidative energy metabolism in the suckling rat (Bailey & Lockwood, 1973; Vernon & Walker, 1968).
Free fatty acids do not cross the placenta freely (Koren & Shafrir, 1964) and are present at lower concentrations in fetal than maternal blood (Blazquez et al., 1975; Girard et al., 1973a). Increases in free fatty acids following birth have been seen; slightly less than a two-fold increase within three or four hours of birth, in the absence of feeding (Girard et al., 1973a; Snell & Walker, 1973b), or a more significant increase maintained for 24 hours when the neonates were allowed to suckle (Blazquez et al., 1974; Dierks-Ventling, 1971; Snell & Walker, 1973b) have been observed. The oxidation of fatty acids in the neonate is vital for the supply of acetyl coenzyme-A required for gluconeogenesis (Ferre, 1978b; Snell, 1974).

Ketone bodies, which are at about the same blood level in mother and fetus (Girard et al., 1973a), remain steady or decrease immediately postnatally but show a marked increase after suckling starts (Ferre et al., 1978a; Girard et al., 1973a; Haymond et al., 1972; Snell & Walker 1973b). Glycerol plasma levels are high in the neonate (Vernon & Walker, 1970) and have been reported to show no change in the first few hours following delivery (Cuezva et al., 1980b) or to show a marked but transient increase, returning to levels similar to those immediately after birth by 6 hours post partum (Girard et al., 1973a).

Miller (1970) has shown that weight gain in the suckling rat is proportional to protein intake, although the calorie content of the milk, of which protein accounts for only 23% (Dymsza et al., 1964), is also crucial to achieve full growth. About 70% of the protein content of milk is used in biosynthesis (Hahn & Koldovsky, 1961), but some amino acids may also serve as gluconeogenic substrates (Snell, 1982a). Alanine is probably quantitatively the most significant amino acid used in glucose formation in the neonatal rat liver (Snell & Walker, 1973a), with glutamate and aspartate also making important contributions (Yeung & Oliver, 1967). Also, an increased gluconeogenic capacity from serine and threonine in the neonate has been reported (Vernon et al., 1968), and confirmed in the case of serine (Snell,
Alanine plasma levels are high in the late fetal and newly born rat, but show a marked decrease in the first few postnatal hours (Cuezva et al., 1980b; Girard et al., 1973a; Haymond et al., 1972; Snell & Walker, 1973a). Little has been published concerning the plasma levels of other amino acids in the perinatal period; Girard et al. (1975) have reported that plasma concentrations of 18 of the 22 amino acids investigated showed decreases up to 16 hours post partum.

1.1.2 GLUCOSE METABOLISM

Following birth, and the loss of the maternal supply of glucose, a period of hypoglycemia is seen in the neonatal animal. The pattern usually seen is a decrease to a nadir at 1 to 2 hours post partum, followed by recovery to late-fetal levels by approximately 3 hours post partum (Butcher & Potter, 1972; Cake et al., 1971; Cuezva et al., 1982a; Dawkins, 1963a; Fernandez et al., 1983; Girard et al., 1972; Girard et al., 1973a; Phillepe & Kitzmiller, 1981; Snell & Walker, 1973b; Sodoyez-Goffaux et al., 1979a). Occasionally, low levels have been seen for up to 5 hours after birth (Ballard, 1971a; Benito et al., 1980; Martin et al., 1981).

The majority of studies performed to investigate this phenomenon have been carried out by caesarian section and removal of the young close to term, in order to synchronise the time of birth for the entire litter. Following delivery, pups are usually placed at constant humidity and temperature and remain unfed until they are killed and their blood glucose level determined (e.g. Girard et al., 1973a; Snell & Walker, 1973b). Gain and Watts (1976) and Gain et al. (1981) have claimed that this does not give an accurate picture of the natural situation. They allowed rats to litter spontaneously, and marked each pup at birth to allow time of delivery of individual pups to be known. The neonates were allowed to remain with the mother and suckle until they were killed. No hypoglycaemia was seen in the six hours
following birth in these studies, nor in that reported by Blazquez et al. (1974), when natural birth was allowed to occur in preference to surgical delivery. However, ether anaesthesia of the neonates was used to obtained blood samples in the work by Gain and coworkers, and this is known to cause elevations in blood glucose levels (DiMarco et al., 1976). Also, postnatal hypoglycemia following natural birth has been noted by other workers (Cake et al., 1971; DiMarco et al., 1976; Sodoyez-Goffaux et al., 1979a). Discrepancies in findings may be due, at least in part, to differences in the temperature at which neonates are kept. Cuezva et al. (1980a) noted only slight postnatal hypoglycemia when neonates were kept at 30°C, compared with 37°C, whilst Kervran et al. (1976) saw no reduction in plasma glucose levels when surgically-delivered neonates were kept at 24°C, although there was marked hypoglycemia at one hour post partum if the temperature was maintained at 37°C. The temperature of the nest in which naturally-born neonates are kept by the dam is 35°C (Pegorier et al., 1978), and so the balance of evidence would appear to support a period of postnatal hypoglycemia as a physiological phenomenon.

Prior to the commencement of suckling, the blood level of glucose is the net result of its utilisation and production by the neonatal tissues. Radioactive labelling studies have shown that glucose utilisation is approximately 14 µmol/min/100g at birth, and decreases during suckling, returning to levels of the same order as that seen immediately after delivery following weaning (Walker & Snell, 1973). The rate of glucose utilisation was reported to be reduced by 2 to 3 hours post partum, which the authors suggested was the result of the changing hormonal environment, and the onset of fatty acid oxidation (Snell & Walker, 1973b); in the same study, glucose production increased markedly in the first two hours post partum, and then dropped again, probably as a result of decreased availability of gluconeogenic substrates. However, other work has shown increased
glucose turnover at 4 and 6 hours post partum (Girard & Guillet, 1975). Recycling of glucose via the Cori and alanine cycle has been reported to be less than 10% of that utilised at any time from birth to day 30 post partum (Vernon & Walker, 1972), however other work has suggested recycling of glucose of approximately 20% in the 1 day old suckling rat (Ferre et al., 1980, 1981b).

Glucose production may be from two possible sources; breakdown of stored glycogen or de novo synthesis from gluconeogenic precursors may both contribute to blood glucose levels. Studies investigating the prenatal accumulation of glycogen, or the postnatal production of glucose, have determined both the levels of the substrates and products of pathways of carbohydrate metabolism, and the activity of the enzymes involved in these pathways. Before describing the findings of such studies, it is appropriate to discuss the enzymes of glucose metabolism and their regulation.

**Enzymes of glucose metabolism**

In addition to synthesis and degradation of enzyme molecules, enzyme activities may be modulated by both stable, covalent, modifications or rapidly-reversible, non-covalent changes (Hems & Whitton, 1980); Van De Werve et al. (1977a) proposed the term 'activation' for the former type of modification and 'stimulation' for the latter.

Glycogen synthase catalyses the conversion of UDP-glucose to glycogen. This enzyme exists in the D (or b) form (in which it may be stimulated by the allosteric modifier glucose-6-phosphate) or in the I (or a) form (active even in the absence of glucose-6-phosphate). Conversion of the I to the D form is by phosphorylation (Lehninger, 1975; Williamson et al., 1981). Conversely, glycogen phosphorylase, which is involved in the breakdown of glycogen to glucose 1-phosphate, is converted from the less active (b) form to the more active (a) form by
phosphorylation (Hems & Whitton, 1980; Williamson et al., 1981). The activation and inactivation of glycogen synthase and phosphorylase are controlled by the activities of kinase and phosphatase enzymes. Phosphorylase b kinase is itself activated by a cyclic AMP-dependent phosphorylation or by Ca\(^{2+}\) (Vandenheede et al., 1977; Cohen, 1982). An inverse relationship between the activities of glycogen synthase and phosphorylase has been noted, which appears to be the result of an inhibitory effect of phosphorylase a on synthase phosphatase (Stalmans et al., 1974). Glucose is known to increase the activity of phosphorylase phosphatase (Stalmans et al., 1974), and this has been suggested to be the mechanism by which glucose activates glycogen synthase (Pines et al., 1976; Stalmans et al., 1974; Williamson et al., 1981). However, there is evidence of a direct stimulation of synthase activity by glucose in adult (Watts et al., 1982c) or day 17 post partum fetal liver (Pines et al., 1976).

Glucose formation by the liver may involve not only glycogen breakdown but also gluconeogenesis. The existence of enzymes unique to gluconeogenesis, rather than enzymes of the glycolytic pathway acting in the reverse direction, is an important feature of regulation of this pathway. Another important feature stems from the intracellular compartmentation of gluconeogenesis which necessitates the transfer of pyruvate from the cytosol into the mitochondria and the export of carbon, in the form of malate or aspartate, from the mitochondria to the cytosol. An increased mitochondrial uptake of pyruvate is associated with a stimulation of gluconeogenesis (Denton & Halestrap, 1979), but there is a general consensus that the primary event is an intramitochondrial response (Haynes, 1985). Pyruvate carboxylase, which catalyses the conversion of pyruvate to oxaloacetate in the mitochondria, is an obvious point of control of gluconeogenesis, being the first enzyme of the gluconeogenic pathway. This enzyme is stimulated allosterically by acetyl coenzyme-A; formation of acetyl coenzyme-A from pyruvate is catalysed by
pyruvate dehydrogenase, which has been shown to be inactivated by phosphorylation (Bailey et al., 1976). Pyruvate carboxylase is also regulated allosterically by ATP (activates) and by glutamate (inhibits). Pyruvate kinase, which catalyses the reaction converting phosphoenol pyruvate to pyruvate has been reported to be inactivated by phosphorylation (Soling et al., 1978; Claus & Pilkis, 1981; Williamson et al., 1981). No evidence of phosphorylation-induced activity changes in phosphoenolpyruvate carboxykinase (PEPCK), which together with pyruvate carboxylase reverses the conversion catalysed by pyruvate kinase, has been reported. The interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate is the next step in the gluconeogenic pathway controlled by different enzymes dependent on the direction of conversion. The gluconeogenic enzyme fructose 1,6-bisphosphatase is activated by phosphorylation (Soling et al., 1978; Williamson et al., 1981), which has been reported to have no effect on (Williamson et al., 1981), or to decrease the activity of (Soling et al., 1978), the glycolytic enzyme phosphofructokinase. Phosphofructokinase activity is also regulated by the concentrations of its substrate, fructose 6-phosphate, and its product, fructose 1,6-bisphosphate (Van Schaftingen et al., 1980a) and is stimulated by fructose 2,6-bisphosphate (Van Schaftingen et al., 1980b). Glucose 6-phosphatase is the final enzyme in the glycogenolytic and gluconeogenic pathways, catalysing the formation of glucose from glucose 6-phosphate. There is no evidence for control of the activity of this enzyme, nor of the opposing glycolytic enzyme, glucokinase, by a phosphorylation-dephosphorylation cycle. Regulation of the interconversion of glucose and glucose 6-phosphate appears to be by synthesis/degradation of the two enzymes involved and by kinetic activation of the enzymes through changes in substrate concentration (Newsholme & Leech, 1983).
Perinatal glycogen synthesis

In all species so far examined, glycogen deposition is seen in the fetal liver towards the end of gestation (Shelley, 1961). Glycogen deposition in the late fetal rat liver was first noted by Dawkins (1963a). In the term rat fetus, glycogen makes up 10% or more of the net weight of the liver. (Bashan et al., 1979; Gain et al., 1981; Girard et al., 1972; Greengard & Dewey, 1970; Pines et al., 1975; Watts et al., 1982a). The hepatic glycogen content is low at day 17 post coitum (Greengard & Dewey, 1970; Pines et al., 1975; Watts et al., 1982b), and increase slowly up to day 19 post coitum, after which a marked increase is seen prior to term (Bashan et al., 1979; Pines et al., 1975; Watts et al., 1982b). Acceleration of glycogen storage appears to be a result of an increase in the activity of the enzyme glycogen synthase due to both increased enzyme synthesis and conversion to the active form by dephosphorylation (Bashan et al., 1979; Devos & Hers, 1974; Pines et al., 1975; Vanstapel et al., 1980; Watts & Gain, 1976). Glycogen synthase is present largely in the inactive (b or D) form in early fetal liver (Devos & Hers, 1974; Pines et al., 1975; Vanstapel et al., 1980); an increased conversion to the active form results from the increase in synthase phosphatase activity, which appears on day 18 post coitum (Devos & Hers, 1974), and increases until day 19 post coitum (Bashan et al., 1979) or until birth (Vanstapel et al., 1980). Burch (1965) reported glycogen synthase activity in late fetal liver to be 400-500% of that normally found in adult liver.

The possibility of glycogen degradation during this period of net deposition is disputed. Devos and Hers (1974) reported glycogen synthase activities only just high enough to account for the rate of glycogen deposition; they suggested any phosphorylase present was in the b form, and thus showed low activity. Le Provost et al. (1980) also found no indication of glycogen degradation from day 19.5 post coitum to term. However, fetal liver glycogen has been described as being in
'dynamic equilibrium' with blood glucose (Bossi & Greenberg, 1972). Gilbert & Bourbon (1978) concluded from radioactive labelling studied that physiological turnover of hepatic glycogen occurs in the late fetal period, despite net deposition. Watts & Gain (1976) proposed a cycle due to the simultaneous presence of synthase a and phosphorylase a in the late fetal liver; they suggested that the glycogen level itself would control the ratio of phosphorylase a to synthase a, and thus the presence of net glycogen synthesis or breakdown.

**Perinatal glycogenolysis**

Following delivery, the glycogen stored in late gestation is rapidly degraded; a 40-50% decrease in five hours has been reported (Benito et al., 1980; Cuezva et al., 1982a; Martin et al., 1981), with glycogen stores 'exhausted' at 12 hours post partum (Girard, 1981) or reduced to 1% or less of term level at 24 hours post partum (Ballard, 1971a). Most data suggest breakdown of less than 10% of hepatic glycogen in the first hour after birth (Cuezva et al., 1982a; Fernandez et al., 1983; Ferre et al., 1978b; Girard et al., 1972, 1973a; Snell & Walker, 1973b) but it was reported that when rats were allowed to litter naturally, the steepest decline in glycogen content occurred between birth and 30 minutes post partum during which period 25% of the glycogen stored in the term fetal liver was lost (Gain & Watts, 1976). This significant glycogen loss in the immediately postnatal period has been confirmed for spontaneously delivered rats in another study by this group (Gain et al., 1981). However, Ferre et al. (1978b) reported no significant glycogenolysis up to two hours post partum in naturally born rats. It is interesting to note that the initial glycogen content, immediately after delivery reported by Gain & colleagues (300 μmol/g wet wt., or 60 mg/g wet wt.) is only 65% of that reported in other studies (Benito et al., 1980; Butcher & Potter, 1972; Cuezva et al., 1980b, 1982a; Fernandez et al., 1983; Girard et al., 1972, 1973a; Snell & Walker, 1973b). If this
was the result of glycogen loss during vaginal delivery, it is surprising that Ferre et al. (1978b) noted starting glycogen levels (94 mg/g wet wt) similar to workers delivering animals surgically. The reasons for the findings of Gain's group are not clear, but the onset of postnatal glycogenolysis only after a delay of one or two hours seems to be a common finding (Benito et al., 1980; Butcher & Potter, 1972; Cuezva et al., 1982a; Ferandez et al., 1983; Ferre et al., 1978b; Girard et al., 1972, 1973a; Snell & Walker, 1973b).

It is generally accepted that an increase in the activity of hepatic glycogen phosphorylase, coupled with decreased glycogen synthase activity, are responsible for postnatal glycogenolysis. Although glucose-6-phosphatase activity also increases sharply after birth, mobilization of liver glycogen has been shown to be independent of this increase (Dawkins, 1963a). There is some debate as to the exact timing of the increase in phosphorylase activity, and whether increase of total phosphorylase present (i.e. enzyme synthesis) or conversion of phosphorylase b to phosphorylase a (i.e. activation by phosphorylation) is responsible. An increase in total phosphorylase activity was seen from day 16 post coitum onwards in one study (Devos & Hers, 1974), and in the last two days of gestation in another (Dawkins, 1963b). An 18-fold increase in total phosphorylase between days 17 and 22 post coitum, and appearance of phosphorylase a from day 20 post coitum (Burch et al., 1963), or an increase of phosphorylase a activity from day 19 post coitum (Pines et al., 1975) have been reported. Another study indicated liver phosphorylase activity reached a peak two days before term (Blazquez et al., 1972). Cake & Oliver (1969) observed 30% of phosphorylase in late fetal liver in the a form, increasing to 45% by four hours post partum, and then decreasing. Girard et al. (1972) noted a postnatal increase in phosphorylase a activity at 1 hour post partum, with high levels maintained at six hours post partum, and Bashan et al. (1979) reported a 50% increase in the activity of phosphorylase a in the first three postnatal hours.
Despite the emphasis on phosphorylase activity changes in neonatal glycogen breakdown, other enzymes are capable of causing glycogenolysis; this aspect of postnatal glycogen metabolism will be discussed in Chapter 5 (section 5.1.3).

**Perinatal gluconeogenesis**

Gluconeogenesis is very low or absent in fetal rat liver (Ballard & Oliver, 1963, 1965; Bossi & Greenberg, 1972; Philippidis & Ballard, 1970) including term and 'post mature' fetuses (Le Provost et al., 1980; Pearce et al., 1974). However, gluconeogenic activity has been reported in fetal liver under conditions of maternal starvation (Bossi & Greenberg, 1972; Girard et al., 1977; Goodner & Thompson, 1967). Higher levels of gluconeogenesis are seen in the suckling rat than the adult (Ballard & Oliver, 1965; Snell, 1981b, Vernon et al., 1968).

There is disagreement as to the timing of onset of gluconeogenesis. Girard et al. (1975) reported the capacity for gluconeogenesis developing immediately after birth, and Snell & Walker (1973b) described initiation of gluconeogenesis between 30 minutes and one hour of birth. However, Medina et al. (1980) have found an insignificant rate of glucose formation from lactate in the first two hours post partum, and Pearce et al. (1974) found only a slight increase in this process during the first postnatal hour, although gluconeogenesis from lactate was marked by three hours post partum.

The widely-accepted cause of postnatal gluconeogenesis is the large increase in cytosolic phosphoenolpyruvate carboxykinase (PEPCK) activity after birth. More than 90% of PEPCK activity in the adult rat liver is cytosolic, whilst in the 17 day post coitum fetus, more than 90% of the very low total activity is located in the mitochondria or nuclei (Ballard & Hanson, 1967). Ballard and Hanson (1967)
observed a 25-fold increase in cytosolic PEPCK activity from negligible levels in
the fetal liver over the first two days post partum; this paralleled the pattern for
the onset of hepatic gluconeogenesis. Increases in activity of 2-fold at 1 hour post
partum, 16-fold at 3 hours post partum and 44-fold at 6 hours post partum have
been observed (Le Provost et al., 1980). Girard (1981) reported that adult levels of
activity were obtained by 12-24 hours post partum, and substrate supply and/or
other factors were then rate-limiting for gluconeogenesis. Although it does appear
that the postnatal increase in PEPCK activity is essential for the initiation of
gluconeogenesis, a number of other factors are believed to also influence the onset
of de novo glucose synthesis.

It appears that the increase in PEPCK activity leads to gluconeogenic
activity only after an increase in the oxidation state of the liver following birth
(Ballard, 1971b; Pearce et al., 1974). It is known that hypoxia causes a high NADH
to NAD$^+$ ratio, and that NADH causes reduced concentrations of oxaloacetate, and
thus hypoxia could inhibit gluconeogenesis if supply of oxaloacetate is rate-limiting
(Ballard, 1971a). Other potential sites of regulation of gluconeogenesis are at the
levels of pyruvate kinase (of major importance in the adult) or pyruvate
carboxylase, which acts in series with PEPCK to reverse the glycolytic step
catalysed by pyruvate kinase. An increase in pyruvate carboxylase activity in
mitochondrial extracts from day 18 post coitum, reaching adult levels by term, has
been reported (Jost & Picon, 1970), suggesting this enzyme is unlikely to be
involved in postnatal onset of gluconeogenesis. However, Bailey et al. (1976)
reported an increase in pyruvate carboxylase activity after birth, and Snell (1974)
described an increase in mitochondrial carboxylation in the suckling rat, compared
with the adult, with such an increase occurring within one hour of birth. There have
been no reports of perinatal changes in pyruvate kinase activity.
1.2 HORMONES IN THE PERINATAL RAT

1.2.1 INSULIN

Fetal plasma insulin increased in the last third of gestation, and achieved levels in excess of maternal levels prior to birth (Blazquez et al., 1970, 1972; Felix et al., 1971; Girard et al., 1973a; Vinicor & Kiedrowski, 1982). Most data suggest an increase in plasma levels from about day 18 post coitum (Blazquez et al., 1974; Cohen & Turner, 1972; Felix et al., 1971; Girard et al., 1974; Kervran & Girard, 1974), with a decrease in the day before birth (Blazquez et al., 1974; DiMarco et al., 1978; Girard et al., 1974; Kervran & Girard, 1974). A sharp drop in the plasma insulin level in the immediate postnatal period appears to be a universal finding (Benito et al., 1980; Blazquez et al., 1974; Cohen & Turner, 1972; Cuezva et al., 1982a; DiMarco et al., 1978; Gain et al., 1981; Gain & Watts, 1976; Girard et al., 1973a; Martin et al., 1981; Sodoyez-Goffaux et al., 1979a). A decrease to approximately 50% of fetal values in 30 minutes (Cuezva et al., 1982a; DiMarco et al., 1978; Sodoyez-Goffaux et al., 1979a) or in the first postnatal hour (Benito et al., 1980; Blazquez et al., 1974; Portha et al., 1978b) have been seen. Lower insulin levels have been reported to be maintained for at least 1 day post partum (Sodoyez-Goffaux et al., 1979a) or up to day 5 post partum, with an increase from day 10 post partum and a sharp increase at weaning to the level in fed adults (Blazquez et al., 1970).

The hyperinsulinemia seen in the rat fetus could be the result of an increase in maternal insulin reaching the fetal circulation, reduced clearance of plasma insulin by the fetus, increased fetal pancreatic secretion of insulin, or any combination of these. Radiolabelling studies have shown that maternal insulin cannot cross the placenta (Girard et al., 1974) and studies of clearance of $^{125}$I-monoiiodoinsulin from prematurely delivered rats indicates that low clearance rates
are unlikely to be the cause of fetal hyperinsulinemia (Sodoyez-Goffaux et al., 1979a). Pancreatic content and secretion of insulin in the perinatal period has been investigated in a number of laboratories. \( \beta \)-cells have been identified on day 16 post coitum (Cohen & Turner, 1972) and rapid increases in pancreatic insulin have been reported between days 16 and 22 post coitum (Hegre et al., 1973), on day 20 post coitum (Blazquez et al. 1972) or between day 20 post coitum and day 2 post partum (Sodoyez-Goffaux et al., 1971). One study using a very sensitive double antibody immunoassay showed insulin to be present in the pancreas at its initial appearance (11 days of gestation) and low but significant pancreatic insulin levels at day 12 post coitum, undergoing a 200-fold increase between days 14 and 19 post coitum (Clark & Rutter, 1972). Several groups have examined the secretory capacity of isolated pancreatic islets in vitro. In one such study, insulin secretion rates increased from three days prior to term to a few hours after birth; despite the postnatal fall in plasma insulin no reduction in the secretory capacity of the pancreatic \( \beta \)-cells was seen after birth (Sodoyez-Goffaux et al., 1979a). Significantly higher insulin release from the pancreas in vitro has been seen for late fetal rats than adults (Blazquez et al., 1970). An increase in pancreatic insulin storage after birth, presumably reflecting continuing synthesis with reduced secretion, has also been reported (Cuezva et al., 1982a; Sodoyez-Goffaux et al., 1979b); neonatal pancreatic insulin has been reported to reach levels three times higher than in the adult (Freie et al., 1975).

### 1.2.2 GLUCAGON

Plasma glucagon levels in the near-term rat fetus have been reported to be lower than maternal levels (Girard et al., 1973a) or in the same range as adult levels (Blazquez et al., 1976). An increase in plasma glucagon occurs in late gestation (DiMarco et al., 1978; Girard et al., 1974), but the exact pattern is
disputed; the presence of cross-reacting substances in radioimmunoassays, resulting in the determination of 'glucagon-like immunoreactivity' (GLI) as well as immunoreactive glucagon (IRG), in some studies (e.g. Blazquez et al., 1974; Girard et al., 1972), may be one of the reasons for lack of agreement.

Most data support a glucagon 'surge' (Sodoyez-Goffaux et al., 1981a) in the immediate postnatal period. An increase in plasma glucagon of as much as four-fold, with a peak at 30 minutes (DiMarco et al., 1978; Girard et al., 1972, 1973a; Kervran et al., 1976; Portha et al., 1978b), one hour (Cuezva et al., 1982a) or two hours (Benito et al., 1980; Martin et al., 1981) post partum has been observed. Following this initial sharp rise, it appears that glucagon levels drop; basal levels have been reported at three hours post partum (Benito et al., 1980; Martin et al., 1981), and six hours post partum (Portha et al., 1978b). However, although a decrease was seen following the postnatal increase in all studies, glucagon levels remained above birth levels for all four (Kervran et al., 1976) or six (Cuezva et al., 1982a; Girard et al., 1972, 1973a) hours investigated in these studies.

Glucagon is unable to cross the placenta (Blazquez et al., 1972; Girard et al., 1974; Greengard & Dewey, 1970). Although no reports of studies on rates of glucagon clearance in the perinatal period have appeared in the literature, it is generally assumed that changes in glucagon concentration reflect pancreatic content and release. Pancreatic glucagon was shown to increase by about 50% from day 18.5 to day 21.5 post coitum (Girard et al., 1973c) and in another report was described as reaching a maximum on day 5 post partum (Edwards et al., 1972). Glucagon secretion has been reported to increase just before birth (Blazquez et al., 1972), but in vitro the pancreas from a 19 day post coitum fetus secreted glucagon (Blazquez et al., 1974). An increase in pancreatic release of glucagon on day 20.5 post coitum was suggested to be the reason for the increase in plasma glucagon
seen on this day (Girard et al., 1973c). Ultrastructural studies show the presence of pancreatic A cells on day 16 post coitum (Perrier, 1970), or day 18 post coitum (Lambert et al., 1969c) and their complete maturation on day 20.5 post coitum (Perrier, 1970).

1.2.3 INSULIN: GLUCAGON RATIO

A great deal of emphasis has been placed in the literature on the changing ratio of insulin: glucagon in the perinatal period, rather than the plasma levels of either hormone per se. The molar ratio of insulin to glucagon was reported as 22.3 on day 21.5 post coitum (Portha et al., 1978b), and as decreasing from 15.6 to 5.9 from day 21 to day 22 post coitum (DiMarco et al., 1978). Delivery by cesarian section on day 21 or 22 post coitum caused a rapid drop of this ratio, reaching a minimum in 30 minutes (DiMarco et al., 1978). In one study, the ratio fell from 10.5 in the term fetus to 1.0 in one hour after delivery, and then stayed low (Girard et al., 1973a); in another, it fell from 10 at birth to 2.2 at 6 hours post partum (Portha et al., 1978b).

1.2.4 ADRENOCORTICOSTEROIDS

Corticosterone is the major circulating adrenocorticosteroid in the rat (Malinowska et al., 1972). Plasma corticosterone concentration increases in pregnancy (Holt & Oliver, 1968), and maternal levels have been reported to be two-fold higher than fetal levels (Milkovic & Milkovic, 1963), or lower than fetal levels from day 17 to 20 post coitum (Dupouy et al., 1975). Diurnal variations in plasma corticosterone concentrations are seen in both the dam and fetus (Cohen, 1976).

Plasma corticosterone concentration has been reported to increase between days 17 and 19 post coitum (Cohen & Brault, 1974; Dupouy et al., 1975), days 17.5
and 19.5 post coitum (Cohen, 1973), days 18.5 and 19.5 post coitum (Roudier et al., 1982), and days 18 and 20 post coitum (Holt & Oliver, 1968). A decrease occurred prior to term (Cohen, 1973; Cohen & Brault, 1974; Dupouy et al., 1975; Holt & Oliver, 1968), or on day 20.5 post coitum with an increase on day 21.5 post coitum (Roudier et al., 1982).

Postnatally, a transient increase in plasma corticosterone concentration is usually seen (Cohen, 1976; Corbier & Roffi, 1974; Dupouy et al., 1975; Eguchi et al., 1976, 1977; Holt & Oliver, 1968; Roudier et al., 1982), although Malinowska et al. (1972) reported a decrease in the first postnatal hour, with a rise at two hours post partum. One study showed an increase in free plasma corticosterone concentration, peaking one hour after delivery, if the pups were delivered by cesarian section on day 21 post coitum, but no significant change in liver corticosterone concentration, or free, bound or total plasma corticosterone concentration, if they were delivered on day 22 post coitum (DiMarco et al., 1978).

Corticosterone is known to cross the placenta (Dupouy et al., 1975), but the observation that following adrenalectomy of a gravid rat on day 14 post coitum, maternal plasma corticosterone level correlated with the number of live fetuses on days 19, 20 and 21 post coitum (Dupouy et al., 1975) demonstrated that fetal plasma levels reflect secretion of the steroid by the fetal adrenal, and not passage from the mother. The half-life of corticosterone in plasma in the first eight hours post partum also suggests that secretion occurs in the neonate, as this could not result from clearance of maternal hormone (Malinowska et al., 1972). The adrenal weight increases between day 17 and 20 post coitum (Dupouy et al., 1975) or day 18 and 21 post coitum (Eguchi & Ariyuki, 1963) and the ratio of adrenal weight to body weight reaches a peak in late pregnancy (Burch, 1965). A significant decrease in the weight of the adrenal gland has been observed at birth (Eguchi & Ariyuki,
1963), and the cortical mass of the adrenal has been reported to decrease postnatally (Josimovich, 1954). Active biosynthesis of cortisol and corticosterone in the rat adrenal has been seen from day 18 post coitum (Kalavsky, 1971), and secretion by the adrenal has been noted from day 19.5 post coitum, with decreased secretion immediately after birth (Josimovich, 1954).

Corticosteroid binding activity of rat plasma at days 3-12 post coitum is only 10-20% of the adult level (Holt & Oliver, 1968), suggesting a greatly increased tissue availability of corticosterone in the postnatal period, compared with the adult.

1.2.5 CATECHOLAMINES

Only three laboratories have reported plasma catecholamine concentrations in the immediately postnatal period, and the findings of these groups differ. Phillipe & Kitzmiller (1981) reported a plasma adrenaline level of 375 pg/ml at birth, increasing to nearly three times this at one hour post partum and to elevenfold the initial value by two hours post partum. Noradrenaline levels were found in this study to be nearly double the adrenaline concentration, and to show similar massive postnatal increases, to 2.5 times and 7.5 times the starting levels at one and two hours post partum, respectively. Cuezva et al. (1982a) by contrast, found higher adrenaline than noradrenaline concentrations in the plasma of newly delivered rats, with adrenaline levels of approximately 4.5 ng/ml being more than ten times higher than those reported by Phillipe & Kitzmiller (1981). Adrenaline, noradrenaline and total catecholamine concentrations were all seen to decrease in the first postnatal hour in the study by Cuezva's group; the minimum adrenaline level determined was seen at 30 minutes post partum, but noradrenaline and total catecholamines showed a further small decrease at one hour post partum. An
increase in all three parameters, with a peak at two hours post partum, and a steady decrease continuing until the final determinations at six hours post partum was seen. The postnatal peak at two hours post partum resulted from catecholamine levels around 40% of those seen immediately after birth. The findings of Jansen et al. (1984) were not dissimilar to those of Cuezva's group. These workers found plasma adrenaline levels of 7.7 ng/ml at birth, decreasing rapidly to 1.3 ng/ml in ten minutes and reading a minimum of 0.8 ng/ml 40 minutes after delivery; a secondary increase in the plasma adrenaline concentration was seen from 40 minutes post partum, reaching a peak of 2.2 ng/ml at two hours post partum and deceasing thereafter. Noradrenaline levels in the plasma declined from 4.4 ng/ml immediately after delivery to 0.9 ng/ml in the first ten minutes of neonatal life and continued to decrease to a plateau of 0.7 to 0.8 ng/ml between 40 minutes at six hours post partum. All of these studies used surgically-delivered rats; a possible explanation of the difference is that the animals were taken by caesarian section on day 21 post coitum by Phillipe & Kitzmiller (1981) and on day 21.5 post coitum by Jansen et al., or day 22 post coitum by Cuezva et al. (1982a). Ben-Jonathon (1978) has reported plasma adrenaline and noradrenaline concentrations on day one and two post partum of 10% or less of the level in the term fetus.

Fuller and Hunt (1967) reported that the volume of the adrenal medulla, the ability to form adrenaline, and the adrenaline to noradrenaline ratio in the adrenal gland all increase in late gestation; Margolis et al. (1966) also described a rapid increase in the adrenaline content of the adrenals just prior to term.

Roffi (1972) noted a rapid neonatal decrease in adrenal adrenaline concentration, with the greatest loss during delivery; this worker suggested that adrenaline is released from the fetal adrenal in response to hypoglycemic or anoxia. Girard and Zeghal (1975), however, felt their work indicated that neonatal
hypoglycemia or cold exposure are not sufficient stimuli for adrenal release of adrenaline and noradrenaline in the neonatal period, and anoxia at the time of parturition is a more significant factor in stimulating adrenaline release from the adrenal medulla. Roffi (1972) also proposed that noradrenaline release, due to activity of the sympathetic nervous system around parturition, is physiological important.

Despite careful study of the adrenal gland during the perinatal period by several investigators, Ben-Jonathon & Maxson (1978) have suggested that catecholamines in the fetal rat are produced by extra-adrenal chromaffin tissue. DeGallardo et al. (1974) have identified such tissue, known as the organ of Zuckerkandl, in the periaortic region, and have described its postnatal involution.
1.3 ADRENERGIC CONTROL OF HEPATIC CARBOHYDRATE METABOLISM IN THE RAT

1.3.1 ADRENERGIC RECEPTORS

A number of reviews are available on the subject of adrenoceptors (e.g. Goodhardt et al., 1984; Insel, 1984; Jenkinson, 1973; Lees, 1981; Lefkowitz et al., 1983; Schmelck & Hanoune, 1980; Stiles et al., 1984).

**Classification**

Adrenoceptors were first classified as α or β by virtue of the relative potency of a number of adrenergic agonists in eliciting physiological responses via such receptors. At α-adrenoceptors, noradrenaline is generally more potent than adrenaline, with isoprenaline relatively very weakly potent. By contrast, at β-adrenoceptors, isoprenaline is more potent than adrenaline, which is equipotent with, or slightly more potent than, noradrenaline (Ahlquist, 1948).

α-adrenoceptors may be further divided into the α₁ and α₂ subtype. This classification has replaced the earlier one of 'pre-synaptic' (now α₂) and 'post-synaptic (now α₁) α-adrenoceptors (Lefkowitz & Hoffman, 1981a). Relative potencies of pharmacological agents was the key to this subtyping of α-adrenoceptors, and has been used to identify two β-adrenoceptor subtypes. Isoprenaline is more potent than adrenaline or noradrenaline at both β-adrenoceptor subtypes; however, whilst adrenaline and noradrenaline are equipotent at β₁-adrenoceptors, adrenaline is ten-fold more potent than noradrenaline at β₂-adrenoceptors (Lands et al., 1967).

**Mechanism of response to adrenoceptor activation**

The effects of catecholamines are mediated via binding to membrane bound adrenoceptors, and the generation of intra-cellular signals, or 'second messengers'
Activation of $\beta$-adrenergic receptors results in stimulation of adenylate cyclase and formation of cyclic AMP, which in turn produces a well-established 'cascade' of events within the cell (Robison et al., 1971). The mechanism by which activation of $\alpha$-adrenergic receptors leads to the observed physiological responses is less well characterised. Inhibition of adenylate cyclase, and hence reduction of intracellular cyclic AMP levels, is believed to follow occupation of the $\alpha_2$-adrenoceptor by an agonist compound (Jakobs & Schultz, 1981; Jakobs et al., 1985; Jard et al., 1981; Lefkowitz & Hoffman, 1981b). The second messenger of $\alpha_1$-adrenoceptor activation appears to be unrelated to the adenylate cyclase-cyclic AMP system; an increase in cytosolic $\text{Ca}^{2+}$ ions appears to be the most significant event triggered by activation of the $\alpha_1$-adrenoceptor (e.g. Assimacopoulos-Jeannet et al., 1977; Keppens et al., 1977; Van de Werve et al., 1977a; Williamson et al., 1985). This increase in cytosolic $\text{Ca}^{2+}$ is believed to result from an initial release of the ion from intracellular, probably mainly endoplasmic reticulum stores (Shears & Kirk, 1984; Somlyo et al., 1985; Williamson et al., 1985), although an activation of $\text{Ca}^{2+}$ entry into the cell from extracellular fluid is probably required to sustain hormonal effects (Joseph et al., 1985). An increase in the hydrolysis of phosphatidylinositol-4,5-bisphosphate is also seen when $\alpha_1$-adrenoceptors are activated, and this has been proposed to be involved in the generation of the calcium signal (Berridge, 1984; Michell, 1975; Michell et al., 1977). One of the products of phosphatidylinositol-4,5-bisphosphate hydrolysis is inositol-1,4,5-trisphosphate which is believed to interact with a receptor on the endoplasmic reticulum membrane to stimulate $\text{Ca}^{2+}$ release into the cytosol (Berridge & Irvine, 1984).

**Modifying factors**

Adrenoceptors may be modified in terms of number, nature and coupling to post-receptor events by a large number of factors (Stiles et al., 1984). Regulation of adrenoceptor populations both in vivo and in vitro may be brought about by an
excess or deficiency of hormones. Catecholamines themselves may produce a reduction in adrenoceptor responsiveness (Perkins, 1982); this so called homologous regulation of adrenoceptors may be by reduction of receptor number ('down-regulation') or decrease in the association of receptor occupation and the cellular response ('uncoupling'), and affects all four adrenoceptor subtypes, but is tissue specific (Insel, 1984). Desensitisation of β-adrenoceptors by catecholamines producing uncoupling has been shown to involve phosphorylation of the receptor molecule (Stadel et al., 1983). Adrenocorticosteroids and thyroid hormones are well known to produce heterologous regulation of adrenoceptors (e.g. Exton et al., 1981; Lai et al., 1982; Malbon, 1980; Preiksaitis et al., 1982; Storm et al., 1984). The type or subtype of adrenoceptor expressed on cells has been shown to change during carcinogenic transformation (Lacombe et al., 1976) or on cell culture (Nakamura et al., 1983; Okajima & Ui, 1982); incubation of rat hepatocytes for as little as four hours has been shown to produce a change in adrenergic receptor subtype specificity (Kunos et al., 1984; Ishac & Kunos, 1986).

In view of the nature of the current study, probably the most relevant factor modifying cellular adrenergic systems is that of age. Changes in the number, type and coupling of adrenoceptors during development have been reported for a range of tissues: rat hepatocytes (Blair et al., 1979a, 1979b; Morgan et al., 1983a), mouse adipocytes (Lai et al., 1981), rat erythrocytes (Limbird et al., 1980), rabbit skeletal muscle (Smith, 1984) and rat lung (Whitsett et al., 1981).

1.3.2 ADRENERGIC EFFECTS ON HEPATIC CARBOHYDRATE METABOLISM IN THE ADULT RAT

Glycogen synthesis

Catecholamines cause a reduction in glycogen synthesis by the liver in vitro (Hornbrook, 1970). $10^{-7}$M adrenaline and $10^{-5}$M phenylephrine did not inactivate glycogen synthase in hepatocytes from fed rats (Hutson et al., 1976), although
adrenaline did inactivate glycogen synthase following exposure to 30 mM glucose in hepatocytes from fed animals (Taniguchi et al., 1980), or 40 mM glucose in hepatocytes from fed or fasted animals (Hutson et al., 1976).

**Glycogenolysis**

Increased hepatic glycogenolysis has been reported to be induced by a number of adrenergic agents. Concentrations of $10^{-8}$M adrenaline or noradrenaline cause hepatic glycogenolysis in starved rats or in in vitro preparations from adult rat liver (Hems, 1977b; Hems & Whitton, 1980). Adrenaline and noradrenaline cause glycogenolysis in isolated adult rat hepatocytes (e.g. Blair et al., 1979a; Exton & Harper, 1975); adrenaline shows a half-maximal effect at $10^{-7}$M (Feliu et al., 1976; Hutson et al., 1976). Phenylephrine also promotes glycogenolysis in isolated adult hepatocytes (e.g. Assimacopoulos-Jeannet et al., 1977; Exton & Harper, 1975; Pointer et al., 1976) with a half-maximal effect at $10^{-6}$M (Hutson et al., 1976), and maximal stimulation at $10^{-5}$M (Cherrington et al., 1976). Isoprenaline at $10^{-5}$M (Blair et al., 1979a) or $1.5 \times 10^{-6}$M (Pointer et al., 1976) increased glycogen breakdown in isolated hepatocytes. Glycogenolysis in perfused liver was seen to be stimulated by adrenaline, noradrenaline and phenylephrine (Exton & Harper, 1975). Glucose output from perfused liver was increased by adrenaline, noradrenaline, phenylephrine and isoprenaline (Sherline et al., 1972).

Increased glycogen phosphorylase activity was seen two seconds after exposure of hepatocytes from fed adult male rats to adrenaline (Blackmore et al., 1983), or following a one minute exposure of hepatocytes isolated from adult female rats to $10^{-6} - 10^{-5}$M adrenaline (Birnbaum & Fain, 1977). Phenylephrine (Assimacopoulos-Jeannet et al., 1977; Birnbaum & Fain, 1977; Hue et al., 1978; Hutson et al., 1976; Van de Werve et al., 1977a) and isoprenaline (Birnbaum & Fain, 1977; Van de Werve et al., 1977a) also activate phosphorylase in isolated
hepatocytes. Adrenaline, noradrenaline, phenylephrine and isoprenaline all activate glycogen phosphorylase in isolated perfused liver (Sherline et al., 1972) and adrenaline and noradrenaline at concentrations of $10^{-8}$ M or higher, increase phosphorylase a in the liver of starved rats (Hems, 1977b). The phosphorylation of glycogen phosphorylase which induces its activation is caused by both α and β-adrenergic agonists by stimulation of the enzyme phosphorylase b kinase (Exton et al., 1983).

**Gluconeogenesis**

Numerous reports document stimulation of gluconeogenesis by sympathomimetic substances. Adrenaline (e.g. Exton & Harper, 1975; Feliu et al., 1976; Tolbert et al., 1973; Tolbert & Fain, 1974), noradrenaline (e.g. Exton & Harper, 1975; Tolbert et al., 1973) and phenylephrine (e.g. Exton & Harper, 1975; Tolbert et al., 1973) increase gluconeogenesis in isolated adult rat hepatocytes. Increased glucose formation from lactate (Fain et al., 1975; Hutson et al., 1976; Kemp & Clark, 1978; Rognstad & Katz, 1977), pyruvate (Fain et al., 1975; Hue et al., 1978) and alanine (Fain et al., 1975) is induced by adrenaline and phenylephrine in isolated hepatocytes. Phenylephrine also stimulates gluconeogenesis from fructose (Hue et al., 1978). The increase in gluconeogenesis due to adrenaline and phenylephrine is dose-dependent; half-maximal stimulation has been seen at $10^{-7}$ M adrenaline or $10^{-6}$ M phenylephrine (Hutson et al., 1976). Maximal stimulation has been reported with $10^{-5}$ M phenylephrine (Cherrington et al., 1976) and $10^{-6}$ M adrenaline (Claus & Pilkis, 1978). The β-agonists isoprenaline and salbutamol have been reported to stimulate gluconeogenesis (Exton & Harper, 1975) or to have no effect on this process in isolated hepatocytes (Fain et al., 1975; Tolbert et al., 1973). Adrenaline, noradrenaline, phenylephrine and isoprenaline cause activation of gluconeogenesis in perfused liver preparations (Exton & Harper, 1975). Adrenaline stimulates the conversion of lactate (Exton et al., 1972), and
phenylephrine that of lactate, pyruvate, alanine, glycerol, sorbitol, dihydroxyacetone or fructose (Taylor et al., 1983), to glucose in this preparation.

Inactivation of pyruvate kinase has been observed in isolated hepatocytes exposed to adrenaline (Chan & Exton, 1978; Feliu et al., 1976; Garrison & Borland, 1979; Kemp & Clark, 1978; Soling et al., 1978), phenylephrine (Chan & Exton, 1978; Garrison & Borland, 1979; Kemp & Clark, 1978) and isoprenaline (Kemp & Clark, 1978). However, in other studies, a lack of effect of phenylephrine on pyruvate kinase activity has been reported (Hue et al., 1978; Thomas & Halestrap, 1981), although absence of Ca$^{2+}$ rendered the enzyme sensitive to phenylephrine inactivation (Hue et al., 1978). Adrenaline has been reported to produce no significant decrease in the flux through pyruvate kinase in hepatocytes from fasted rats, and to increase the flux through this enzyme in hepatocytes from fed rats (Rognstad & Katz, 1977). Adrenaline and noradrenaline stimulate mitochondrial ATPase, and have therefore been proposed to have an analogous action to glucagon in increasing the ATP to ADP ratio and thus promoting pyruvate carboxylation (Titheradge et al., 1979). Kneer et al. (1974) concluded from his studies that stimulation of gluconeogenesis from galactose and fructose by adrenaline is due to a decreased flux through phosphofructokinase and an increased flux through fructose 1,6-bisphosphatase.

Adrenoceptor mediating effect

It appears from a number of observations that adrenergic effects on hepatic carbohydrate metabolism are mediated by different receptors in adult male and female rats. Birnbaum & Fain (1977) and Studer & Borle (1982) concluded that stimulation of glycogenolysis by adrenergic agonists in the liver of the mature female rat is mediated by both α- and β-adrenergic pathways, while in the male only the α-adrenergic path is of any significance.
1.3.3 ADRENERGIC EFFECTS ON HEPATIC CARBOHYDRATE METABOLISM IN THE IMMATURE RAT

Glycogen synthesis

Isoprenaline at $10^{-5}$ or $10^{-6}$M inhibited the synthesis of glycogen by 45% and 26%, respectively, in hepatocytes isolated from day 20 post coitum fetal rats and cultured for three days (Freemark & Handwerger, 1984). In the same study, phenylephrine at $10^{-5}$M produced only a 20% inhibition of glycogen synthesis, and at $10^{-6}$M had no significant effect. When two subcutaneous doses of 20 µg/100 g adrenaline were given to pregnant rats on day 18.5 post coitum, and fetuses obtained by caesarian section one or three hours later, fetal liver glycogen was reduced to 10-15% of control levels (Goldwater & Stetten, 1974). As active synthesis of glycogen would be expected in the fetal liver at this stage of gestation, the effect of adrenaline is probably at least partly due to inhibition of glycogen synthesis.

Glycogenolysis

0.5 µg/g bodyweight adrenaline administered subcutaneously immediately or one hour after natural birth produced a decrease in the glycogen content of the liver at six hours post partum (Kotoulas & Phillips, 1971). In hepatocytes freshly isolated from term fetal rats, $10^{-6}$M phenylephrine produced a 22% or 33% increase, and $5 \times 10^{-6}$M isoprenaline a 68% or 83% increase, in glycogen breakdown (Hühn et al., 1983; Schülze et al., 1984). In hepatocytes freshly isolated from 'young' (27-35 days old) rats, $10^{-5}$M adrenaline, isoprenaline or phenylephrine all increased glucose production from glycogen, and a combination of α- and β-antagonists was required to block this effect (Blair et al., 1979a). In hepatocytes isolated from fetal rats on day 18 post coitum, and cultured for three days, $10^{-8}$M adrenaline produced maximum glycogenolytic effects after 4 hours; 60% of glycogen was degraded, with newly-synthesised glycogen being broken down preferentially (Moncany & Plas, 1980). Adrenaline has also been shown to reduce
the glycogen content in liver explants made from term fetal rats and incubated for 18 or 40 hours (Eisen et al., 1973a). Adrenaline at $2 \times 10^{-7} \text{M}$ or higher concentrations or $10^{-6} \text{M}$ isoprenaline activated glycogen phosphorylase in term fetal liver explants after 18 hours incubation; $10^{-5} \text{M}$ phenylephrine had very little effect on this enzyme (Sherline et al., 1974).

**Gluconeogenesis**

A three to four-fold increase in hepatic PEPCK activity has been reported in isolated fetal liver explants exposed to $10^{-4} \text{M}$ isoprenaline (Wicks, 1969). The appearance of this enzyme in the perinatal period may be induced from day 16 post coitum onwards by the intrauterine administration of 5 or 10 μg adrenaline or noradrenaline to fetuses (Yeung & Oliver, 1968a).

**Adrenoceptor mediating effects**

A number of workers have proposed that, in the perinatal rat, adrenergic effects on hepatic carbohydrate metabolism are mediated predominantly, if not exclusively, by β-adrenoceptors (e.g. Itoh et al., 1984; McMillian et al., 1983; Moncany & Plas, 1980; Sherline et al., 1974). However, studies cited in favour of such a conclusion often have used isolated liver tissue, and have studied adrenergic effects after a long incubation period or a period of culture; in view of the known emergence of β-adrenoceptor mediated effects on culture, or even following a four hour incubation, of adult liver tissue (see the description of modifying factors in section 1.3.1), some caution must be exercised before reaching such a conclusion. In studies using freshly isolated term fetal hepatocytes, isoprenaline was seen to produce a greater stimulation of glycogen breakdown and glucose release than phenylephrine (Hühn et al., 1983; Schlüze et al., 1984), but as the β-adrenergic agonist was present at a five-fold higher concentration than the α-stimulating compound, this does not provide evidence for a more significant β-mediated pathway than α-mediated. The work of Blair et al. (1979a), would seem to indicate
a significant contribution of β-adrenoceptor activation in adrenergic stimulation of glycogenolysis in fresh hepatocytes from young rats, with a decrease in this contribution with ageing. However, there is no study reported in the literature which gives clear evidence of a β-adrenergic pathway modulating hepatic carbohydrate metabolism in the perinatal rat.
1.4 **AIMS OF THE STUDY**

1.4.1 To characterise the hepatic adrenoceptor population during the immediately perinatal period in the rat.

1.4.2 To assess the effects of adrenergic agents on carbohydrate metabolism in hepatocytes freshly isolated from the perinatal rat.

1.4.3 To investigate the effects of adrenergic agents, when administered in vivo, on carbohydrate metabolism in the immediately postnatal period in the rat.

1.4.4 To draw conclusions, from the above, regarding the contribution of hepatic adrenergic pathways in the recovery from postnatal hypoglycemia in the laboratory rat.
CHAPTER 2

CHARACTERISATION OF THE HEPATIC ADRENOCEPTOR POPULATION IN THE PERINATAL RAT
2.1 INTRODUCTION

2.1.1 RECEPTOR THEORY

Paul Ehrlich (1913) is believed to have been the first worker to use the term 'receptor' to describe the structure on a cell surface with which a substance (in his work, a chemotherapeutic drug) must react to produce a cellular response, although Langley's (1906) description of a 'receptive substance' mediating the response of muscle to curare slightly predated this. In 1937, Clark attempted the first mathematical analysis of a drug-receptor interaction. The concept that a drug showing agonist activity must, as well as binding to the receptor, have some inherent feature which an antagonist compound lacks and which produces a biological response was discussed in the literature some twenty years later (Ariens, 1954; Stephenson, 1956). The terms 'intrinsic activity' and 'efficacy' were proposed to describe this feature of an agonist drug by Ariens and Stephenson, respectively.

Today, a receptor may be defined as a cellular macromolecule with which a drug interacts to bring about a characteristic biological response. Schematically, this may be represented as:

\[
\begin{align*}
D + R & \rightarrow DR \\
& \rightarrow DR^* \\
\end{align*}
\]

where: D : free drug  
R : free receptor  
DR : drug-receptor complex  
DR* : drug-receptor complex with receptor in activated form

The affinity of D for R will influence the formation of DR and DR* equally, the efficacy of D will influence the proportion of drug-receptor complex of the form DR*. Thus, response depends on both affinity and efficacy.
2.1.2 TECHNIQUES OF RECEPTOR CHARACTERISATION

Pharmacological approach

In early work concerned with the characterisation of receptors, information was obtained by the use of an indirect approach. Understanding of the nature of receptors was gained by study of the biological response to drugs and establishment of structure-activity relationships (for review see Albert, 1971). As discussed in Chapter 1 (section 1.3.1), it was this approach which allowed the initial classification and subclassification of adrenoceptors.

The potency orders of highly-subtype selective agonist and antagonist drugs is still a very useful tool in investigation of the nature of adrenoceptor populations, but there are a number of drawbacks to the use of a purely pharmacological approach to receptor characterisation. Because this technique makes use of a biological response in receptor characterisation, it follows that receptors may be investigated only in an environment in which the response pathway is intact. Thus, it is essential to establish that the response studied results solely from interaction with the receptor of interest, and that any influences of other effector systems are eliminated or recognised. A second point of note is that this approach may provide a great deal of qualitative information, but is limited in the quantitative data which may be obtained. Although relative receptor populations in different tissues may be established by means of relative potencies of a drug in these tissues, no information on the absolute number of receptor molecules per unit mass of tissue can be obtained.

Purification

The most direct way to classify a receptor is to isolate the receptor protein in a pure form. A number of cell-surface hormone receptors have been isolated,
and detailed information of their primary, secondary and tertiary structure obtained. Both α- and β-adrenoceptors have been isolated using membrane solubilisation with detergent, followed by purification by affinity chromatography (Kunos, 1984; Stiles et al., 1984; Strosberg et al., 1981). Covalent labelling and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) have also been used to investigate the molecular nature of adrenoceptors (Kunos, 1984; Strosberg et al., 1981).

Although this approach may provide a great deal of detailed information regarding the structure of receptors, down to the level of the constituent amino acids, it is technically demanding and great care is required in the extrapolation of findings to the nature and function of receptors in intact tissues and cells. Proteolysis of receptor molecules during their isolation and purification can produce erroneous data, and any influence of the membrane environment on receptory tertiary structure is lost. Unlike the pharmacological approach, information regarding the size of a receptor population in a particular tissue may be gained by this technique.

Affinity labelling

A relatively new approach to investigating adrenoceptor structure is affinity labelling (Stiles et al., 1984). Affinity labelling reagents are analogues of physiological ligands, containing reactive groups. The photoaffinity probe \(^{125}\text{I}\)-p-azidobenzylcarazolol has been used in identification of the polypeptide structure of β-adrenoceptors (Caron et al., 1985).

Radioligand binding

The nature and number of cell-surface receptors may be investigated by the use of the radioligand binding technique (for review see Lefkowitz & Hoffman,
1981a). This involves incubating membranes containing the receptor(s) of interest with a radioactively labelled compound (the 'radioligand') with known affinity for the receptor. Radioligand not bound to the membrane is then removed, usually by a process of rapid filtration under vacuum and extensive washing. Measurement of the radioactivity associated with the membrane then allows determination of the size of the receptor population and interference with the binding of the radioligand by compounds with well-defined affinity for various types of receptor indicates the nature of the receptor. One limitation of this technique is that it provides no information regarding the molecular structure of the receptor under investigation.

ASSAY TECHNIQUES AND ANALYSIS

Radioligand binding assays may be considered to be of two basic types: equilibrium (protein dilution profile, inhibition profile and saturation assay) or kinetic (association and dissociation time course).

Protein dilution profile

This is usually the first type of assay performed with any radioligand, in order to determine a working concentration of protein to be used in subsequent assays. Varying amounts of membrane are incubated with a fixed concentration of radioligand; total binding and 'non-specific' binding (that remaining in the presence of an excess of an agent competing for the ligand binding site) are determined at equilibrium for each protein concentration. Any protein concentration within the range at which the concentration of ligand specifically bound is linearly related to protein concentration is considered to be a suitable working concentration.

Inhibition profile

This approach is a means of characterising the radioligand binding site in terms of interaction with a range of unlabelled compounds. Thus, both compounds
with known affinity for adrenoceptors, and compounds with no known affinity for such receptors, may be investigated. Hofstee plots (see Fig. 2.1a) of the data can be made, and the concentration of agent required to displace 50% of the bound radioligand (IC$_{50}$) calculated. From this, the equilibrium dissociation constant of the displacing agent ($K_i$) may be calculated using the Cheng and Prusoff equation (Cheng & Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{L}{K_D}}$$

where $L$ = concentration of radioligand (M)

$K_D$ = equilibrium dissociation constant of radioligand (M)

From their $K_i$ values, the relative potencies of compounds in displacing a radioligand can be established, and thus the receptor type and subtype being labelled can be inferred. The amount of radioligand bound at equilibrium to a fixed concentration of protein, in the presence of varying concentrations of the compound under study, is measured.

**Saturation assay**

Data from this assay may be used to calculate the equilibrium dissociation constant ($K_D$) for the radioligand and the receptor concentration ($B_{max}$); these are usually obtained from a Scatchard representation of the data (see Fig. 2.1b). The assay is carried out by incubating a fixed concentration of protein with varying concentrations of radioligand, and measuring total ligand bound, and that bound in the presence of excess of an agent competing for the ligand binding site (non-specific binding) when the reaction has reached equilibrium. Specific binding is calculated as the difference between total and non-specific binding.
FIGURE 2.1  
Graphical analyses of binding data

(a)  Hofstee plot of inhibition profile

Radioligand displaced/Conc. displacing drug

(b)  Scatchard plot of saturation assay

\[\text{Gradient} = -\frac{K_A}{K_D}\]

'Bond' radioligand refers to specific binding.
Association time course

This type of assay demonstrates the time taken to reach equilibrium and allows calculation of the association rate constant ($k_1$) (for details see section 2.3.4). Fixed concentrations of ligand and protein are incubated together for various time periods; total and non-specific binding are determined, and specific binding calculated, at each time point.

Dissociation time course

From this type of assay, the dissociation rate constant ($k_{-1}$) can be calculated (for details see section 2.3.4). Using this approach, excess competing ligand is added to a fixed concentration of radioligand and protein at equilibrium. Measurements are made of total and non-specific binding at zero time and varying times after addition of the competitive compound. The specific binding is calculated and used in calculation of $k_{-1}$.

When association and dissociation rate constants are known, the association constant ($K_A$) and dissociation constant ($K_D$) may be calculated:

$$K_A = \frac{k_1}{k_{-1}} \quad , \quad K_D = \frac{1}{K_A}$$

RADIOLIGANDS FOR ADRENOCEPTORS

Radioligand binding assays of adrenoceptors have been carried out for some years now (for review see Hoffman & Lefkowitz, 1980). The variety of ligands available for such assays has increased enormously, with many of the more recently developed compounds having the advantages of good specificity for the receptor, and/or high specific activity (Nahorski, 1981; Lefkowitz & Hoffman, 1981b). One difference between $\alpha$- and $\beta$-adrenoceptors is that whilst radioligands are available with selectivity for one or the other $\alpha$-adrenoceptor subtype, only non-subtype
selective β-adrenoceptor radioligands are available. Therefore, in characterising
the subtypes present in the α-adrenoceptor population of a tissue, both labelling of
different subtypes directly by use of selective ligands or non-selective labelling of
both subtypes and displacement from one subtype by inclusion of an excess of an
unlabelled subtype-selective competitive agent, may be used. In radioligand binding
assays of β-adrenoceptors, however, only the latter approach is possible.

[^3^H] -Dihydroergocryptine is the most commonly used non-subtype selective
α-adrenoceptor radioligand (e.g. Aggerbeck et al., 1978; El-Refai & Exton, 1980;
Guellaen et al., 1978). [^3^H] prazosin, an α₁-selective antagonist is the ligand
usually employed to label α₁-adrenoceptors (e.g. Goodhardt et al., 1982; Hoffman
et al., 1981), although the tritiated antagonist compound WB-4101 has also been
used (Lefkowitz & Hoffman, 1981b, Up Pritchard et al., 1977). For direct selective
labelling of α₂-adrenoceptors, [^3^H] yohimbine, also an antagonist at its preferred
receptor, is available (Hoffman et al., 1981).

The first reports of successful labelling of β-adrenoceptors were made in
1974; ligands used were [^3^H] -dihydroalprenolol (Lefkowitz et al., 1974), [^125^I] -
hydroxybenzylpindolol (Aurbach et al., 1974) and [^3^H] -propranolol (Atlas et al.,
1974). Other ligands available for radioligand binding assays of β-adrenoceptors are
[^125^I] -iodocyanopindolol (Engel et al., 1981),[^125^I] -pindolol (Barovsky &
Brooker, 1980) and[^3^H] isoprenaline (Hadjiivanova et al., 1984).

Tritiated forms of adrenaline and noradrenaline are available for radioligand
binding assay, and some workers have argued that the correlation of binding of
these ligands with physiological parameters indicates their superiority in such
assays (El-Refai et al., 1978, 1979). However, antagonist compounds are generally
preferred as radioligands due to the commonly held view that, unlike agonists, their
affinity for adrenoceptors is not affected by guanyl nucleotides (see section 2.4.3). The physiological ligands for adrenoceptors also have the disadvantage of relatively poor type and subtype selectivity.

2.1.3 AIMS OF THE EXPERIMENTS

Carbohydrate metabolism in the rat liver has been shown to be affected by adrenergic compounds via activation of both α-adrenoceptors (e.g. Exton & Harper, 1975; Kemp & Clark, 1978) and β-adrenoceptors (e.g. Blair et al., 1979a; Studer & Borle, 1982). Such effects are predominantly α₁-mediated in the adult male rat (see section 1.3.1). Effects of adrenergic agents on hepatic carbohydrate metabolism during the perinatal period must involve activation of adrenoceptors; characterisation of the adrenoceptor population in the liver during the period around the time of birth would therefore be useful in determining the potential role of the adrenergic system in the modulation of fetal and neonatal carbohydrate metabolism.

The aim of the experiments described in this chapter was to determine the number, type and subtype of hepatic adrenoceptors in the perinatal rat. Membranes were prepared from fetal rat livers at one and two days prior to term (days 20 and 21 post coitum) and at term (day 22 post coitum) and from neonatal rats at 24 and 48 hours after delivery. Thus, any change in the size or nature of the hepatic adrenoceptor population during this crucial developmental period could be demonstrated.
2.2 MATERIALS & METHODS

2.2.1 MATERIALS

NaCl, MgCl₂, KHCO₃, the disodium salt of ethylenediaminetetraacetic acid (EDTA Na₂) and sucrose were all obtained from BDH Chemicals Ltd., Poole, Dorset and were analytical (AR) grade. MgSO₄ was from Fisons, Loughborough and was also AR grade. Trizma base, (-)-isoprenaline(-)-bitartrate, (+)-adrenaline, (+)-noradrenaline HCl, yohimine HCl and DL-propranolol HCl were from Sigma Chemical Company, Poole, Dorset. Atenolol, ICI 118 551, prazosin HCl, phentolamine mesylate, ketanserin, atropine sulphate, spiperone, mepyramine, ranitidine HCl, and syrosingopine were supplied by the Biochemical Pharmacology Department, Glaxo Research Ltd., Ware, Herts. Pico-fluor 30 scintillant fluid was obtained from United Technologies Packard, Illinois, USA. Glaxo Operations UK supplied ascorbic acid.

(-)²-[Propyl-2,3-³H] 1-dihydroalprenolol ([³H]-DHA), [(9,10(n)-³H]9,10-] dihydroergocryptine ([³H]-DHE), and (-)¹²⁵I-iodocyanopindolol ([¹²⁵I]-ICYP) were obtained from Amersham International plc, Amersham, Bucks. (-)¹²⁵I-iodopindolol ([¹²⁵I]-IP) was synthesised by Nia Cook at the Department of Pharmacology and Therapeutics, University of Leicester, using the method of Barovsky and Brooker (1980).¹²⁵I-NaI for the synthesis was obtained from Amersham International plc and pindolol from the Biochemical Pharmacology Department, Glaxo Research Ltd.

2.2.2 ANIMALS

AHA strain rats from the breeding unit of Glaxo Research Ltd. were used. 'Adult' male and female rats weighed 200-300g, 'young adult' rats weighed 140-160 g. 'Weanlings' were 21 days post partum, at which age they were withdrawn
from the dams. The age of fetal rats was determined from the time of mating; the day that a vaginal plug was discovered was considered to be day 1 post coitum. 'Near-term' fetal rats were day 20 or 21 post coitum, the precise day of mating not being known.

Parturition usually occurred overnight between days 21 and 22 post coitum; results reported for day 22 post coitum are for naturally-born neonates killed shortly after the complete litter (10 to 14 pups) had been delivered. 24 and 48 hour post partum neonates were obtained by caesarian delivery on day 21 of gestation; umbilical cords were broken by a sharp tug close to the body, as this procedure mimicked the 'natural' situation and prevented the blood loss seen when cords were cut, and also avoided the time-consuming suturing of cords. Neonates were then fostered for the appropriate period to a lactating female. All neonates so treated had fed and all survived. Older neonates had been born naturally. All animals from a litter were weighed together and a mean weight per fetus or pup calculated.

In one experiment to investigate the possible effects of catecholamine depletion on ligand binding to hepatic adrenoceptors, adult female rats were dosed with syrosingopine. 40 mg syrosingopine was mixed with 0.2 ml glacial acetic acid, 0.8 ml propanol-1,2-diol, 1 ml absolute ethanol and 18 ml distilled water to give a syrosingopine concentration of 2 mg/ml. Dosing was by the intra-peritoneal route and 0.5 mg/kg was administered 24 hours prior to killing.

Adult, young adult and weanling rats were killed by cervical dislocation; fetuses and neonates were killed by decapitation.

All animals were given food and water ad libitum.
2.2.3 LIVER MEMBRANE PREPARATION

The standard method used to prepare liver membranes is described in section A below. However, in an attempt to overcome difficulties encountered for some assays, liver membranes were prepared by several methods, also detailed below. Single adult livers, pooled livers from one litter of weanlings or day 7 post partum neonates, or pooled livers from 1-4 litters of fetuses or young neonates were used, to give a total weight of 10-30 g of tissue.

A. This method was based on that of Witkin and Harden (1981). 'Homogenisation medium' was 145 mM NaCl, 2 mM MgCl$_2$, 20 mM Tris, pH 7.5. 'Storage medium' was 250 mM sucrose, 5 mM MgCl$_2$, 50 mM Tris, pH 7.5. All steps were carried out at 4°C.

1. Livers were rinsed in homogenisation medium, scissor-minced where necessary and homogenised in about 40 volumes of homogenisation medium using a Silverson type homogeniser (Vortmix mixer/emulsifier) followed by a Dounce homogeniser (Jencons Scientific Supplies).

2. The homogenate was centrifuged at 2,000 g for 10 minutes (MSE Coolspin).

3. The pellet was discarded and the supernatant centrifuged at 40,000 g for 15 minutes (MSE Europa 65).

4. The supernatant was discarded, the pellet resuspended in homogenisation medium using the Dounce homogeniser and centrifuged at 40,000 g for 15 minutes (MSE Europa 65).

5. Step 4 was repeated.

6. The supernatant was discarded and the pellet resuspended in storage medium using the Dounce homogeniser.
1 ml aliquots were taken into capped polypropylene tubes (Sarstedt). Membranes were used freshly prepared or were snap-frozen in liquid \( N_2 \) and used after storage at -60\(^\circ\)C to -80\(^\circ\)C.

This method gave a membrane yield of 5-30 mg protein/g tissue.

B. This method was adapted from a standard membrane preparation method used in the Biochemical Pharmacology Department of Glaxo Research Ltd. 'Medium' was 50 mM Tris, 10 mM MgCl\(_2\), pH 7.4. All steps were carried out at 4\(^\circ\)C.

1. Livers were rinsed in medium, scissor-minced where necessary and homogenised in about 2.5 volumes of medium using a Silverson type homogeniser (Vortmix mixer/emulsifier) followed by a Dounce homogeniser (Jencons Scientific Supplies).

2. The homogenate was filtered through four layers of cheese-cloth, then centrifuged at 8,000 g for 10 minutes (MSE Coolspin).

3. The pellet was discarded and the supernatant centrifuged at 40,000 g for 20 minutes (MSE Europa 65).

4. The supernatant was discarded, the pellet resuspended in medium using the Dounce homogeniser and centrifuged at 70,000 g for 20 minutes (MSE Europa 65).

5. Step 4 was repeated.

6. The supernatant was discarded and the pellet resuspended in medium using the Dounce homogeniser.

7. 1 ml aliquots were taken into capped polypropylene tubes (Sarstedt). Membranes were used freshly prepared or after storage at -60\(^\circ\)C.

This method gave a membrane yield of 2-3 mg protein/g tissue.
C. This method was based on that of Neville (1968). 'Medium' was 1 mM NaHCO₃. All steps were carried out at 4°C.

(1) Livers were rinsed in medium, scissor-minced where necessary and homogenised in about 6 volumes of medium using a Silverson type homogeniser (Vortmix mixer/emulsifier) followed by a Dounce homogeniser (Jencons Scientific Supplies).

(2) The homogenate was filtered through four layers of cheese-cloth, then centrifuged at 1,500 g for 10 minutes (MSE Coolspin).

(3) The supernatant was discarded and the pellet resuspended in medium using the Dounce homogeniser.

(4) Step 2 was repeated.

(5) The supernatant was discarded and the pellet resuspended in sufficient 69% (w/w) sucrose in medium to give 44% (w/w) sucrose.

(6) 10 ml aliquots of crude membrane suspension were placed in 13 ml centrifuge tubes and gently overlain with 3 ml of 42.3% (w/w) sucrose in medium.

(7) The tubes were left to stand for 30 minutes, then centrifuged at 100,000 g for 2 hours (MSE Europa 65).

(8) The membranes floating on the top of the sucrose gradient were aspirated, resuspended in medium using the Dounce homogeniser and centrifuged at 8,000 g for 10 minutes (MSE Coolspin).

(9) The supernatant was discarded and pellet resuspended in medium using the Dounce homogeniser.

(10) 1 ml aliquots were taken into capped polypropylene tubes (Sarstedt). Membranes were used freshly prepared or after storage at -60°C.

Very low membrane yields (< 0.1 mg protein/g tissue) were obtained with this method.
D. This method was based on that of Kaumann and Birnbaumer (1974). 'Medium' was 1 mM KHCO₃. All steps were carried out at 4°C.

(1) Livers were rinsed in medium, scissor-minced where necessary and homogenised in about 40 volumes of medium using a Silverson type homogeniser (Vortmix mixer/emulsifier) followed by a Dounce homogeniser (Jencons Scientific Supplies).

(2) The homogenate was filtered through two layers of gauze then centrifuged at 2,000 g for 10 minutes (MSE Coolspin).

(3) The pellet was discarded and the supernatant centrifuged at 40,000 g for 15 minutes (MSE Europa 65).

(4) The supernatant was discarded, the pellet resuspended in medium using the Dounce homogeniser and filtered through two layers of gauze, then centrifuged at 40,000 g for 15 minutes (MSE Europa 65).

(5) Step 4 was repeated.

(6) The supernatant was discarded and the pellet resuspended in medium using the Dounce homogeniser.

(7) 1 ml aliquots were taken into capped polypropylene tubes (Sarstedt). Membranes were used freshly prepared or after storage at -60°C.

This method gave a membrane yield of about 10 mg protein/g tissue.

2.2.4 RADIALIGANDS USED IN BINDING ASSAYS

[^3H]-Dihydroalprenolol

An aliquot of liver membrane preparation containing 0.05 - 2.0 mg of protein was incubated with[^3H]-DHA at ligand concentrations in the range 0.15 - 20.0 nM for saturation assays and 1.5 - 3.0 nM for time course, protein dilution and displacement assays. A total volume of 400 µl was incubated in the presence of
50 mM Tris, 10 mM MgCl₂ and 100 μg/ml ascorbic acid at pH 7.4. Phentolamine was included in all incubations (usually at 0.1 mM) to reduce binding to α-receptors. Displacing drugs were dissolved in the incubation mixture. Specific binding was defined as that binding displaced by 2 x 10⁻⁶M isoprenaline.

Disposable polypropylene tubes (Sarstedt) were used for all incubations. Incubation was always in triplicate and was started by addition of membranes, except in protein dilution assays when addition of ligand started the incubation. Incubation was for 20 minutes, or for varying times up to 20 minutes when the time course of association was being investigated, and was always at 30°C. Incubation was terminated by addition of 3 ml of ice-cold 50 mM Tris buffer at pH 7.4, followed immediately by filtration under vacuum through GF/B glass microfibre filters (Whatman); a Millipore 1225 sampling manifold was used. Assay tubes were washed with 3 ml ice-cold buffer and the filters washed with a further 2 x 5 ml ice-cold buffer.

Filters were placed in disposable scintillation vials (Packard, Illinois, USA) and 10 ml of Pico-fluor 30 scintillant fluid added. Bound [³H]-DHA was counted in a scintillation counter (Mark III, Model 688, Tracor Analytic). Aliquots of [³H]-DHA were counted to allow calculation of the concentration of ligand present in incubations.

[³H]-Dihydroergocryptine

An aliquot of liver membrane containing 0.1 - 0.5 mg protein was incubated with [³H]-DHE at ligand concentrations in the range 4.5 - 15.0 nM. A total volume of 400 μl was incubated in the presence of 50 mM Tris and 10 mM MgCl₂ at pH 7.6. Displacing drugs were dissolved in the incubation mixture (in the presence of ascorbic acid where appropriate).
Disposable polypropylene tubes (Sarstedt) were used for all incubations. Incubation was always in triplicate and was started by addition of membranes, except in protein dilution assays when addition of ligand started the incubation. Incubation was for 10 minutes, or for varying times up to 15 minutes when the time course of association was being studied, and was always at 37°C. Incubation was terminated by the addition of 3 ml ice-cold 50 mM Tris buffer at pH 7.6 followed immediately by filtration under vacuum through GF/B glass microfibre filters (Whatman); a Millipore 1225 sampling manifold was used. Assay tubes were washed with 3 ml ice-cold buffer and the filters washed with a further 2 × 5 ml ice-cold buffer.

Filters were placed in disposable scintillation vials (Packard) and 10 ml of Pico-fluor 30 scintillation fluid added. Bound $[^3\text{H}]$-DHE was counted in a scintillation counter (Mark III, Model 688, Tracor Analytic). Aliquots of $[^3\text{H}]$-DHE were counted to allow calculation of the concentration of ligand present in incubations.

$[^{125}\text{I}]-\text{Iodocyanopindolol}$

An aliquot of liver membrane containing 0.10 - 0.15 mg protein was incubated with $[^{125}\text{I}]-\text{ICYP}$ at ligand concentrations in the range of 12-500 nM for saturation assays and 70-115 nM for time course and displacement assays. A total volume of 150 μl was incubated in the presence of 120 mM Tris, 5 mM MgSO$_4$ and 1.3 mM EDTA at pH 7.6. Displacing drugs were dissolved in the incubation medium (in the presence of ascorbic acid where appropriate). Specific binding was defined as that binding displaced by 2 × 10$^{-6}$ M isoprenaline.

Disposable polypropylene tubes (Sarstedt) were used for all incubations. Incubation was always in triplicate, and was started by addition of membranes. Incubation was for 30 minutes, or for varying times up to 45 minutes when the time
course of association was being studied, and was at 37°C. Incubation was
terminated by addition of 1 ml of ice-cold washing buffer (10 mM Tris, 5 mM
MgCl₂, pH 8.0) followed by immediate filtration under vacuum through GF/B glass
microfibres (Whatman); a Millipore 1225 sampling manifold was used. Assay tubes
were washed with 2 x 1 ml ice-cold washing buffer and the filters washed with a
further 2 x 5 ml ice-cold washing buffer.

Filters were folded into semi-circles and placed in disposable polypropylene
tubes (Sarstedt). Bound [¹²⁵I]-ICYP was counted in a gamma-counter (Model
1185R, Searle Analytic Inc.). Aliquots of [¹²⁵I]-ICYP were placed in 2 ml
polystyrene tubes, capped and counted to allow calculation of the concentration of
ligand present in incubation.

[¹²⁵I] Iodopindolol

[¹²⁵I]-IP binding was assayed using a method adapted from that of
McMillian et al. (1983). An aliquot of liver membrane containing 0.09 - 0.40 mg
protein was incubated with [¹²⁵I]-IP at ligand concentrations in the range 10 -
600 pM for saturation assays and 40 - 90 pM for all other assays. A total volume of
150 µl was incubated in the presence of 145 mM NaCl, 20 mM Tris, 2 mM MgCl₂
and 1 mM ascorbic acid at pH 7.5. Displacing drugs were dissolved in the incubation
mixture. Specific binding was defined as that binding displaced by 2 x 10⁻⁶M
isoprenaline. In some saturation assays 5 x 10⁻⁷M atenolol or 5 x 10⁻⁸M ICI 118 551
was present; these concentrations were calculated to occupy 90 - 100% of β₁ or β₂
adrenoceptors respectively (Cook et al., 1984).

Disposable polypropylene tubes (Sarstedt) were used for all incubations.
Incubation was at 25°C, was always in triplicate and was started by addition of
membranes, except in protein dilution assays, when addition of ligand started the
incubation. Incubation was for 45 minutes when protein dilution profiles, saturation
assays or inhibition assays were being performed, for varying times by up to 45 minutes when the time course of association was being studied, or for 45 minutes followed by varying times up to 20 minutes after addition of isoprenaline to give a concentration of $5 \times 10^{-6} \text{M}$ when the dissociation time course was being studied. Incubation was terminated by addition of 2 ml ice-cold washing buffer (145 mM NaCl, 10 mM Tris, pH 7.5), followed by immediate filtration under vacuum through GF/B glass microfibre filters (Whatman); a Millipore 1225 sampling manifold was used. Assay tubes were washed with 2 ml ice-cold washing buffer and filters were washed with a further 2 x 5 ml ice-cold buffer.

Filters were folded into semi-circles and placed in disposable polypropylene tubes (Sarstedt). Bound [$^{125}\text{I}$]-IP was counted in a gamma counter (Model 1185R, Searle Analytic Inc.). Aliquots of [$^{125}\text{I}$]-IP were placed in 2 ml polystyrene tubes, capped and counted to allow calculation of the concentration of ligand present incubations.

2.2.5 ANALYSIS OF DATA

All statistical analyses were carried out using paired Student's t-test.
2.3 RESULTS

2.3.1 \(^{3}\text{H} \) -DIHYDROALPENOL binding assay

\(^{3}\text{H} \) -DHA binding assays were carried out using liver membranes prepared by each of the methods detailed in the 'Materials and Methods' section. Adult male, adult female, young adult male, weanling male, near-term fetal, and catcholamine-depleted adult female rat liver membranes were investigated. No difference was apparent in results obtained when using liver membranes prepared by the various methods or when animals of different ages or sexes were used. Similar results were obtained with either freshly-prepared liver membranes or membranes used after storage at -60\(^\circ\)C.

Non-specific binding was extremely high in all assays, usually representing between 60 and 70% of total binding, and never being less than 40%.

Protein dilution profile

Total binding of \(^{3}\text{H} \) -DHA increased with increasing protein concentration over the range studied, but correlation of specific binding and protein concentration was generally poor due to non-specific binding being high and variable.

Association time course

Examination of the association time course showed a typical hyperbolic increase in total ligand binding over the 20 minutes studied. However, non-specific binding was high and showed an erratic increase with time and thus specific binding was low and did not show a typical association time course (Fig. 2.2).

Dissociation time course

Due to poor results in other assays, this assay was not attempted with this radioligand.
FIGURE 2.2  
Time course of association of $[^3\text{H}]$-dihydroalprenolol

Total binding (□—□) and non-specific binding (△—△) were determined at each time point; specific binding (▲—▲) was calculated as the difference between these (see text for details).
Inhibition profile

Displacement curves with isoprenaline, adrenaline, and noradrenaline gave inconsistent results. The range of $K_i$ values obtained are given in Table 2.1, but these were highly variable, and on some occasions no $K_i$ value could be obtained due to the variable degree of binding which could be defined as specific.

Saturation assay

Only one saturation assay was attempted with $[^3]$-DHA. Total, non-specific and specific binding all increased with increasing $[^3]$-DHA concentration, but the high and variable nature of the non-specific binding made any analysis of the data impossible.

Investigation of high non-specific binding

In an attempt to reduce the high non-specific binding, the concentration of phentolamine included in the assay was varied between 0.05 and 1.0 mM; a concentration of 0.1 mM phentolamine resulted in the greatest reduction in non-specific binding. EDTA at concentrations of 0.5 or 1.0 mM had no consistent effect on binding.

To investigate whether the high non-specific binding was due to binding of $[^3]$-DHA to filters, a single assay was carried out by the centrifugation method, in which ligand bound to membrane is separated from free ligand by rapid centrifugation to form a membrane pellet. Using this method, non-specific binding was never less than 95% of total; this assay method was obviously unsuitable. This finding suggests that it is unlikely that binding of the ligand to filters was the cause of the high non-specific binding found using the filtration technique.
### TABLE 2.1

$[^3H]$-DHA displacement from rat liver membranes

<table>
<thead>
<tr>
<th>Displacing drug</th>
<th>Experimental results</th>
<th>Literature values$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)isoprenaline</td>
<td>13.5 - 52.0</td>
<td>12 - 60</td>
</tr>
<tr>
<td></td>
<td>(n = 3)</td>
<td></td>
</tr>
<tr>
<td>(-)adrenaline</td>
<td>397</td>
<td>120 - 484</td>
</tr>
</tbody>
</table>

Values obtained from inhibition profiles (see text for details).

$^a$ Aggerbeck et al., 1978 and 1980; Dax et al., 1981; Guellaen et al., 1978; McMillian et al., 1983; Malbon, 1980 and 1981; Munnich et al., 1981.

### TABLE 2.2

$[^3H]$-DHA binding data in cat ventricle membranes

<table>
<thead>
<tr>
<th></th>
<th>Experimental results - cat heart</th>
<th>Published values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cat heart$^a$</td>
</tr>
<tr>
<td>$K_D$ (nM)</td>
<td>1.9, 1.5</td>
<td>4.34</td>
</tr>
<tr>
<td>$B_{max}$ (fmol/mg protein)</td>
<td>29.3, 26.6</td>
<td>80 ± 6</td>
</tr>
</tbody>
</table>

Values obtained from saturation assays (see text for details).


$^b$ Dax et al., 1981; Kawai and Arinze, 1983; Minneman and Molinoff, 1980; Munnich et al., 1981.

NA Not applicable
To investigate the possibility that uptake of the ligand by the incubation tube was leading to high non-specific binding, a fixed amount of ligand in incubation buffer was placed in the tube and incubated for varying times up to 40 minutes at 30°C. A fixed aliquot was removed from the tube at the end of the incubation, and counted. The absence of any dependence of the recoverable radioactivity on the length of incubation confirms that there is no significant uptake of [3H]-DHA by the tube.

In order to ascertain whether the problems encountered with [3H]-DHA binding assays in liver membranes were due to the nature of liver tissue or to poor experimental technique, [3H]-DHA binding assays were carried out using membranes prepared from cat ventricle by method D described in section 2.2.3 of Materials and Methods. Using cat heart membrane, non-specific binding was still high, but was always less than 50%, and in saturation assays increased linearly with increasing [3H]-DHA concentration. Results of Scatchard analysis of [3H]-DHA saturation binding data in cat ventricle membranes are given in Table 2.2. K_D values found were somewhat lower than the only published value found for this ligand in heart membranes (Sharma and Corr, 1983) but were of the same order as published values for other tissues and were in close agreement with values found for this tissue by other workers in the Biochemical Pharmacology Department of Glaxo Research Ltd (unpublished results). B_max values were considerably lower than the value reported by Sharma and Corr (1983) for this tissue, but were in good agreement with results obtained previously by other works at Glaxo.

### 2.3.2 [3H]-DIHYDROERGOCRYPTINE BINDING ASSAY

[3H]-DHE binding assays were carried out using liver membranes prepared by method A in section 2.2.3 of Materials and Methods. Near-term fetal and day 7 post partum neonatal liver membranes were used. No difference was apparent in
results obtained from fetal or neonatal animals. Similar results were obtained with freshly-prepared liver membranes or membranes used after storage at -60°C.

**Protein dilution profile**

Total binding of $[^3H] - $DHE to liver membranes increased proportionally with increasing protein or ligand concentration. However, failure to differentiate specific and non-specific binding in this assay system makes interpretation of these results impossible. Major problems arose in the $[^3H] - $DHE assay in defining any binding as specific. Phentolamine (a non-subtype-selective α-antagonist) is most commonly used to define specific binding in $[^3H] - $DHE binding assays reported in the literature, either at 10 μM (Clarke et al., 1978; El-Refai & Exton, 1980; Hoffmann et al., 1979) or at 100 μM (Aggerbeck et al., 1978; Butlen et al., 1980; Guellaen et al., 1978). In the experimental work reported here, inclusion of drugs with known affinity for α-receptors resulted, in the majority of instances, in an increase in ligand binding to liver membranes.

**Investigation of the effects of α-antagonists on binding**

Investigation of the effect varying phentolamine concentrations showed a concentration-dependent change in the binding of $[^3H] - $DHE to fetal or neonatal liver membranes. In one experiment, $10^{-7} - 10^{-5}$ M phentolamine displaced $[^3H] - $DHE, but the % displacement decreased with increasing drug concentration and a maximum displacement of 20% was obtained with $10^{-7}$ M; with phentolamine concentrations of $10^{-5} - 10^{-3}$ M, ligand binding increased proportionally with increasing phentolamine concentration and was about 160% of control at the top phentolamine concentration (Fig. 2.3). On repeating this experiment, only 1% ligand displacement was seen at $10^{-7}$ M phentolamine, and a concentration-related increase in binding, with a maximum level of 163% of control, was seen for phentolamine concentrations up to $10^{-3}$ M (Fig. 2.3).
Findings from two experiments are shown (see text for details).

Findings from two typical experiments are shown (see text for details).
The effects of varying concentrations of the α₂-antagonist yohimbine were also studied. This drug increased \([^{3}\text{H}]\)-DHE binding to liver membranes in a concentration-dependent manner at low concentrations, but at higher concentrations ligand displacement was seen which was proportional to the level of yohimbine in the incubation. Increases up to 120% and decreases down to 55% of control binding levels were observed, but the concentrations of yohimbine causing these changes varied from experiment to experiment. However, yohimbine above \(10^{-4}\text{M}\) always resulted in a displacement of \([^{3}\text{H}]\)-DHE from liver membranes, and yohimbine below \(10^{-6}\text{M}\) always resulted in an enhancement of \([^{3}\text{H}]\)-DHE binding to liver membranes. The binding changes observed were always concentration-related (Fig. 2.4).

2.3.3 \([^{125}\text{I}]\)-IODOCYANOPINDOLOL BINDING ASSAY

\([^{125}\text{I}]\)-ICYP binding assays were carried out using adult female liver membranes prepared by the method B described in section 2.2.3 of Materials and Methods, and day 7 post partum neonatal liver membranes prepared by method A described in section 2.2.3 of Materials and Methods. No difference was apparent in results obtained from neonatal or adult tissues. Similar results were obtained with freshly-prepared tissue or tissue used after storage at -60°C. Non-specific binding was typically 15-30% of total binding.

Inhibition profile

A normal inhibition profile was obtained with isoprenaline in one experiment; analysis of this gave a \(K_i\) for isoprenaline of 2.7 nM. However, a second displacement assay showed very irregular displacement.

Saturation assay

Scatchard analysis of saturation binding data obtained in one experiment gave
a $K_D$ for $^{125}\text{I}$-ICYP of 170 pM, which is high compared with published values, which range from 13 to 66 pM (Brodde, 1982; Brodde et al., 1981; Engel et al., 1981; Heitz et al., 1983; Wolfe & Harden, 1981; Zini et al., 1983) and on repeating the assay the data obtained showed a scattered distribution on a Scatchard plot.

**Association time course**

The time course of association of $^{125}\text{I}$-ICYP to liver membranes was investigated, as the inconsistency of the results obtained might have resulted from reactions not reaching equilibrium. It was observed that although the rate of increase of binding was fairly low by 25 minutes, a small increase in total and specific binding could still be seen up to 45 minutes. A typical graph of the association time course is shown in Figure 2.5.

**Dissociation time course**

Due to poor results in the association time course, this assay was not attempted with this radioligand.

**2.3.4 $^{125}\text{I}$-IODOPINDOLOL BINDING ASSAY**

$^{125}\text{I}$-IP binding assays were carried out using liver membranes from day 20, 21, and 22 post coitum fetal rats and 24 and 48 hours post partum neonatal rats, prepared as detailed in method A of section 2.2.3 of Materials and Methods. Similar results were obtained with freshly-prepared tissue or with tissue used after storage at -60°C.

Non-specific binding was low at all times in this assay, representing 5-15% of total binding in a typical saturation assay.

Protein dilution profiles, the time course of association and dissociation,
FIGURE 2.5

Time course of association of $^{125I}$ iodocyanopindolol

Total binding (□—□) and non-specific binding (△—△) were determined at each time point; specific binding (▲—▲) was calculated as the difference between these (see text for details).

FIGURE 2.6

Protein dilution profile of $^{125I}$ iodopindolol binding

Total binding (□—□) and non-specific binding (△—△) were determined at each protein concentration; specific binding (▲—▲) was calculated as the difference between these (see text for details).
inhibition profiles for a range of adrenergic agents and investigation of the inhibitory effects of compounds with affinity for receptors other than the β-adrenoceptor, were carried out using membranes from day 20 post coitum fetuses. Limited time and ligand availability prevented a complete range of assays from being carried out at all ages, but dissociation constants for displacing agents (Kᵢ) obtained from inhibition profiles, and dissociation constants for [¹²⁵I]-IP (Kᵢ) obtained from saturation assays (Tables 2.3 and 2.4), confirmed that it was reasonable to assume that [¹²⁵I]-IP was binding to the same receptor population at each age.

Protein dilution profile

Total and specific ligand binding increased linearly with increasing protein concentration over the protein concentration range studied; non-specific binding also increased linearly with protein concentration, but was always < 10% of total binding in protein dilution profiles (Fig. 2.6).

Association time course

The time course of association of [¹²⁵I]-IP with day 20 post coitum fetal liver membranes showed a typical hyperbolic increase of total and specific binding, reaching a plateau at around 25 minutes; non-specific binding showed a sharp increase in the first minute, and then a slight linear increase over the remainder of the time studied (Fig. 2.7).

The association of a radioligand with its receptor is a second order reaction; however, if less than 10% of the ligand present is specifically bound at equilibrium, the reaction becomes pseudo-first order, and is described by the rate equation:
### TABLE 2.3

<table>
<thead>
<tr>
<th>Displacing drug</th>
<th>Age:</th>
<th>K&lt;sub&gt;I&lt;/sub&gt; (nM)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)Isoprenaline</td>
<td>210 (n=2)</td>
<td>-</td>
</tr>
<tr>
<td>(+)Adrenaline</td>
<td>350 (n=2)</td>
<td>-</td>
</tr>
<tr>
<td>(+)Noradrenaline</td>
<td>5100</td>
<td>-</td>
</tr>
<tr>
<td>Atenolol</td>
<td>14000 (n=2)</td>
<td>14900</td>
</tr>
<tr>
<td>ICI 118551</td>
<td>5.6 ± 2.4 (n=3)</td>
<td>6.1 (n=2)</td>
</tr>
</tbody>
</table>

Values obtained from inhibition profiles (see text for details).

<sup>a</sup> K<sub>I</sub> is shown as mean ± SEM.
TABLE 2.4  
[\textsuperscript{125}I]-IP binding data in perinatal rat liver membranes: $K_D$

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>$66.4 \pm 26.0$ (n=4)</td>
<td>$85.8 \pm 4.7$ (n=3)</td>
<td>$81.2 \pm 19.7$ (n=3)</td>
<td>$72.9 \pm 10.6$ (n=3)</td>
<td>$69.9 \pm 5.2$ (n=3)</td>
</tr>
<tr>
<td>$\beta$-Receptors</td>
<td>$94.7$ (n=2)</td>
<td>$81.8 \pm 5.5$ (n=3)</td>
<td>$86.2 \pm 20.9$ (n=3)</td>
<td>$101.7 \pm 19.4$ (n=3)</td>
<td>$76.4 \pm 6.1$ (n=3)</td>
</tr>
</tbody>
</table>

Values obtained from saturation assays (see text for details).

$K_D$ is shown as mean ± SEM.

TABLE 2.5  
[\textsuperscript{125}I]-IP binding data in perinatal rat liver membranes: $B_{max}$

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>$13.0 \pm 2.6$ (n=4)</td>
<td>$18.3 \pm 1.3$ (n=3)</td>
<td>$30.4 \pm 2.4$ (n=3)</td>
<td>$21.6 \pm 2.4$ (n=3)</td>
<td>$19.9 \pm 1.2$ (n=3)</td>
</tr>
<tr>
<td>$\beta$-Receptors</td>
<td>$12.3$ (n=2)</td>
<td>$15.3 \pm 2.0$ (n=3)</td>
<td>$28.5 \pm 1.6$ (n=3)</td>
<td>$19.8 \pm 1.7$ (n=3)</td>
<td>$18.5 \pm 3.7$ (n=3)</td>
</tr>
<tr>
<td>$\beta_2$-Receptors</td>
<td>$12.3$ (n=2)</td>
<td>$15.3 \pm 2.0$ (n=3)</td>
<td>$28.5 \pm 1.6$ (n=3)</td>
<td>$19.8 \pm 1.7$ (n=3)</td>
<td>$18.5 \pm 3.7$ (n=3)</td>
</tr>
</tbody>
</table>

Values obtained from saturation assays (see text for details).

$B_{max}$ is shown as mean ± SEM.

* Significantly different from Day 22 p.c., $p < 0.05$
** Significantly different from Day 22 p.c., $p < 0.01$
*** Significantly different from Day 22 p.c., $p < 0.005$
**** Significantly different from Day 22 p.c., $p < 0.001$
Total binding (○—○) and non-specific binding (△—△) were determined at each time point; specific binding (▲—▲) was calculated as the difference between these. The inset shows a linear representation of the data (see text for details).
\[ \ln \left( \frac{B_e}{B_{e-Bt}} \right) = k_1 \cdot t \left( \frac{L \cdot B_{max}}{B_e} \right) \]

Where:
- \( B_e \) = concentration of ligand specifically bound at equilibrium (moles per mg protein)
- \( B_{t} \) = concentration of ligand specifically bound at time \( t \) (moles per mg protein)
- \( L \) = concentration of radioligand at zero time (M)
- \( B_{max} \) = receptor concentration (moles of ligand specifically bound per mg protein)
- \( k_1 \) = association rate constant.

From this equation, it can be seen that if \( \ln \left( \frac{B_e}{B_{e-Bt}} \right) \) is plotted against time (in seconds), a straight line is obtained of gradient \( k_1 \cdot \left( \frac{L \cdot B_{max}}{B_e} \right) \) (Fig 2.7 inset).

When the association time course data shown in Figure 2.7 was expressed in this way an association rate constant \( (k_1) \) of \( 1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) was obtained.

**Dissociation time course**

When isoprenaline was added to an incubation mixture containing membrane from day 20 post coitum livers with \( [ \text{I}^{125} \text{I}] \)-IP bound at equilibrium, such that a concentration of isoprenaline of \( 5 \times 10^{-6} \text{ M} \) was achieved, the ligand was displaced from the membrane in a time-dependent manner (Fig. 2.8), with 50% of the specifically bound ligand displaced in approximately 12 minutes. The dissociation of a radioligand from its receptor is a first order reaction described by the equation

\[ \log \left( \frac{B_e}{B_t} \right) = \frac{k_{-1} \cdot t}{2.303} \]
FIGURE 2.8 Time course of dissociation of \(^{125}\text{I}\) - Iodopindolol

Legend as for Figure 2.7
Where \( B_e \) = concentration of ligand specifically bound at equilibrium (moles per mg protein)

\( B_t \) = concentration of ligand specifically bound at time \( t \) (moles per mg protein)

\( k_{-1} \) = dissociation rate constant.

Thus, if \( \log \left( \frac{B_e}{B_t} \right) \) is plotted against time (in seconds), a straight line is obtained of gradient \( \frac{-1}{2.303} \) (Fig. 2.8 inset). When the dissociation time course data shown in Figure 2.8 was expressed in this way, a dissociation rate constant of \( 9.1 \times 10^{-4} \text{ s}^{-1} \) was obtained.

The half time of dissociation may be calculated from \( k_{-1} \) as follows:

\[
t_{0.5} = \frac{0.693}{k_{-1}} = 13 \text{ min}
\]

**Calculation of dissociation constant from rate constants**

Using the values determined for \( k_1 \) and \( k_{-1} \) the dissociation constant \( (K_D) \) may be obtained:

\[
K_D(M) = \frac{k_{-1} \text{ (s}^{-1}\text{)}}{k_1 \text{ (M}^{-1}\text{s}^{-1})}
\]

The dissociation constant determined from kinetic analysis is therefore

\[
\frac{9.1 \times 10^{-4}}{1.5 \times 10^7} \text{ (s}^{-1}\text{ M}^{-1}\text{s}^{-1}) = 6.07 \times 10^{-11} \text{ (M)}
\]

or 60.7 pM
Inhibition profile

Compounds with known affinity for β-adrenoceptors displaced $[^{125}\text{I}]$-IP from fetal and neonatal liver membranes in a concentration-dependent manner and when a full range of compounds was investigated using day 20 post coitum membranes, these showed relative potencies consistent with $\beta_2$-adrenoceptor binding (Table 2.3) (for review, see Lefkowitz and Hoffman, 1981a).

A typical sigmoidal displacement curve of $[^{125}\text{I}]$-IP by isoprenaline, and the Hofstee plot of these data, are shown in Figure 2.9. Displacement curves obtained using the $\beta_1$-subtype selective antagonist atenolol or the $\beta_2$-subtype-selective antagonist ICI 118 551 showed slight inflections, and Hofstee plots of the same data were curved (e.g. Fig. 2.10); the significance of these findings will be considered in the Discussion section of this Chapter.

As a further confirmation that $[^{125}\text{I}]$-IP was binding β-adrenergic receptors, compounds with pharmacological activity other than for β-adrenoceptors were investigated for their ability to displace the ligand. The following antagonistic compounds (the receptors at which they act is given in brackets) had no significant effect on $[^{125}\text{I}]$-IP binding when present at $10^{-6}$ or $10^{-5}$M: phentolamine ($\alpha$-adrenoceptor), ketanserin (5HT receptor), atropine sulphate (muscarinic cholinergic receptor), spiperone ($D_2$ dopamine receptor), mepyramine ($H_1$ histamine receptor), ranitidine ($H_2$ histamine receptor).

Saturation assay

A typical saturation profile of $[^{125}\text{I}]$-IP binding is shown in Figure 2.11, together with the Scatchard plot of these data. $K_D$ and $B_{max}$ values obtained by Scatchard analysis of saturation assays are shown in Tables 2.4 and 2.5. Values for $\beta_2$-receptors' were obtained using data from assays in which $5 \times 10^{-7}$M atenolol
**FIGURE 2.9**

Inhibition profile of $[^{125}\text{I}]$-iodopindolol binding by isoprenaline

The data shown are from a typical experiment. The inset shows the Hofstee plot of the data (see text for details).
FIGURE 2.10  Inhibition profile of [125I] - iodopindolol binding

(a)  Atenolol

(b)  ICI 118 551

Legend as for Figure 2.9
The data shown are for a typical experiment. Total binding (□—□) and non-specific binding (△—△) were determined at each concentration of radioligand; specific binding (∗—∗) was calculated as the difference between these. The inset shows a Scatchard plot of the data (see text for details).
(calculated to displace 90-100% of \( \beta_1 \)-adrenoceptor-bound \([^{125}\text{I}]\)-IP) was included in all incubations. The inclusion of ICI 118 551 at 5 \( \times \) \( 10^{-8} \) M (calculated to displace 90-100% of \( \beta_2 \)-adrenoceptor-bound ligand) resulted in very low binding levels and very high \( K_D \) values for \([^{125}\text{I}]\)-IP, and saturation assays to obtain \( K_D \) and \( B_{\text{max}} \) values for \( \beta_1 \)-adrenoceptors were therefore not attempted. \( K_D \) values obtained ranged from 66.4 pm (at day 20 post coitum) to 85.8 pm (at day 21 post coitum) for total \( \beta \)-receptors and from 76.4 pm (at 48 hours post partum) to 101.7 pm (at 24 hours post partum). No statistically significant difference was found for the \( K_D \) value obtained for total \( \beta \)-adrenoceptors, compared with \( \beta_2 \)-adrenoceptors, at any age studied, or for the \( K_D \) value obtained at different perinatal ages, and there was no obvious age-related trend in the \( K_D \) values obtained. The variation in \( K_D \) observed was probably due to the small numbers of assays performed at each age. \( B_{\text{max}} \) values, however, showed a very clear age-dependent variation. Both total \( \beta \)- and \( \beta_2 \)-adrenoceptors reached a maximum concentration at term (day 22 post coitum), with \( B_{\text{max}} \) values for \( \beta \)- and \( \beta_2 \)-adrenoceptors at all other ages studied being statistically significantly less than the \( B_{\text{max}} \) value at day 22 post coitum (Table 2.5).
2.4 DISCUSSION

2.4.1 OVERVIEW

$^{125}$I-Iodopindolol proved a suitable ligand for labelling β-adrenoceptors in fetal and neonatal liver membranes. The high specific activity and the selective nature of $^{125}$I-IP probably accounts for the successful use of this ligand. Attempts to quantify rat hepatic adrenoceptors using $^3$H-dihydroalprenolol or $^{125}$I-iodocyanopindolol for β-receptors or $^3$H-dihydroergocryptine for α-receptors, were unsuccessful. It seems probable that the relatively small number of publications concerning radioligand labelling of hepatic adrenoceptors, commented on by Kawai & Arinze (1983), reflects the difficulties encountered with these more commonly used radioligands in the liver.

Four methods of preparation of liver membranes for use in binding assays with $^3$H-DHA were investigated. None of these methods resulted in preparations showing reduced non-specific binding of $^3$H-DHA. Method A detailed in section 2.2.3 was used to prepare membranes for the majority of the work reported in this chapter, including all of that using $^{125}$I-iodopindolol. This is a fairly crude method of preparation, but gives the highest yield of membrane protein per unit weight of liver, and avoids the problem of variation of recovery of membrane protein with age which can occur using more extensive purification methods (McMillian et al., 1983).

2.4.2 $^3$H-DIHYDROALPRENOLOL BINDING ASSAY

Although Aggerbeck et al. (1978), Guellaen et al. (1978) and Munnich et al. (1981) have described the use of $^3$H-DHA to characterise β-adrenoceptors in adult rat liver membranes, an acceptable binding assay could not be established in the present experimental work, even when the reported methodology was followed closely. Reports concerning the problems of using $^3$H-DHA in binding assays
have appeared in the literature; however, none of the sources of error discussed in these publications appears to provide an explanation of the findings reported here. Nahorski & Richardson (1979) described how the use of the β-adrenergic antagonists (-)alprenolol or (-)propranolol for the determination of the specific element of the binding of \[^3H\]DHA to bovine lung membrane resulted in displacement of the radioligand from more than one binding site. The β-adrenergic agonist (-)isoprenaline was used to determine specific binding in the assays using \[^3H\]DHA reported in this chapter; Nakorski & Richardson showed this compound to displace \[^3H\]DHA from a single, homogeneous binding site. The occurrence of binding of \[^3H\]DHA to membrane sites from which it may be displaced nonstereospecifically has been described in cardiac tissue (Krawietz & Erdmann, 1979) and in liver tissue (Dax et al., 1981). Dax et al. (1981) concluded that quantitation of liver β-adrenergic receptors in normal rats is impossible using \[^3H\]DHA; however these workers found that two relevant factors influenced the incidence of sites binding \[^3H\]DHA in a nonstereospecific manner: animal age and the presence of the α-adrenergic antagonist phentolamine. The nonstereospecific element of radioligand binding increased with age, and was therefore probably of relatively minor significance in the liver membranes used in those experiments discussed here, in which membranes were prepared from rats during the perinatal period, but problems were still encountered with these preparations. Dax and coworkers found that addition of phentolamine at a concentration of 5 x 10^{-6}M or higher eliminated a large proportion of the nonstereospecifically displaceable binding; the assays carried out with \[^3H\]DHA in the current work all included phentolamine at 10^{-4}M. As the values for \[^3H\]DHA binding parameters in cat ventricle membranes were of the order expected, it would appear that the failure to obtain satisfactory binding data for \[^3H\]DHA in rat liver membranes is due to the nature of the tissue rather than to any technical problems.
Findings made when investigating \(^{3}H\)-DHE binding to liver membranes were unexpected; there is no report in the literature of \(\alpha\)-antagonists enhancing \(^{3}H\)-DHE binding. Butlen \textit{et al.} (1980) used \(^{3}H\)-DHE to label hepatic \(\alpha\)-adrenoceptors in tissue from perinatal rats, and reported no difficulties, although non-specific binding did represent up to 40\% of total binding. No displacement curves were shown in this paper. Aggerbeck \textit{et al.} (1978, 1980), Clarke \textit{et al.} (1978) and Guellaen \textit{et al.} (1978) investigated \(\alpha\)-adrenoceptors in liver membranes from adult rats using \(^{3}H\)-DHE, and presented plots showing dose-related displacement of \(^{3}H\)-DHE by \(\alpha\)-adrenergic agents. Non-specific binding was 20-25\% of total in the assays reported by Aggerbeck and Guellaen and coworkers, and was reported to be 35-40\% of total by Clarke's group.

No obvious explanation can be found as to why the present results differ so greatly from those published, however the concentration-dependence of the effects of phentolamine and yohimbine in increasing \(^{3}H\)-DHE binding together with the effects of phentolamine on glucose production by perinatal hepatocytes \textit{in vitro} (see Chapter 3), suggest that the effects seen were not simply artefactual. Increased ligand binding due to conformational changes in \(\alpha\)-receptors following binding of the \(\alpha\)-antagonists to adjacent adrenoceptors is one possible explanation for the observed phenomenon, but no evidence is available of such cooperativity. The possible involvement of nucleotides in affinity changes must also be considered. Reduction in the affinity of agonists for \(\beta\)-adrenoceptors, resulting from the binding of guanine nucleotides to the receptor, was first recognised by Rodbell \textit{et al.} (1971) and is now well documented (for review see Lefkowitz & Hoffman, 1981a). Goodhardt \textit{et al.} (1982) reported a similar regulation of agonist binding to hepatic \(\alpha_{1}\)-adrenoceptors, and Hoffman \textit{et al.} (1980, 1981) to hepatic \(\alpha_{2}\)-adrenoceptors by guanyl nucleotides. GTP may be included in a binding assay to
ensure a constant high level of nucleotide; although it was not included in the $^3$H-DHE binding assay, the ligand and displacing drugs were all antagonists. Nucleotides have been widely shown to have no effect on the affinity of antagonists for $\beta$-adrenoceptors (Lefkowitz & Hoffman, 1981a). Although Wolfe & Harden (1981) reported an increase in the affinity of antagonists for $\beta$-adrenoceptors by GTP, no confirmation of this observation has appeared in the literature.

2.4.4 $^{125}$I-IODOCYANOPINDOLOL BINDING ASSAY

Initial work with $^{125}$I-ICYP produced promising results, but gave an unexpectedly high $K_D$ value for the ligand, a rather low $K_i$ value for isoprenaline, and replication of findings was poor. The association time course showed that specific binding of $^{125}$I-ICYP had not reached a plateau after 45 minutes of incubation (Fig. 2.7), and thus it is likely that failure to reach equilibrium during the time of the assay was responsible for the poor results obtained. Engel et al. (1981) and Brodde et al. (1981) found that $^{125}$I-ICYP binding reached equilibrium only after one hour; even longer delays in attaining equilibrium have been encountered by other workers using this ligand (S. Nahorski, personal communication). Engel et al. (1981) have also reported a slow component of the dissociation of this ligand with less than 50% dissociation in eight hours. The large cyano grouping on the $^{125}$I-ICYP molecule may impede its access to the binding site on the $\beta$-adrenoceptor, thus increasing the time to reach equilibrium. This delay in reaching equilibrium must produce some doubt as to the physiological significance of findings made with $^{125}$I-ICYP.

2.4.5 $^{125}$I-IODOPINDOLOL BINDING ASSAY

Summary of findings

$^{125}$I-IP has been described as a useful radioligand for characterising $\beta$-adrenoceptors in tissues in which other ligands have proved unsuitable (Ezrailson et
The binding of \( ^{125}I \)-IP to perinatal rat liver membranes was rapid, saturable, reversible and showed displacement characteristics typical of binding to \( \beta \)-adrenoceptors. This radioligand showed no selectivity for \( \beta_1 \)- or \( \beta_2 \)-adrenoceptors. The reaction between \( ^{125}I \)-IP and its binding site was characterised as a second order reaction between a univalent, homogeneous ligand and a homogeneous, non-interacting receptor population by the linearity of the Scatchard plots (mean ± SEM of regression coefficients = 0.99 ± 0.01) and the proximity of the Hill coefficient to unity (mean ± SEM of Hill coefficients = 1.00 ± 0.1) (Weiland & Molinoff, 1981). The dissociation constant (\( K_D \)) was very similar whether obtained by Scatchard analysis of saturation assays (mean ± SEM = 75 ± 7pM) or by kinetic analysis (61 pM) and was of the same order as published values, which range from 30-100 pM, (Brooker and Barovsky, 1981; Wolfe and Harden, 1981; Moylan et al., 1982; Hedberg et al., 1983; McCarthy, 1983; McMillian et al., 1983; Moretti-Rajas et al., 1983), although only one of these published values (68 ± 4 pM) was obtained in liver (McMillian et al., 1983).

**Type and subtype of adrenoceptors**

The classification of adrenergic receptors is discussed in Chapter 1 (section 1.3.1). As potency orders are used in the classification of adrenoceptors, and the affinity of a compound for a receptor is a factor in the potency of that compound, the relative abilities of adrenergic agents in displacing a radioligand from an adrenoceptor indicate the type and subtype of the receptor. The potency order of adrenergic agonists in displacing \( ^{125}I \)-IP bound to membranes prepared from fetal rat liver on day 20 post coitum (Table 2.3) was characteristic of binding to \( \beta_2 \)-adrenoceptors. The relative abilities of the \( \beta_1 \)-adrenergic antagonist, atenolol, and the \( \beta_2 \)-adrenergic antagonist, ICI 118 551 to displace \( ^{125}I \)-IP confirm that radioligand binding was to predominantly \( \beta_2 \)-adrenoceptors. However, the observation of inflections in displacement curves obtained with subtype-selective
\(\beta\)-antagonists together with non-linearity of Hofstee plots of such displacement data (Fig. 2.10) suggest that binding was to both subtypes of \(\beta\)-adrenoceptors, although \(\beta_2\)-adrenoceptors were far more numerous.

**Size of adrenoceptor population**

A number of approaches were used to assess the relative population sizes of the receptor subtypes. As described in Section 2.2.4 of Materials and Methods, some saturation assays with \(\text{[}^{125}\text{I}]\)-IP were performed in the presence of concentrations of the \(\beta_1\)-adrenergic antagonist atenolol or the \(\beta_2\)-adrenergic antagonist ICI 118 551 calculated to displace \(\text{[}^{125}\text{I}]\)-IP from more than 90% of \(\beta_1\)- or \(\beta_2\)-adrenoceptors respectively, so that the \(\beta\)-adrenoceptor population being assayed was effectively purely \(\beta_2\) or \(\beta_1\). However, when ICI 118 551 was included at \(5 \times 10^{-8}\text{M}\), such a large proportion of the receptors was occupied by this \(\beta_2\)-antagonist that \(\text{[}^{125}\text{I}]\)-IP binding characteristics were totally altered and no significant data could be obtained. The binding characteristics of the radioligand did not appear to be significantly affected by blocking \(\beta_1\)-adrenoceptors with \(5 \times 10^{-7}\text{M}\) atenolol, as evidenced by the lack of significant difference in \(K_D\) values obtained in the absence or presence of the \(\beta_1\)-antagonist (Table 2.4). By comparing \(B_{\text{max}}\) values obtained in saturation assays performed with and without the inclusion of atenolol, the proportion of the total \(\beta\)-adrenoceptor population which consisted of \(\beta_1\)-adrenoceptors could be calculated (Table 2.6, column 1). An alternative approach was to use a computerised curve-fitting program for the analysis of the inhibition profiles of \(\text{[}^{125}\text{I}]\)-IP resulting from incubating the radioligand with liver membranes in the presence of increasing concentrations of \(\beta_1\)- or \(\beta_2\)-selective antagonists. In this program, the theoretical curve obtained for a one, two or three site binding model, with relative proportions and dissociation constants for each binding site entered during the analysis, is compared with the experimentally-obtained curve (DeLean et al., 1978). The model producing the closest fit to the experimental curve is identified and thus the relative population sizes of the
TABLE 2.6

$\beta_2$-Adrenoceptors as % of total $\beta$-adrenoceptors in perinatal rat liver

<table>
<thead>
<tr>
<th>Age</th>
<th>[1] From $B_{\text{max}}$ values</th>
<th>From computer analysis of inhibition profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 20 p.c.</td>
<td>95</td>
<td>-</td>
</tr>
<tr>
<td>Day 21 p.c.</td>
<td>84</td>
<td>82</td>
</tr>
<tr>
<td>Day 22 p.c.</td>
<td>94</td>
<td>93</td>
</tr>
<tr>
<td>24h p.p.</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>48h p.p.</td>
<td>93</td>
<td>-</td>
</tr>
</tbody>
</table>

The % of total $\beta$-adrenoceptors comprising $\beta_2$-adrenoceptors was determined experimentally from saturation assays or by computer analysis of inhibition profiles (see text for details).

TABLE 2.7

$\beta$-Adrenoceptor population in the perinatal rat liver

<table>
<thead>
<tr>
<th>B$\text{max}$ (fmoles/mg protein)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age:</td>
</tr>
<tr>
<td>McMillian et al.</td>
</tr>
<tr>
<td>Present study</td>
</tr>
</tbody>
</table>

Values obtained from saturation assays and from McMillan et al. (1983).

$^a$ $B_{\text{max}}$ is shown as mean ± SEM.

ND Not determined.
binding sites in the experimental tissue are obtained. Using this curve-fitting program to analyse displacement data obtained with atenolol and ICI 118 551 confirmed the presence of two binding sites. Assuming these to be $\beta_1$ and $\beta_2$-adrenoceptors, the relative subtype population sizes (Table 2.6, columns [2] and [3]) were in good agreement with those obtained by comparing $B_{\text{max}}$ values. $\beta_2$-adrenoceptors therefore appear to form approximately 85-95% of the total $\beta$-adrenoceptor population in the perinatal rat liver.

2.4.6 PUBLISHED STUDIES OF HEPATIC ADRENOCEPTORS IN THE PERINATAL RAT

The work reported in this chapter was carried out to investigate the type and number of adrenoceptors in the rat liver around the time of parturition. The only previous published work on this topic are the papers by Butlen et al. (1980) and by McMillian et al. (1983)

Investigation of $\alpha$-adrenoceptors

The work by Butlen was concerned with $\alpha$-adrenoceptors in perinatal rat liver, and was referred to earlier in this Discussion, when considering the $[^3\text{H}]$-DHE binding assay. These workers found that $\alpha$-adrenoceptors in the fetal rat liver were mainly of the $\alpha_1$ subtype; that these were homologous with $\alpha$-adrenoceptors found in the adult rat liver was strongly suggested by the identical dissociation constants of $[^3\text{H}]$-DHE at the different ages. The number of $\alpha$-adrenoceptors was seen to decrease 4-fold between the late fetal and early postnatal period, followed by an increase in the neonatal liver.

In contrast to the findings of Butlen's group, McMillian et al. reported that $\alpha_2$-adrenoceptors were more numerous than the $\alpha_1$-subtype during late gestation. Binding of the $\alpha_1$-selective ligand $[^3\text{H}]$-prazosin indicated changes in the $\alpha_1$-adrenoceptor number around the time of birth very similar to those observed by
Butlen et al.: decrease at birth followed by an increase during the postnatal period. Labelling of $\alpha_2$-adrenoceptors with $[^3H]$-rauwolscine showed at ten-fold decrease in $\alpha_2$-adrenoceptor number between day 17.5 post coitum and day 0.5 post partum, with this trend continuing throughout the postnatal period.

Investigation of $\beta$-adrenoceptors

The paper by McMillian et al. is the only published investigation of $\beta$-adrenoceptors in the perinatal rat liver. The dissociation constant ($K_D$) for the binding of $[^{125}\text{I}]-\text{IP}$ to liver membranes prepared from fetal or neonatal rats reported from this study by McMillian's group (68 ± 4 pM) is not significantly different from that found in the current work (75 ± 7 pM is the mean ± SEM $K_D$ from saturation assays for total $\beta$-adrenoceptors at all ages studied). The mean $K_D$ for total $\beta$-adrenoceptors is compared with the $K_D$ value in the paper by McMillian et al., as the assays carried out by McMillian and coworkers using $[^{125}\text{I}]-\text{IP}$ would have determined binding to both subtypes of $\beta$-adrenoceptors. The assumption by McMillian et al. that the binding sites being labelled by $[^{125}\text{I}]-\text{IP}$ were of the $\beta_2$-subtype appears to have been based on the relative ability of adrenergic agonists and antagonists in displacing the radioligand; as discussed earlier in this section, $\beta_1$-adrenoceptors make up 5-15% of the total hepatic $\beta$-adrenoceptor population during the perinatal period. The displacement characteristics of various compounds reported by McMillian's group were in agreement with those reported here (Section 2.3.4 and Table 2.3) for adrenaline, noradrenaline, phentolamine and atropine. The $K_i$ of 33 nM for isoprenaline found by McMillian's group is, however, somewhat lower than that of 210 nM found in the current investigation (Table 2.3). A possible explanation for this difference is discussed later.

It is not possible to make direct comparisons of $B_{max}$ values determined in the present work with those reported by McMillian et al., as animals of different
ages were used. As shown in Table 2.7, McMillian et al. found a steady decrease in receptor numbers with increasing age, whereas in the present study an increase up to term, followed by a decrease after birth, was the pattern seen. $B_{\text{max}}$ values shown are the mean ± SEM of three or four determinations at each age for the present study and the mean ± SEM (derived from the histogram presenting the results) of 'three to six' determinations at each age for the work by McMillian et al.

A number of aspects of the methodology used by McMillian et al. for the quantitative analysis of $[^{125}\text{I}]$-IP binding to liver membranes are open to criticism.

1. $[^{125}\text{I}]$-IP binding was measured after incubation for 20 minutes at 25°C. In the present study, when the time course of the association of $[^{125}\text{I}]$-IP with its binding site was studied, equilibrium was not reached until approximately 25 minutes (also at 25°C). McMillian et al. do not report any kinetic investigations, but it would appear highly likely that equilibrium had not been reached at the time that ligand binding was measured; this is particularly likely in inhibition studies, as the presence of a displacing drug would have increased the time taken to reach equilibrium (Molinoff et al., 1981).

2. Only six concentrations of $[^{125}\text{I}]$-IP were used in each saturation assay; this is a small number if a meaningful plot is to be produced. By contrast, ten to twelve concentrations of radioligand were used for each saturation assay in the current study. Bürgisser (1984) considered six data points to be sufficient for Scatchard analysis if the saturation binding curve is of "good quality". As no indication is given of the saturation characteristics or of the range of regression coefficients obtained for the Scatchard plots of McMillian's group's results, it is difficult to assess the extent to which this limited number of ligand concentrations may have affected the results obtained.
(3) Bürgisser (1984) stated that in order to obtain accurate parameters from analysis of saturation assays, at least 90% of the binding sites must be occupied at the top concentration of radioligand. To achieve this, a concentration of nine to ten-fold the ligand $K_D$ is required. The highest concentration of $^{125}\text{I}$ IP used by McMillan et al. was 200 pM, which is approximately three-fold the $K_D$ obtained. As can be seen in Figure 2.11, saturation of binding sites was obtained only at concentrations of $^{125}\text{I}$ IP of around 400 pM and above in the present study. The $B_{max}$ values quoted in the paper by McMillan et al., derived from 'saturation' assays in which saturated was not achieved, must be considered to be inaccurate.

(4) McMillian et al. quoted indirect Hill coefficients of $0.72 \pm 0.04$ obtained from indirect Hill plots of inhibition profiles. If a displacing compound is acting competitively, an indirect Hill plot of 1 should be obtained (Weiland & Molinoff, 1981). Competitive inhibition of binding is a prerequisite for the application of the Cheng & Prusoff equation (Cheng & Prusoff, 1973); the $K_i$ values reported by McMillian et al. should more accurately be described as $K_{0.5}$ values (i.e. the concentration of inhibitory agent required to saturate 50% of the available receptor sites). This observation may explain the discrepancy of the $K_i$ for isoprenaline determined in the present work and that reported by McMillian et al.

2.4.7 HEPATIC CELLULAR COMPOSITION AND ADRENOCEPTORS

Dramatic changes occur in the proportion of the various cell types found in the liver during gestation and immediately postnatally (Nagel, 1968; Greengard et al., 1972). The volume of the liver made up by hepatocytes increases from 60-85% between day 18 post coitum and term (haematopoietic and reticulo-endothelial cells account for the bulk of the remaining cells), whilst the mean volume of individual parenchymal cells increases three-fold. The proportion of the liver volume composed of hepatocytes shows no change compared with term at day 2.
post partum (Greengard et al., 1972). As seen in Table 2.7, the number of β-adrenoceptors (per unit weight of protein) in membranes prepared from liver shows a greater than 100% increase between day 20 post coitum and term, with a decrease of approximately 30% in the two days following birth. If β-adrenoceptors are present on hepatocytes, then the increase in β-adrenoceptor number seen in late gestation could be at least partly accounted for by the increase in the proportion of membrane protein derived from parenchymal cells. As radioligand binding assays using intact isolated hepatocytes are impractical due to the known endocytosis of adrenoceptor-bound ligands in intact cell systems (Hadjiivanova et al., 1984; Hertel et al., 1983; Lefkowitz et al., 1983; Reinhart et al., 1984), to investigate the possible influence of changing hepatic cellular composition further, attempts were made to prepare membranes from hepatocytes which had been isolated from rat liver during the perinatal period, and to perform $^{125I}$-IP binding assays on these membranes. Isolation of hepatocytes was carried out using the methodology described in Section 3.2.3 of Materials and Methods in Chapter 3; this procedure produced a cell suspension in which > 95% of the cells (by number) were hepatocytes. Membranes were prepared from the isolated cells using method C described in section 2.2.3 of Materials and Methods. Unfortunately, membrane yields from isolated hepatocytes were extremely low (< 0.01 mg protein/g liver tissue), and this approach therefore required a prohibitively large number of animals. Further investigation is required to assess the potential effect of changes in the proportion of liver tissue consisting of parenchymal cells on changes in the β-adrenoceptor population in the perinatal period.

2.4.8 SIGNIFICANCE OF FINDINGS

The presence of a significant population of β-adrenoceptors in the fetal and neonatal rat liver might be expected in view of the reported involvement of the β-adrenoceptor in the mediation of adrenergic effects on carbohydrate metabolism in younger animals (Sherline et al., 1974; Blair et al., 1979a,b; Moncany & Plas, 1980;
Morgan et al., 1983a). However, as discussed in Chapter 1, these observations must be considered carefully in view of the observed change in the adrenoceptor type of adult rat hepatocytes from α to β during culturing (Okajima & Ui, 1982; Nakamura et al., 1983; Kunos et al., 1984). As the effects of adrenergic agents were tested in cultured fetal liver explants in the work by Sherline et al. (1974), and in fetal hepatocytes cultured for three days in the experiments of Moncany and Plas (1980), only the observations of Blair et al. (1979a,b) and Morgan et al. (1983), using freshly isolated hepatocytes can be considered truly relevant. The present study confirms the observation of McMillian et al. (1983), that a substantial number of hepatic β2-adrenoceptors are present in the perinatal rat. This work also shows that β1-adrenoceptors are present in the liver during this period, although these account for only 15% or less of the total β-adrenoceptor population.

The effects of adrenergic agents on perinatal hepatic carbohydrate metabolism are considered in subsequent chapters, both in isolated hepatocytes in vitro and the whole animal in vivo. The significance of the findings reported in this chapter for known changes in hepatic carbohydrate metabolism in the perinatal period will be considered in Chapter 5.
CHAPTER 3

ADRENERGIC EFFECTS ON CARBOHYDRATE METABOLISM
IN HEPATOCYTES FROM PERINATAL RATS
3.1 INTRODUCTION

3.1.1 ADVANTAGES AND DISADVANTAGES OF ISOLATED CELLS

Isolated cells are a very valuable tool in the investigation of metabolic pathways. Cells in vitro may be exposed to known concentrations of agents for a fixed length of time, with none of the complications of absorption, distribution, metabolism and excretion of the agents which arise when in vivo administration is used. Similarly, measurement of levels of the substrates, intermediates or products of metabolism is greatly simplified in cell preparations. Therefore, isolated hepatocytes may provide a great deal of information on the potential of the liver to metabolise carbohydrate compounds, and to respond to adrenergic agents by modulation of carbohydrate metabolism.

It must, of course, always be borne in mind that the very advantages of working with isolated cells in vitro are also the limitations in interpretation of observations made and extrapolation of findings to the in vivo situation. In addition to the lack of influences of other tissues and organs on isolated hepatocytes, disruption of the liver and preparation of parenchymal cells in suspension obviously results in loss of liver architecture and intra-organ influences. The enzyme content and subcellular structure of hepatocytes from the periportal and perivenous zones of the liver are known to differ in adult liver (Jungermann & Katz, 1982). However, in the present work this loss of hepatic zonation on preparation of isolated hepatocytes is unlikely to be of significance, as heterogeneity of the hepatocyte population has been reported to develop gradually during the first weeks of postnatal life (Jungermann & Katz, 1982) and would therefore not be established at the ages investigated.
3.1.2 ISOLATION OF HEPATOCYTES

Techniques for hepatocyte isolation from adult rats

Adult rat hepatocytes have been prepared by a range of techniques, but the use of enzymes to digest intercellular material and thus isolate cells has been shown to be preferable to methods of mechanical disruption (Lipson et al. 1972). Howard et al. (1967) reported successful isolation of adult rat hepatocytes using collagenase and hyaluronidase to digest the inter-parenchymal tissue of the liver. Berry and Friend (1969) produced a greater yield of hepatocytes, with high viability, by perfusing the liver with medium containing these two enzymes, and recycling the medium for maximum effect. This method has been adapted to include perfusion with Ca$^{2+}$-free medium to reduce cell adhesion, followed by the medium containing collagenase in the presence of Ca$^{2+}$, which is a cofactor in this enzyme's action (Seglen, 1972 & 1973a).

Seglen (1973b) reported that no reduction in the dispersal of liver cells resulted from omission of hyaluronidase and inclusion of hyaluronidase has been shown to reduce the glycogen content of the isolated cells (Wagle, 1974). There are many descriptions of variations on the techniques of Seglen in the literature (e.g. Capuzzi et al., 1971; LeCam et al., 1976; Van Bezooijen et al., 1974; Zahlten & Stratman, 1974). Perfusion of the liver with an enzyme solution appears to produce a higher yield of cells of better viability than liver slicing followed by incubation in an enzyme solution (Gustavsson & Mörland, 1980). Contamination of the cell preparation with non-parenchymal cells may be reduced to 1-2% by the use of differential centrifugation (Seglen, 1973b). The method used to isolate adult rat hepatocytes for use in the current investigation was based on that of Seglen (1973b).

Techniques for hepatocyte isolation from perinatal rats

Isolation of hepatocytes from fetal or neonatal rats requires a different
approach to that used for adults, as the size of the animals restricts perfusion of individual livers. Fetal hepatocytes have been isolated following perfusion of a lysozyme-containing perfusate into the maternal dorsal aorta (Hommes et al., 1971). Perfusion of the liver via the vena cava with a collagenase solution, prior to hepatocyte isolation, has been described at day 1 post partum (Ferre et al., 1981a) and day 5 post partum (Ziegler, 1980), but such a technically demanding procedure would be unsuitable for the frequent preparation of hepatocytes from large numbers of fetal and neonatal animals.

Plas et al. (1973) described a combination of enzymic and mechanical disruption of livers of day 15 post coitum fetal rats, in which trypsinisation was combined with shaking of liver tissue with glass beads. However, methods involving chopping or mincing of the liver, followed by incubation with trypsin (Huhn et al., 1983) or collagenase (De Sante et al., 1984; Freemark & Handwerger, 1984; Leffert & Paul, 1972) appear to be more satisfactory for the preparation of hepatocytes from rats during the perinatal period. Therefore, a method involving chopping of fetal livers into small pieces and shaking in media, first in the absence of calcium, and then in the presence of calcium and collagenase, was developed in this laboratory. This is described in detail in Section 3.2.3.

3.1.3. AIMS OF THE EXPERIMENTS

If adrenergic mechanisms are involved in the physiological stimulation of hepatic glucose production and the consequent recovery of plasma glucose concentration in the neonatal rat, hepatocytes isolated from rats around the time of parturition would be expected to show sensitivity of glucose production rates to the stimulatory effect of adrenergic agonists, as shown by many workers for adult cells (e.g. Assimacopoulos-Jeannet et al., 1977; Exton & Harper, 1975; Rognstad & Katz, 1977).
By investigating the adrenergic responsiveness of cells isolated from fetal rats one and two days prior to term (day 20 and 21 post coitum) and at term (day 22 post coitum) and neonatal rats one and two days after delivery, changes in the sensitivity of pathways of glucose production to adrenergic stimulation with age were demonstrated. The magnitude of the response at various concentrations of adrenergic agonists was established at the five perinatal ages to determine the susceptibility of hepatic glucose production to adrenergic influence at these ages. Exposure to agonist and antagonist compounds with known selectivity for α- or β-adrenoceptors provided evidence of the nature of the adrenoceptor involved.

Hepatocytes were also prepared from term fetal rats obtained from dams which have gestational diabetes. It is well established that maternal diabetes produces changes in neonatal carbohydrate metabolism in the rat (e.g. Cuezva & Patel, 1980b; Snell, 1982b), and it is of great interest to establish whether changes in adrenergic sensitivity, particularly if this may be shown to be of physiological significance, may occur in this pathological condition.
3.2 MATERIALS & METHODS

3.2.1 MATERIALS

Phosphate buffered salt tablets (Dulbecco's formula, modified: without Ca²⁺ or Mg²⁺) and trypan blue solution (0.5% in 0.85% saline) were obtained from Flow Laboratories, Rickmansworth, Herts. Collagenase was from Sigma Chemical Company Ltd., Poole, Dorset (Type IV), Worthington, Flow Laboratories, Irvine, Ayrshire, Scotland, or Boehringer Corp., Lewes, East Sussex; no difference in cell yield or viability was apparent using the enzyme from different sources. Streptozotocin and desoxyribonuclease 1 (DNase 1) were from Boehringer Corp., Lewes, East Sussex. Ethylene glycol-bis-(β-amino ethyl ether) N,N,N',N'-tetraacetic acid (EGTA), defatted bovine serum albumin, L(+)lactic acid, sodium pyruvate, amyloglucosidase (suspension in 3.2 M (NH₄)₂SO₄), type II oyster glycogen, Dowex-2 anion exchange resin (200-400 dry mesh), (+)adrenaline, (+)noradrenaline HCl, (-)isoprenaline (+)bitartrate, DL-propranolol HCl, and L-phenylephrine HCl were from Sigma Chemical Company Ltd., Poole, Dorset. Phentolamine mesylate was from Ciba Laboratories, Horsham, West Sussex, and ICI Ltd., Alderley Edge, Cheshire, kindly provided the propanolol HCl, atenolol and ICI 118 551. Syperonic NX detergent was supplied by Durham Chemical Distributors Ltd., Birtley, Tyne & Wear. Toluene and glacial acetic acid were from May & Baker Ltd., Dagenham, Essex. 1,4-Di-(2-(4-methyl-5-phenyloxazolyl))-benzene (dimethyl POPOP) was obtained from Fisons Scientific Apparatus, Loughborough, Leics. 2,5-Diphenyloxazole (PPO) was from Koch-Light Laboratories Ltd., Colnbrook, Bucks. L[U-¹⁴C]-lactic acid was from Amersham International plc, Amersham, Bucks. All other reagents were from BDH Chemicals Ltd., Poole, Dorset.

3.2.2 ANIMALS

Rats of a random-bred Wistar albino strain, were obtained from the University of Surrey Animal Unit. Each male was housed overnight with 5-10
females and pregnancy was established by palpation 10 to 20 days after mating, or by the presence of sperm in a vaginal smear (when diabetes was to be induced during pregnancy). The day following overnight housing with the male was taken as day 1 post coitum. Diabetes was induced in some pregnant animals by intraperitoneal injection of 45 mg/kg streptozotocin in 50 mM sodium citrate buffer, pH 4.5, on day 5 post coitum. Diabetes was confirmed by a plasma glucose level above 15 mM at the time of killing (day 22 post coitum).

Fetal rats were obtained by caesarian section at a time between 9 and 10.30 a.m. on the appropriate gestational day (day 20, 21 or 22 post coitum). Parturition usually occurred overnight between days 22 and 23 post coitum. Neonates were delivered naturally and were killed between 1.30 and 2.30 p.m. of day 1 or 2 post partum. All neonatal animals used had fed and appeared healthy. All the young from a litter were weighed together and a mean weight per fetus or pup calculated.

Maternal rats were killed by cervical dislocation; fetal and neonatal rats were killed by decapitation. Anaesthesia was not used for any of the animals.

All animals were given food and water ad libitum.

3.2.3 PREPARATION OF HEPATOCYTES FROM ADULT RATS

Hepatocytes were prepared from adult male and female rats using collagenase perfusion of the liver (Gettings, 1985).

3.2.4 PREPARATION OF HEPATOCYTES FROM FETAL OR NEONATAL RATS

Solutions

(A) Phosphate buffered salt solution, pH 7.5; made from tablets according to
manufacturer's instructions; without \( \text{Ca}^{2+} \) or \( \text{Mg}^{2+} \), but containing 5 mM glucose.

(B) As solution A but also containing 0.5 mM EGTA.

(C) 5 mg collagenase and 1 mg DNase 1 dissolved in 10 ml of Hanks balanced salt solution (137 mM \( \text{NaCl} \), 5.4 mM KCl, 0.44 mM \( \text{KH}_2\text{PO}_4 \), 0.34 mM \( \text{Na}_2\text{HPO}_4 \), 5.5 mM glucose), 0.2 ml 250 mM \( \text{CaCl}_2 \) added and pH adjusted to 7.5 by addition of 2.8% \( \text{NaHCO}_3 \).

Procedure

(1) Livers were taken into ice-cold solution A, then chopped into 0.5 mm\(^2\) pieces using a McIlwain automatic tissue chopper (Mickle Laboratory Engineering Company Ltd., Gomshall, Surrey) and placed in a 250 ml glass conical flask with approximately 10 ml of solution A. Liver material from a single litter (8-14 pups) was placed in one flask.

(2) The flask was placed in a water bath at 37°C and shaken for 10 minutes at 40 strokes/min; the supernatant was then decanted off the liver pieces and discarded.

(3) 10 ml fresh solution A was added to the flask.

(4) Step 2 was repeated.

(5) 10 ml solution B was added to the flask and step 2 was repeated.

(6) Step 5 was repeated.

(7) 10 ml solution C was added to the flask, which was placed in the water bath at 37°C and shaken for 45 minutes at 90 strokes/min.

(8) The supernatant was poured through 'Nybolt' nylon bolting cloth, mesh width 125 \( \mu \)m (John Stranier and Co., Manchester), and the remaining liver material agitated gently with a pasteur pipette to loosen any detached cells. Free cells were then washed through the cloth with about 20 ml of solution A.
The cell suspension was placed in a 50 ml Falcon tube (Benton-Dickinson, Oxford) and centrifuged at approximately 10 g for two minutes, at room temperature, in a bench top centrifuge (MSE minor 'S' or IEC Centra-3R).

After centrifugation the supernatant was removed using a pasteur pipette, and was discarded.

The cells were resuspended in about 15 ml solution A by gentle sucking in and out of a pasteur pipette.

The washing procedure (steps 9-11) was repeated twice.

3.2.5 HEPATOCYTE INCUBATION

Incubation medium

The medium for incubation was a Krebs-Ringer bicarbonate buffer, pH 7.5, (118.5 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.18 mM KH₂PO₄, 1.18 mM MgSO₄, 2.5 mM NaHCO₃) containing 2%(w/v) defatted bovine serum albumin. 10 mM lactate and 1 mM pyruvate were present as gluconeogenic precursors. 20 µg/ml ascorbic acid was included when an adrenergic agent susceptible to oxidation was to be used. In some concentration-reponse experiments, when hepatocytes isolated from day 22 post coitum fetal livers were being used, the medium contained L [ U-¹⁴C ]- lactic acid such that the specific activity of the lactate pool was approximately 20 nCi/ml.

Procedure

The hepatocytes were washed a further two times with approximately 10 ml of incubation medium, using gentle centrifugation and resuspension as detailed in steps 9-11 of the hepatocyte isolation procedure. The cells were resuspended in an appropriate volume of incubation medium (see below) and filtered through 'Nybolt' bolting cloth, mesh width 125 µm, to remove any clumped cells. Aliquots of the cell suspension were placed in 20 ml polypropylene vials
(Packard, Illinois, USA) and incubation was carried out by placing the vials in a closed polythene box containing water at 37°C to a depth of about two cm, standing in a water bath at 37°C. The hepatocyte suspensions were incubated in an atmosphere of 95% O₂/5% CO₂ by continual gassing of the box. The vials were shaken at 50 strokes/min and at the end of the incubation period the cell suspensions were poured into 7.5 ml glass tubes on ice, and centrifuged immediately at 2,000 g for 10 minutes at 4°C (Beckman J6B or IEC Centra-3R centrifuge).

An aliquot of the supernatant obtained from centrifugation of the cell suspension following incubation was retained (for glucose analysis) in all experiments; in some initial concentration-response experiments using hepatocytes isolated from fetal rats on day 22 post coitum, all of the supernatant was retained for total glucose and [³¹⁴C] glucose analysis. Glycogen levels were determined in most experiments in which hepatocytes were isolated from fetuses on day 22 post coitum. When glycogen was to be assayed the cell pellet was retained. Cell pellets and supernatants were kept on ice; if glycogen or glucose could not be assayed within 24 hours, the samples were stored at -20°C.

**Hepatocyte viability**

In all experiments, two samples of the hepatocyte suspension were taken during the aliquoting procedure to check cell yield and viability. This was carried out in a 'Neubauer' cell counting chamber (Weber Scientific International Ltd., Lancing, East Sussex) under a light microscope, using the trypan blue exclusion method to assess viability. The cell suspension was seen to consist predominantly of hepatocytes, with other cell types (mainly erythrocytes) accounting for less than 5% of the cells (by number). Using this method, viability was never less than 95% in the freshly prepared cell suspension.
A number of other methods are available for the determination of cell viability, including metabolic integrity, the ability to exclude succinate (Mapes & Harris, 1975), and the retention of lactate dehydrogenase or ATP by isolated cells (Dickson & Pogson, 1977). As glucose production by the hepatocytes was determined in the course of the experiments reported in this chapter, metabolic integrity was automatically assessed in addition to trypan blue exclusion.

Pogson et al. (1983) suggested that the assay of ATP content with luciferin-luciferase (Stanley & Williams, 1969) is a convenient method, giving more reliable results than dye exclusion. As this method ideally requires a luminometer, which was not available in the Biochemistry Department of the University of Surrey, it could not be used routinely. However, cell viability over a four and six hour time-course was assessed using both the trypan blue exclusion and ATP content methods, to allow comparison of these two methods. The assay of ATP was carried out in Dr. R. Paterson's laboratory at Wellcome Research, Beckenham, Kent.

The ATP content of freshly isolated fetal hepatocytes was 3.7 nmoles/10^6 cells for the cells prepared for the four-hour time course, and 2.9 nmoles/10^6 cells for the six-hour time course. ATP content of these two preparations dropped to 3.6 and 2.6 nmoles/10^6 cells after 30 minutes of incubation; the lowest level measured was 2.1 nmoles/10^6 cells after 6 hours of incubation. Dickson & Pogson (1977) stated that 8-13 nmol ATP/mg dry wt. was an acceptable range for viable adult rat hepatocytes. Cornell (1983) recommended that adult rat hepatocytes with an ATP content below 2 μmol/g wet wt. should not be used. Using the figures of Schulze et al. (1984) of 3.2mg wet wt. or 0.8mg dry wt. per 10^6 fetal rat hepatocytes, the figures of Dickson & Pogson equate to 6.4-10.4 nmol ATP /10^6 cells, with the level proposed by Cornell being the lowest value in this range. Although the ATP levels estimated for the cell preparations in the current work
are below this range, it is important to remember that these values are for fetal hepatocytes; Philippidis & Ballard (1970) reported ATP concentrations in fetal rat liver in vivo to be less than 50% of the adult value. This, together with the observation that ATP levels remained relatively constant during incubations over several hours, whilst other workers have reported a rapid loss of ATP when hepatocytes were isolated from starved rats (Dickson & Pogson, 1977) or were incubated in anoxic conditions (Cornell, 1983), suggest that those ATP levels indicated viable fetal rat hepatocytes.

Hepatocyte viability (expressed as the % of the zero time value) over the time periods studied, as assessed by trypan blue exclusion and ATP content was:

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Trypan Blue Exclusion</th>
<th>ATP Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>97</td>
<td>94</td>
</tr>
<tr>
<td>1 hr</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
<td>2 hr</td>
<td>93</td>
<td>83</td>
</tr>
<tr>
<td>4 hr</td>
<td>82</td>
<td>75</td>
</tr>
<tr>
<td>6 hr</td>
<td>75</td>
<td>72</td>
</tr>
</tbody>
</table>

A limited number of conclusions may be drawn from these data, due to the comparative assessments being made using only two cell preparations and incubations. However, it does appear from these investigations that viability as determined using the two methods, is not significantly different. Viability was always slightly higher when assessed using trypan blue exclusion, rather than ATP content, but this difference was never greater than 10%, and the pattern and rate of loss of viability during the incubations was similar whichever method was used. The comparison of trypan blue exclusion and cell ATP content as methods for the assessment of hepatocyte viability therefore confirmed the former method to be a valid means of detecting loss of viability of a degree which would affect the outcome of experiments.
Concentration-response experiments were carried out using hepatocytes prepared from fetal, neonatal and adult male and female rats. In this series of experiments, glucose production in hepatocytes freshly isolated from rats and incubated with various concentrations of adrenergic agonists was investigated. Hepatocytes were incubated in the absence of any agent or in the presence of an agonist compound at one of four or five concentrations, over a concentration range of four or five orders of magnitude.

The agents investigated, and the concentration ranges used were: adrenaline \((10^{-10}-10^{-6} \text{M})\), noradrenaline \((10^{-9}-10^{-5} \text{M})\), isoprenaline \((10^{-10}-10^{-6} \text{M})\), and phenylephrine \((10^{-7}-10^{-3} \text{M})\) for fetal and neonatal hepatocytes; adrenaline \((10^{-9}-10^{-6} \text{M})\) and isoprenaline \((10^{-10}-10^{-6} \text{M})\) for adult hepatocytes. Hepatocytes were resuspended in 70 or 90 ml of incubation medium and 1.8 ml aliquots of the cell suspension were placed in each of 35 20 ml polypropylene vials. 0.2 ml of the incubation medium alone was added to ten of these cell suspensions to act as control incubations. The other cell suspensions received 0.2 ml of the incubation medium containing the hormone under investigation at a concentration 10-fold higher than the final concentration to which the hepatocytes were to be exposed; 5 vials were used at each concentration. In experiments in which the rate of glycogenolysis was to be determined as well as total glucose production, hepatocytes from day 22 post coitum fetuses were resuspended in 90 ml of the incubation medium and, in addition to aliquots for incubation, a further ten 1.8 ml aliquots of the cell suspension were taken into 7.5 ml glass tubes on ice. These were immediately centrifuged at 2,000 g for 10 minutes at 4°C (Beckman J6B or IEC Centra-3R centrifuge). The supernatant was discarded and the cell pellet retained for glycogen analysis; this allowed the glycogen content of the hepatocytes prior to incubation to be determined. Incubation in these experiments
was at 37°C for 30 minutes at a shaking speed of 50 strokes/min.

Cell viability was assessed, using the trypan blue exclusion method described in 3.2.5, in one control incubation at the end of the incubation period. Viability was always greater than 90% at this time.

**Response-blocking experiments**

In this series of experiments, the effects of adrenergic antagonists on glucose production by hepatocytes isolated from rats on day 21.5 post coitum, and incubated in the absence of any stimulating agent or in the presence of 10^{-6} M adrenaline, was investigated.

Hepatocytes were resuspended in 70 ml of the incubation medium for use in these experiments. 1.8 ml aliquots of the cell suspension were placed in each of 35 20 ml polypropylene vials. 0.2 ml of the incubation medium was added to ten of these cell suspensions to act as control incubations. Five vials received 0.1 ml of the incubation medium containing 2 \times 10^{-5} M adrenaline (as 10^{-6} M adrenaline had been seen to elicit maximal stimulation of glucose production), plus 0.1 ml of the incubation medium. Two antagonist compounds were investigated in each experiment of this series. Ten vials received 0.1 ml of the incubation medium containing one of the antagonists at 20-fold the final concentration required; 0.1 ml of the incubation medium was added to five of these vials and the other five received 0.1 ml of the incubation medium containing 2 \times 10^{-5} M adrenaline. The remaining ten vials contained the second antagonist under investigation, again in the presence or absence of an optimal concentration of adrenaline. Incubation in these experiments was at 37°C for 30 minutes at 50 strokes/min.

Cell viability was assessed, using the trypan blue exclusion method described in section 3.2.5, in one control incubation at the end of the incubation period.
Viability was always greater than 90% at this time.

**Time-course experiments**

In this series of experiments, the production of glucose by hepatocytes isolated from fetal rats on day 22 post coitum was investigated at time intervals over two or three hours of incubation. Incubation was for two hours in the absence of hormone or in the presence of $10^{-6}$M adrenaline (which was shown in concentration-response experiments to result in maximal stimulation of glucose production), or for three hours in the absence of hormone or in the presence of $10^{-5}$M adrenaline, noradrenaline or glucagon.

**TWO HOUR TIME-COURSE**

In the two hour time-course experiments, hepatocytes were resuspended in 100 ml of incubation medium and 1.8 ml aliquots of the cell suspension were placed in each of 40 20 ml polypropylene vials. 0.2 ml of the incubation medium was added to 20 of these cell suspensions to act as control incubations. The other 20 cell suspensions received 0.2 ml of $10^{-5}$M adrenaline in the incubation medium, so that the hepatocytes were exposed to $10^{-6}$M adrenaline during incubation. A further ten 1.8 ml aliquots of the cell suspension were taken into 7.5 ml glass tubes on ice. These were immediately centrifuged at 2,000 g for 10 minutes at 4°C (Beckman J6B or IEC Centra-3R centrifuge). The supernatant was discarded and the cell pellet retained for glycogen analysis. At 30, 60, 90 and 120 min of incubation, five vials containing hepatocytes in control medium and five vials containing hepatocytes in medium plus $10^{-6}$M adrenaline were removed from the incubation bath, the cell suspension taken into 7.5 ml glass tubes on ice, and treated as described above. All cell pellets were retained for glycogen analysis.

**THREE HOUR TIME-COURSE**

In the three hour time-course experiments, during the final stages of
preparation of hepatocytes from day 22 post coitum fetal rats, the cell suspension was split into two approximately equal aliquots, and these were centrifuged as detailed in step 9 of the hepatocyte preparation procedure (3.2.4). The supernatants were removed, and one cell pellet resuspended in incubation medium alone, and the other in incubation medium containing $10^{-5}$ M adrenaline, noradrenaline or glucagon. 3 ml aliquots of the cell suspensions were placed in 20 ml polypropylene vials. At one, two and three hours of incubation, three or five vials containing hepatocytes in control medium and three or five vials containing hepatocytes in medium plus hormone were removed from the incubation bath and the cell suspension taken into 7.5 ml glass tubes on ice, and treated as detailed above. Supernatants were taken for glucose determination, but cell pellets were not retained for glycogen analysis in this series of experiments.

**HEPATOCYTE VIABILITY**

In both types of time-course experiment, cell viability was assessed, using the trypan blue exclusion method described in section 3.2.5, in one control incubation at each time point. Viability did not drop below 85% at any time point studied.

**Effect of maternal diabetes**

In this series of experiments, glucose production by hepatocytes isolated on day 22 post coitum from fetal rats of diabetic mothers, and incubated in the absence of any agent or in the presence of adrenaline in the concentration range $10^{-10}$-$10^{-3}$ M, was investigated.

Hepatocytes were resuspended in 70 or 90 ml of the incubation medium for use in these experiments. 1.8 ml aliquots of the cell suspension were placed in each of 35 20 ml polypropylene vials. 0.2 ml of the incubation medium was added to ten of these cell suspensions to act as control incubations. The other cell suspensions received 0.2 ml of the incubation medium containing adrenaline at a concentration
10-fold higher than the final concentration to which the hepatocytes were to be exposed; five vials were used for each concentration. In some experiments a further ten 1.8 ml aliquots of the cell suspension were taken into 7.5 ml glass tubes on ice. These were immediately centrifuged at 2,000 g for 10 minutes at 4°C (Beckman J6B or IEC Centra-3R centrifuge). The supernatant was discarded and the cell pellet retained for glycogen analysis. Incubation in these experiments was at 37°C for 30 minutes at 50 strokes/min.

3.2.7 ASSAYS

Glucose assay

Glucose in the supernatant incubation medium was measured directly using the hexokinase - glucose-6-phosphate dehydrogenase method on a Cobas-Bio centrifugal autoanalyser (Roche, Welwyn Garden City, Herts). No deproteinisation was required. Reagent kits (Glucose 'rapid', Roche Diagnostics, Welwyn Garden City, Herts, or Centrifichem glucose assay, Baker Instruments, Allentown, PA, USA) were used according to the manufacturer's instructions. Appropriate glucose standards were included for each set of determinations; these were in the range 0.05 - 0.20 mM glucose in distilled water.

Glycogen assay

The cell pellet was sonicated in 1 ml of distilled water using a sonic probe (type 1130A, Dawes Instruments Ltd., London). A 0.2 ml aliquot of the sonicate was placed in a 7.5 ml glass tube and mixed with 1.0 ml of 0.2 M acetate buffer, pH 4.8, containing 74 i.u./ml amyloglucosidase. The tubes were shaken at 30 strokes/min in a water bath at 37°C for 4 hours, and were vortex mixed approximately half way through this time. A distilled water blank and 0.2 ml aliquots of glycogen standards (0.1-1.0 mg/ml oyster glycogen in distilled water) were treated identically. At the end of the incubation, all tubes were centrifuged at 2,000 g for 10 minutes at room temperature (Beckman J6B or IEC Centra-3R centrifuge) to
remove any sediment. Glucose in the supernatant was measured using the Cobas-Bio autoanalyser (see above), and the glycogen content of the cell pellets calculated from the standard curve obtained. Linear standard plots were obtained; using the methodology described the highest glycogen standard concentration resulted in a glucose concentration in the range 0.89-0.95 mM, indicating 97-100% of the glycogen was hydrolysed to glucose. Preliminary analyses showed negligible values of free glucose in the cell sonicates, and therefore incubation of samples in the absence of amyloglucosidase was not carried out routinely.

\[ ^{14}\text{C} \]-Glucose assay

Separation of \[^{14}\text{C}\]lactate and \[^{14}\text{C}\]glucose was achieved using ion exchange chromatography; the method was based on that of Hammerstedt (1980). 1 ml of the supernatant incubation medium was deproteinised by mixing with 10 ml 0.5 M NaOH and 0.84 ml of 10% (w/v) ZnSO\(_4\)•7H\(_2\)O. The precipitate was removed by centrifuging at 3,000 g for 15 minutes at room temperature (Beckman J6B centrifuge). 2 ml of deproteinised sample was added to a column of Dowex-2 ion-exchange resin, in the formate form, packed in a pasteur pipette. 6 ml of distilled water was added to the pressure-head tubing. The eluate was collected in glass scintillation vials and freeze dried. 1 ml distilled water and 10 ml scintillant fluid (23g PPO, 1 g dimethyl POPOP, 3.3 l toluene, 1.67 l Syperonic NX detergent) were added to the vials, and \[^{14}\text{C}\] -glucose counted using a Rack-Beta liquid scintillation counter (type 1217, LKB), after thorough mixing.

When known amounts of L\[\text{U}^{14}\text{C}\] -lactic acid and D \[^{14}\text{C}\] -glucose in incubation medium were treated as described above, and then applied to the Dowex-2 ion-exchange column, 97% of the lactate was retained, and 86% of the glucose was collected in the eluate.

3.2.8 ANALYSIS OF DATA

All statistical analyses were carried out using paired Student's t-test.
3.3 RESULTS

3.3.1 CONCENTRATION-RESPONSE EXPERIMENTS

Glucose production

CONTROL PRODUCTION RATE

In the various experiments described below the control glucose production rate (in the absence of hormonal stimuli) in hepatocytes isolated during the perinatal period was greatest in those cells prepared from fetal rats on day 22 post coitum just prior to term (Tables 3.1, 3.2, 3.3 and 3.4). The control rate one day after delivery was 30-50% of that immediately before birth, and recovered in the following day by 20-30%, except in the experiments investigating the effects of isoprenaline. The glucose production rate by unstimulated hepatocytes from day 22 post coitum fetuses was 26-46% of the control glucose production rate in adult male hepatocytes, and 60-71% of that in hepatocytes from adult female rats.

EFFECTS OF ADRENALINE

Glucose production in hepatocytes freshly isolated from fetal rats on day 20, 21 or 22 post coitum, from neonatal rats on day 1 or 2 post partum, or from adult male or female rats, showed a concentration-dependent stimulation by adrenaline (Table 3.1 and Fig. 3.1). The control glucose production rate in cells isolated from fetal rats on day 20 post coitum was very low, and showed no statistically significant increase due to adrenaline. Because of the problems of measuring this low rate of glucose production, only two experiments were carried out using hepatocytes isolated from day 20 post coitum fetuses.

The glucose production rate showed no statistically significant increase above the control level at adrenaline concentrations below $10^{-8}$M at any age. This concentration was the lowest producing a significant stimulation of glucose
### Effect of adrenaline on glucose production in hepatocytes freshly isolated from fetal, neonatal or adult rats

<table>
<thead>
<tr>
<th>Concentration of Adrenaline (M)</th>
<th>0</th>
<th>10^{-10}</th>
<th>10^{-9}</th>
<th>10^{-8}</th>
<th>10^{-7}</th>
<th>10^{-6}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 20 p.c.</td>
<td>52 ± 6</td>
<td>48 ± 7</td>
<td>53 ± 9</td>
<td>61 ± 10</td>
<td>68 ± 11</td>
<td></td>
</tr>
<tr>
<td>(n = 20)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>Day 21 p.c.</td>
<td>110 ± 12</td>
<td>114 ± 20</td>
<td>120 ± 19</td>
<td>126 ± 18</td>
<td>135 ± 27</td>
<td></td>
</tr>
<tr>
<td>(n = 40)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td></td>
</tr>
<tr>
<td>Day 22 p.c.</td>
<td>432 ± 8</td>
<td>428 ± 18</td>
<td>503 ± 19</td>
<td>565 ± 22</td>
<td>583 ± 25</td>
<td></td>
</tr>
<tr>
<td>(n = 60)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td></td>
</tr>
<tr>
<td>Day 1 p.p.</td>
<td>164 ± 10</td>
<td>144 ± 26</td>
<td>172 ± 18</td>
<td>190 ± 17</td>
<td>217 ± 14</td>
<td></td>
</tr>
<tr>
<td>(n = 60)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td></td>
</tr>
<tr>
<td>Day 2 p.p.</td>
<td>304 ± 19</td>
<td>298 ± 22</td>
<td>320 ± 30</td>
<td>367 ± 38</td>
<td>378 ± 42</td>
<td>424 ± 39</td>
</tr>
<tr>
<td>(n = 60)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td>(n = 30)</td>
</tr>
<tr>
<td>Adult male</td>
<td>1664 ± 132</td>
<td>1660 ± 203</td>
<td>1723 ± 203</td>
<td>2103 ± 234</td>
<td>2350 ± 260</td>
<td></td>
</tr>
<tr>
<td>(n = 30)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
<td></td>
</tr>
<tr>
<td>Adult female</td>
<td>738 ± 31</td>
<td>598 ± 54</td>
<td>742 ± 52</td>
<td>931 ± 56</td>
<td>1088 ± 79</td>
<td></td>
</tr>
<tr>
<td>(n = 30)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
<td></td>
</tr>
</tbody>
</table>

Incubation was for 30 min. n = total number of incubations from 3 adult cell preparations, or 4-6 perinatal cell preparations, except Day 20 p.c. (see text for details).

*Glucose production rate is shown as mean ± SEM.*

* Significantly higher than control, p < 0.1
** Significantly higher than control, p < 0.025
*** Significantly higher than control, p < 0.005

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
FIGURE 3.1  Effect of adrenaline on glucose production in hepatocytes freshly isolated from fetal, neonatal or adult rats.

Glucose production rate is shown as a % of the control rate.
production in hepatocytes isolated from fetuses on day 22 post coitum, whilst $10^{-7}$M adrenaline was the threshold concentration from stimulation of glucose production in cells from adult females, and $10^{-6}$M was required for any statistically significant effect at any other age studied (Table 3.1). It would appear from the data in Table 3.1 that adrenaline is producing an increase in the glucose production rate at concentrations below those marked as causing significant increases, but that the large variation in basal production rate observed from one preparation to another (see discussion in section 3.4.1) prevented these effects from being of statistical significance. For hepatocytes isolated at all ages investigated, the maximal stimulation observed was at $10^{-6}$M adrenaline; this was the highest concentration investigated (Fig. 3.1). Maximal stimulation of the glucose production rate by adrenaline was 31-47%, depending on the age of the animal from which the cells were made (Table 3.5). The adrenaline concentrations calculated to produce 50% of the maximal increase in production ($EC_{50}$) varied from 13-200 nM, depending on age (Table 3.5).

**EFFECTS OF NORADRENALINE**

Noradrenaline produced a concentration-related stimulation of the glucose production rate in hepatocytes freshly isolated from fetal or neonatal rats (Table 3.2 and Fig. 3.2). Glucose production was not significantly affected by noradrenaline at concentrations below $10^{-7}$M, at any age; $10^{-7}$M noradrenaline increased the glucose production rate above the control rate in hepatocytes isolated on day 22 post coitum, or on day 2 post partum. A ten-fold higher concentration of the drug was required to elicit a statistically significant increase in cells prepared from day 1 post partum neonates, and a concentration of $10^{-5}$M was the lowest producing a significant stimulation of the glucose production rate in cells prepared from day 21 post coitum fetuses (Table 3.2). At all ages, the maximal stimulation observed was that produced by $10^{-5}$M noradrenaline, which
TABLE 3.2

**Effect of noradrenaline on glucose production in hepatocytes freshly isolated from fetal or neonatal rats**

<table>
<thead>
<tr>
<th>Glucose Production Rate (nmoles/10^6 cells/h)^a</th>
<th>0</th>
<th>10^-9</th>
<th>10^-8</th>
<th>10^-7</th>
<th>10^-6</th>
<th>10^-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Noradrenaline (M):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21 p.c.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>274 ± 13 (n = 30)</td>
<td>246 ± 16 (n = 15)</td>
<td>253 ± 16 (n = 15)</td>
<td>271 ± 24 (n = 15)</td>
<td>294 ± 16 (n = 15)</td>
<td>*333 ± 22 (n = 15)</td>
<td></td>
</tr>
<tr>
<td>Day 22 p.c.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>459 ± 19 (n = 30)</td>
<td>444 ± 20 (n = 15)</td>
<td>471 ± 20 (n = 15)</td>
<td>525 ± 19 (n = 15)</td>
<td>620 ± 26 (n = 15)</td>
<td>640 ± 23 (n = 15)</td>
<td></td>
</tr>
<tr>
<td>Day 1 p.p.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>188 ± 8 (n = 40)</td>
<td>189 ± 13 (n = 20)</td>
<td>188 ± 15 (n = 20)</td>
<td>224 ± 23 (n = 20)</td>
<td>285 ± 31 (n = 20)</td>
<td>302 ± 28 (n = 20)</td>
<td></td>
</tr>
<tr>
<td>275 ± 8 (n = 40)</td>
<td>285 ± 14 (n = 20)</td>
<td>275 ± 14 (n = 20)</td>
<td>334 ± 22 (n = 20)</td>
<td>474 ± 21 (n = 20)</td>
<td>477 ± 27 (n = 20)</td>
<td></td>
</tr>
</tbody>
</table>

Incubation was for 30 min. n = total number of incubations from 3 or 4 cell preparations (see text for details).

a  Glucose production rate is shown as mean ± SEM.

*  Significantly higher than control, p < 0.025

**  Significantly higher than control, p < 0.01

*** Significantly higher than control, p < 0.005

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
FIGURE 3.2 Effect of noradrenaline on glucose production in hepatocytes freshly isolated from fetal or neonatal rats.

Hepatocytes were prepared from day 21 p.c. fetal, day 22 p.c. fetal, day 1 p.p. neonatal or day 2 p.p. neonatal rats.

Incubation was for 30 minutes, in the absence of any adrenergic agent or in the presence of noradrenaline as shown (see text for details).

Glucose production rate is shown as a % of the control rate.
was the highest concentration investigated (Fig. 3.2). The maximal stimulation of the glucose production rate by noradrenaline was 22-73%, and the EC$_{50}$ for noradrenaline ranged from 180-260 nM, depending on age (Table 3.5).

**EFFECTS OF ISOPRENALEINE**

A concentration-related increase in the glucose production rate in hepatocytes freshly isolated from fetal, neonatal and adult female rats was observed during incubation in the presence of the $\beta$-adrenergic agonist, isoprenaline (Table 3.3 and Fig. 3.3). Isoprenaline concentrations below $10^{-8}$ M did not elicit statistically significant increases in the glucose production rate at any age. A concentration of $10^{-8}$ M produced a significant stimulation of glucose production in hepatocytes prepared at all ages except day 1 post partum, for which $10^{-7}$ M isoprenaline was required to produce an elevation of the glucose production rate, or adult male hepatocytes, when no significant increase in glucose production was observed at any concentration of isoprenaline investigated (Table 3.3). For hepatocytes prepared from neonatal rats on day 1 post partum, or from adult male rats, the greatest response to isoprenaline was at $10^{-6}$ M, the highest concentration investigated. However, at other perinatal ages, a decrease from the maximal stimulation observed was seen at this concentration; the increase in glucose production was maximal at $10^{-7}$ M isoprenaline for cells prepared on day 22 post coitum or day 2 post partum and at $10^{-8}$ M for cells isolated on day 21 post coitum (Fig. 3.3). Maximal increases over the control glucose production rate by isoprenaline varied from 29-57%, and the EC$_{50}$ for this agent was 1.7 - 90 nM, depending on the age studied (Table 3.5).

**EFFECTS OF PHENYLEPHRINE**

The $\alpha$-adrenergic agonist phenylephrine produced a concentration-dependent stimulation of the glucose production rate in hepatocytes freshly isolated from
**TABLE 3.3**

*Effect of isoprenaline on glucose production in hepatocytes freshly isolated from fetal, neonatal or adult rats*

<table>
<thead>
<tr>
<th>Concentration of Isoprenaline (M):</th>
<th>0</th>
<th>10⁻¹⁰</th>
<th>10⁻⁹</th>
<th>10⁻⁸</th>
<th>10⁻⁷</th>
<th>10⁻⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21 p.c.</td>
<td>219 ± 17</td>
<td>227 ± 25</td>
<td>246 ± 27</td>
<td>*282 ± 28</td>
<td>272 ± 20</td>
<td>263 ± 23</td>
</tr>
<tr>
<td>(n = 40)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
</tr>
<tr>
<td>Day 22 p.c.</td>
<td>541 ± 24</td>
<td>590 ± 33</td>
<td>621 ± 49</td>
<td>*675 ± 55</td>
<td>740 ± 49</td>
<td>722 ± 46</td>
</tr>
<tr>
<td>(n = 40)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
</tr>
<tr>
<td>Day 1 p.p.</td>
<td>254 ± 17</td>
<td>242 ± 27</td>
<td>298 ± 31</td>
<td>316 ± 40</td>
<td>**365 ± 40</td>
<td>387 ± 35</td>
</tr>
<tr>
<td>(n = 40)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
</tr>
<tr>
<td>Day 2 p.p.</td>
<td>230 ± 10</td>
<td>221 ± 14</td>
<td>226 ± 20</td>
<td>**351 ± 29</td>
<td>362 ± 29</td>
<td>329 ± 31</td>
</tr>
<tr>
<td>(n = 40)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
</tr>
<tr>
<td>Adult male</td>
<td>1180 ± 42</td>
<td>1180 ± 72</td>
<td>1224 ± 44</td>
<td>1202 ± 32</td>
<td>1257 ± 61</td>
<td>1262 ± 96</td>
</tr>
<tr>
<td>(n = 20)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Adult female</td>
<td>758 ± 10</td>
<td>790 ± 35</td>
<td>*803 ± 22</td>
<td>963 ± 49</td>
<td>1122 ± 43</td>
<td>1069 ± 31</td>
</tr>
<tr>
<td>(n = 20)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
</tbody>
</table>

Incubation was for 30 min. n = total number of incubations from 2 adult cell preparations, or 4 perinatal cell preparations (see text for details).

**a** Glucose production rate is shown as mean ± SEM.

* Significantly higher than control, p < 0.05

** Significantly higher than control, p < 0.01

*** Significantly higher than control, p < 0.005

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
FIGURE 3.3 Effect of isoprenaline on glucose production in hepatocytes freshly isolated from fetal, neonatal or adult rats.

Hepatocytes were prepared from adult male (△), adult female (▼), day 21 p.c. fetal (○), day 22 p.c. fetal (□), day 1 p.p neonatal (●), or day 2 p.p neonatal (■) rats.

Incubation was for 30 minutes, in the absence of any adrenergic agent, or in the presence of isoprenaline as shown (see text for details).

Glucose production rate is shown as a % of the control rate.
fetal and neonatal rats, although at a higher concentration range than the other catecholamines investigated (Table 3.4 and Fig. 3.4). $10^{-6}$M phenylephrine produced a statistically significant increase in the glucose production rate in hepatocytes prepared on day 22 post coitum, but $10^{-5}$M phenylephrine was the lowest concentration having any significant effect on glucose production in cells prepared at the other ages investigated (Table 3.4). At all ages except day 2 post partum, $10^{-4}$M phenylephrine produced a maximal response; in cells isolated from neonates on day 2 post partum, increasing the agonist concentration ten-fold caused a slight but non-significant increase in the glucose production rate, but hepatocytes prepared at other ages showed slightly less stimulation of glucose production when exposed to this higher concentration (Fig. 3.4). The maximal stimulation of the glucose production rate was 27-61% and the $EC_{50}$ ranged from 770-5000 nM, depending on age (Table 3.5).

**Glycogen breakdown**

When hepatocytes were prepared from fetal rats at term (day 22 post coitum) and used in concentration-response experiments, the glycogen content of the cells before and after incubation was determined in addition to the glucose production (Table 3.6 and Fig. 3.6). Glycogenolysis in hepatocytes freshly isolated from term fetal rats was stimulated in a concentration-dependent manner by catecholamines (Table 3.7). Although a clear trend was observed in the glycogen breakdown rate in the presence of increasing concentrations of any of the four adrenergic agonists investigated, a statistically significant decrease in the glycogen content of the cells after incubation in the presence of an agent, compared with that after incubation in control medium, was seen only for $10^{-5}$M noradrenaline or $10^{-4}$M phenylephrine (Table 3.6). The lack of statistical significance for the effects of the other two agents, or for the response to $10^{-6}$M noradrenaline, is attributable to the large inter-experimental variation in glycogen content (Table 3.6) and control glycogen breakdown rate (Table 3.7). Maximal responses of glycogenolysis to
<table>
<thead>
<tr>
<th>Concentration of Phenylephrine (M):</th>
<th>0</th>
<th>10⁻⁷</th>
<th>10⁻⁶</th>
<th>10⁻⁵</th>
<th>10⁻⁴</th>
<th>10⁻³</th>
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</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21 p.c.</td>
<td>199 ± 10 (n = 40)</td>
<td>191 ± 12 (n = 20)</td>
<td>210 ± 16 (n = 20)</td>
<td>*250 ± 17 (n = 20)</td>
<td>255 ± 14 (n = 19)</td>
<td>244 ± 14 (n = 20)</td>
</tr>
<tr>
<td>Day 22 p.c.</td>
<td>409 ± 12 (n = 50)</td>
<td>408 ± 19 (n = 25)</td>
<td>**472 ± 16 (n = 25)</td>
<td>507 ± 18 (n = 25)</td>
<td>522 ± 18 (n = 25)</td>
<td>513 ± 16 (n = 25)</td>
</tr>
<tr>
<td>Day 1 p.p.</td>
<td>128 ± 7 (n = 40)</td>
<td>134 ± 8 (n = 20)</td>
<td>131 ± 12 (n = 20)</td>
<td>**170 ± 11 (n = 20)</td>
<td>183 ± 12 (n = 20)</td>
<td>174 ± 8 (n = 20)</td>
</tr>
<tr>
<td>Day 2 p.p.</td>
<td>207 ± 9 (n = 40)</td>
<td>214 ± 14 (n = 20)</td>
<td>224 ± 14 (n = 20)</td>
<td>**293 ± 15 (n = 20)</td>
<td>329 ± 21 (n = 20)</td>
<td>334 ± 12 (n = 20)</td>
</tr>
</tbody>
</table>

Incubation was for 30 min. n = total number of incubations from 4 or 5 cell preparations (see text for details).

a  Glucose production rate is shown as mean ± SEM.

*  Significantly higher than control, p < 0.01

**  Significantly higher than control, p < 0.005

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
**FIGURE 3.4**

**Effect of phenylephrine on glucose production in hepatocytes freshly isolated from fetal or neonatal rats.**

Hepatocytes were prepared from day 21 p.c. fetal (○—○), day 22 p.c. fetal (□—□), day 1 p.p. neonatal (●—●), or day 2 p.p. neonatal (■—■) rats.

Incubation was for 30 minutes, in the absence of any adrenergic agent, or in the presence of phenylephrine as shown (see text for details).

Glucose production rate is shown as a % of the control rate.
TABLE 3.5

Sensitivity of glucose production in freshly isolated rat hepatocytes to the effects of adrenergic agonists

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>20 p.c.</th>
<th>21 p.c.</th>
<th>22 p.c.</th>
<th>1 p.p.</th>
<th>2 p.p.</th>
<th>Adult male</th>
<th>Adult female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADRENALINE:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. Stim.</td>
<td>31</td>
<td>41</td>
<td>35</td>
<td>32</td>
<td>39</td>
<td>41</td>
<td>47</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>81</td>
<td>200</td>
<td>13</td>
<td>14</td>
<td>17</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>NORADRENALINE:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. Stim.</td>
<td>-</td>
<td>22</td>
<td>39</td>
<td>60</td>
<td>73</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>-</td>
<td>260</td>
<td>180</td>
<td>200</td>
<td>230</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ISOPRENALINE:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. Stim.</td>
<td>-</td>
<td>29</td>
<td>37</td>
<td>52</td>
<td>57</td>
<td>6</td>
<td>48</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>-</td>
<td>1.7</td>
<td>2.0</td>
<td>3.7</td>
<td>2.7</td>
<td>-</td>
<td>90</td>
</tr>
<tr>
<td>PHENYLEPHRINE:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. Stim.</td>
<td>-</td>
<td>27</td>
<td>27</td>
<td>43</td>
<td>61</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>-</td>
<td>2900</td>
<td>770</td>
<td>4500</td>
<td>5000</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Max. Stim. = Maximal stimulation of glucose production rate observed (expressed as % above control glucose production rate).

b EC<sub>50</sub> = Concentration of agonist calculated to result in 50% of the maximal stimulation observed (expressed as nM).
<table>
<thead>
<tr>
<th>Agonist</th>
<th>Glycogen Content (µg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Incubation</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>508 ± 12 (n=40)</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>461 ± 8 (n=20)</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>597 ± 42 (n=40)</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>466 ± 22 (n=16)</td>
</tr>
</tbody>
</table>

Statistical evaluation was carried out on the mean of the replicate values for separate preparations. Significantly lower than control, *p < 0.05*, **p < 0.0005**. Glucose production rate is shown as mean ± SEM. The effect of adrenergic agonists on glycogen breakdown in hepatocytes freshly isolated from term fetal rats was evaluated. Incubation was for 30 min. n = total number of incubations from 2-4 cell preparations (see text for details).
FIGURE 3.5  
Effect of adrenergic agonists on glucose production in hepatocytes freshly isolated from term fetal rats.

Hepatocytes were isolated from day 22 p.c. fetal rats and incubated for 30 minutes, in the absence of any adrenergic agent, or in the presence of various concentrations of adrenaline (-----A), noradrenaline (——O), isoprenaline (□——)， or phenylephrine (●—●), as shown (see text for details).

FIGURE 3.6  
Effect of adrenergic agonists on glycogen breakdown in hepatocytes freshly isolated from term fetal rats.

Legend as for Figure 3.5.
TABLE 3.7

Effect of adrenergic agonists on glycogen breakdown rate in hepatocytes freshly isolated from term fetal rats

<table>
<thead>
<tr>
<th>Concentration of Catecholamine (M)</th>
<th>0</th>
<th>10^{-10}</th>
<th>10^{-9}</th>
<th>10^{-8}</th>
<th>10^{-7}</th>
<th>10^{-6}</th>
<th>10^{-5}</th>
<th>10^{-4}</th>
<th>10^{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonist</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenaline</td>
<td>230</td>
<td>-</td>
<td>258</td>
<td>262</td>
<td>276</td>
<td>282</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>198</td>
<td>-</td>
<td>174</td>
<td>204</td>
<td>208</td>
<td>276</td>
<td>258</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>396</td>
<td>402</td>
<td>440</td>
<td>496</td>
<td>450</td>
<td>458</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>338</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>404</td>
<td>376</td>
<td>444</td>
<td>464</td>
<td>440</td>
</tr>
</tbody>
</table>

Incubation was for 30 min.

a Glycogen breakdown rate was calculated from the mean glycogen content before and after incubation.
stimulation by catecholamines were seen at \(10^{-6}\)M adrenaline or noradrenaline, \(10^{-8}\)M isoprenaline and \(10^{-4}\)M phenylephrine (Fig. 3.6). The maximal stimulation produced, together with the concentration of agent calculated to produce 50% of the maximal stimulation observed (EC\(_{50}\)), for each of the agents investigated is shown in Table 3.8.

**Gluconeogenesis**

In all experiments investigating the production of glucose by hepatocytes in vitro, 10 mM lactate and 1 mM pyruvate were present in the incubation medium as gluconeogenic substrates. In some initial experiments investigating the effect of adrenaline or glucose production in hepatocytes isolated from term fetal rats, the lactate pool was labelled with \(^{14}\)C. Using ion exchange chromatography to remove any radioactive substrate, \(^{14}\)C \(\Delta\)-glucose release into the medium could be determined to allow an estimation of the rate of gluconeogenesis. Glucose production via de novo synthesis from gluconeogenic precursors was not present at a detectable level in hepatocytes freshly isolated from fetal rats on day 22 p.c..

### 3.3.2. RESPONSE-BLOCKING EXPERIMENTS

In this series of experiments, incubating hepatocytes isolated from fetal rats on day 21.5 post coitum in the presence of \(10^{-6}\)M adrenaline stimulated the glucose production rate to 141% of the control rate (Table 3.9 and Fig. 3.7). Incubation with the \(\beta\)-adrenergic antagonist propranolol alone at \(10^{-5}\)M had no significant effect on the glucose production rate; this concentration of propranolol effectively blocked the increase in glucose production elicited by \(10^{-6}\)M adrenaline.

Surprisingly, the \(\alpha\)-adrenergic antagonist phentolamine alone at \(10^{-5}\)M caused a significant increase above the control glucose production rate. When \(10^{-5}\)M phentolamine was present in the incubation medium together with \(10^{-6}\)M
### TABLE 3.8

**Sensitivity of glycogen breakdown in hepatocytes freshly isolated from term fetal rats to the effects of adrenergic agonists**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Max. Stim.</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenaline</td>
<td>23</td>
<td>1.0</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>39</td>
<td>160</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>25</td>
<td>1.1</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>37</td>
<td>3200</td>
</tr>
</tbody>
</table>

- **Max. Stim.** = Maximal stimulation of glucose breakdown rate observed (expressed as % above control glycogen breakdown rate).
- **EC<sub>50</sub>** = Concentration of agonist calculated to result in 50% of the maximal stimulation observed (expressed as nM).
TABLE 3.9

Effect of adrenergic antagonists on stimulation by adrenaline of glucose production in hepatocytes freshly isolated from near-term fetal rats

<table>
<thead>
<tr>
<th>Medium</th>
<th>Glucose Production Rate&lt;sup&gt;a&lt;/sup&gt; (nmole/10&lt;sup&gt;6&lt;/sup&gt; cells/h)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>278 ± 8</td>
<td>39</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt;M Adrenaline</td>
<td>**391 ± 17</td>
<td>22</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt;M Propranolol</td>
<td>264 ± 13</td>
<td>15</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt;M Adrenaline +</td>
<td>280 ± 15</td>
<td>17</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt;M Propranolol</td>
<td>264 ± 12</td>
<td>15</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt;M Phentolamine</td>
<td>**413 ± 21</td>
<td>17</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt;M Adrenaline +</td>
<td>**563 ± 34</td>
<td>17</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt;M ICI 118 551</td>
<td>262 ± 4</td>
<td>5</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt;M Adrenaline +</td>
<td>288 ± 7</td>
<td>5</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt;M ICI 118 551</td>
<td>266 ± 12</td>
<td>5</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt;M Atenolol</td>
<td>329 ± 20</td>
<td>5</td>
</tr>
</tbody>
</table>

Incubation was for 30 min. n = total number of incubations from 1-4 cell preparations (see text for details).

<sup>a</sup> Glucose production rate is shown as mean ± SEM

* Significantly higher than control, p < 0.05

** Significantly higher than control, p < 0.001

<sup>b</sup> Significantly higher than 10<sup>-6</sup>M adrenaline, p < 0.001

*Statistical evaluation was carried out on the mean of the replicate values for separate preparations.*
Hepatocytes were isolated from day 21.5 p.c. fetal rats and incubated for 30 minutes in the absence of any adrenergic agent, or in the presence of various agents, as shown (see text for details).
adrenaline, the glucose production rate was slightly more than double the control rate, and was significantly higher than the rate in the presence of $10^{-6}\text{M}$ adrenaline alone. The $\beta_1$-adrenergic antagonist atenolol and the $\beta_2$-adrenergic antagonist ICI 118 551 alone, each at $10^{-5}\text{M}$, produced no significant change in the glucose production rate compared with the control rate. ICI 118 551 at $10^{-5}\text{M}$ reduced the stimulation of glucose production caused by $10^{-6}\text{M}$ adrenaline to 5% above the control level, at which level the glucose production rate was not significantly different from the control rate. Atenolol also reduced the effect of adrenaline, but glucose production in the presence of both $10^{-6}\text{M}$ adrenaline and $10^{-5}\text{M}$ atenolol was still significantly higher than control rates of glucose production.

3.3.3. TIME-COURSE EXPERIMENTS

Two hour time-course

GLUCOSE PRODUCTION

The amount of glucose produced by hepatocytes isolated from fetal rats on day 22 post coitum and incubated for 30, 60, 90 or 120 min is shown in Table 3.10. The glucose production rate calculated for each incubation period is shown in Figure 3.8. The production rate was highest in the first 30 min of incubation, and decreased in each subsequent 30 min time period throughout the two hour incubation when the cells were incubated in control medium. When $10^{-6}\text{M}$ adrenaline was present in the incubation medium, a similar pattern of decreasing glucose production rate was seen until 90 min; the glucose production rate in the 90-120 min time period in hormone-exposed hepatocytes increased compared with that in the previous time period, and was of the same order as that in the 30-60 min incubation period (Fig. 3.8).

Significantly more glucose was produced in the presence of adrenaline than in its absence at each time point investigated (Table 3.10). The effect of $10^{-6}\text{M}$
TABLE 3.10

Effect of $10^{-6}$M adrenaline on glucose production in hepatocytes freshly isolated from term fetal rats

<table>
<thead>
<tr>
<th>Length of Incubation (min)</th>
<th>Glucose Production$^a$ (nmoles/10$^6$ cells)</th>
<th>Glucose produced in the presence of $10^{-6}$M Adrenaline (as % of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control medium</td>
<td>$10^{-6}$M Adrenaline</td>
</tr>
<tr>
<td></td>
<td>(n=15)</td>
<td>(n=15)</td>
</tr>
<tr>
<td>30</td>
<td>$293 \pm 14$</td>
<td>$333 \pm 23$</td>
</tr>
<tr>
<td></td>
<td>($n=15$)</td>
<td>($n=15$)</td>
</tr>
<tr>
<td>60</td>
<td>$434 \pm 23$</td>
<td>$546 \pm 28$</td>
</tr>
<tr>
<td></td>
<td>($n=15$)</td>
<td>($n=15$)</td>
</tr>
<tr>
<td>90</td>
<td>$533 \pm 36$</td>
<td>$661 \pm 38$</td>
</tr>
<tr>
<td></td>
<td>($n=15$)</td>
<td>($n=15$)</td>
</tr>
<tr>
<td>120</td>
<td>$618 \pm 44$</td>
<td>$877 \pm 56$</td>
</tr>
<tr>
<td></td>
<td>($n=15$)</td>
<td>($n=15$)</td>
</tr>
</tbody>
</table>

$n$ = total number of incubations from 3 cell preparations (see text for details).

$^a$ Glucose production is shown as mean $\pm$ SEM

* Significantly higher than control at this time point, $p < 0.05$

** Significantly higher than control at this time point, $p < 0.01$

*** Significantly higher than control at this time point, $p < 0.005$

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
FIGURE 3.8  
Effect of 10^{-6} M adrenaline on glucose production in hepatocytes freshly isolated from term fetal rats.

Hepatocytes were isolated from day 22 E.S. fetal rats and incubated for 30, 60, 90 or 120 minutes in the absence of any adrenergic agent (O—O), or in the presence of 10^{-6} M adrenaline (●—●) (see text for details).

FIGURE 3.9  
Effect of 10^{-6} M adrenaline on glycogen breakdown in hepatocytes freshly isolated from term fetal rats.

Legend as for Figure 3.8.
adrenaline on glucose production increased throughout the two hour time-course, whilst the effect of the agent on the glucose production rate showed a drop in the 60-90 min time period, and a large increase in the final incubation period (Fig. 3.8).

GLYCOGEN BREAKDOWN

The glycogen content of hepatocytes from term fetal rats over a two hour time-course incubation is shown in Table 3.11, and the glycogen breakdown rate calculated for the 0-30 min, 30-60 min, 60-90 min and 90-120 min time periods is shown in Figure 3.9. Whether the cells were incubated in the presence or absence of adrenaline, the glycogen breakdown rate was highest in the first 30 min of incubation, and decreased during the next incubation period. An increase was observed during the 60-90 min incubation period in control incubations, but a decrease was seen in adrenaline-exposed cells, and the glycogen breakdown rate in the presence or absence of adrenaline was lowest in the final 30 min of incubation (Fig. 3.9). When incubated in control medium, 24%, 33%, 45% and 56% of the glycogen initially present in the cells was degraded after 30, 60, 90 and 120 min of incubation, respectively.

The glycogen content of hepatocytes exposed to $10^{-6}$M adrenaline throughout the incubation was not statistically significantly lower than that of cells from a control incubation at any time point investigated during the two hour time-course (Table 3.11). However, it is clear that adrenaline did promote glycogenolysis in these cells; the absence of statistical significance is attributable to the large inter-experimental variation in the initial glycogen content of the hepatocytes. The stimulation of the glycogen breakdown rate by $10^{-6}$M adrenaline was greatest in the incubation period 30-60 min; in the following two incubation periods less glycogen breakdown was observed in the presence of adrenaline than in its absence (Table 3.11 and Fig. 3.9).
TABLE 3.11

Effect of 10^{-6}M adrenaline on glycogen breakdown in hepatocytes freshly isolated from term fetal rats

<table>
<thead>
<tr>
<th>Length of Incubation (min)</th>
<th>Glycogen content^a (μg/10^6 cells)</th>
<th>Glycogen content in the presence of 10^{-6}M Adrenaline (as % of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control medium</td>
<td>Medium + 10^{-6}M Adrenaline</td>
</tr>
<tr>
<td>0</td>
<td>654 ± 44</td>
<td>654 ± 44</td>
</tr>
<tr>
<td></td>
<td>(n=29)</td>
<td>(n=29)</td>
</tr>
<tr>
<td>30</td>
<td>497 ± 49</td>
<td>443 ± 41</td>
</tr>
<tr>
<td></td>
<td>(n=15)</td>
<td>(n=12)</td>
</tr>
<tr>
<td>60</td>
<td>438 ± 69</td>
<td>337 ± 30</td>
</tr>
<tr>
<td></td>
<td>(n=13)</td>
<td>(n=13)</td>
</tr>
<tr>
<td>90</td>
<td>361 ± 36</td>
<td>291 ± 31</td>
</tr>
<tr>
<td></td>
<td>(n=14)</td>
<td>(n=13)</td>
</tr>
<tr>
<td>120</td>
<td>288 ± 45</td>
<td>250 ± 36</td>
</tr>
<tr>
<td></td>
<td>(n=14)</td>
<td>(n=13)</td>
</tr>
</tbody>
</table>

n = total number of incubations from 3 cell preparations (see text for details).

^a Glycogen content is shown as mean ± SEM.

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
Three hour time-course

When glucose production in hepatocytes freshly isolated from term fetal rats was investigated over three hours of incubation, the glucose production rate decreased in each successive incubation period, and in the period two to three hours was low or negative, both in cells incubated in control medium and cells incubated in the presence of a hormone (Table 3.12). The effects of three hormones, adrenaline, noradrenaline and glucagon, were seen to differ greatly in this preparation. $10^{-5}$M adrenaline produced an increase in glucose production at each time point studied throughout the incubation, although the greatest (and the only statistically significant) effect was seen in the first hour. The same concentration of noradrenaline by contrast, showed an increasing stimulation of glucose production during the three hour time-course, attaining a significant level only in the final hour of incubation. In contrast, $10^{-5}$M glucagon did not stimulate glucose production significantly at any time throughout the three hours of incubation.

3.3.4. EFFECT OF MATERNAL DIABETES

Glucose production

The control glucose production rate in hepatocytes freshly isolated from fetal rats on day 22 post coitum was approximately 70% greater when the fetuses were obtained from dams in which diabetes had been induced on day 5 post coitum than when the fetuses were from normal dams (Table 3.13 vs. Table 3.1). Adrenaline had no significant effect on the glucose production rate in hepatocytes prepared from term fetal rats of gestationally diabetic dams. Adrenaline concentrations in
<table>
<thead>
<tr>
<th>ADRENALINE</th>
<th>NORADRENALINE</th>
<th>GLUCAGON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of Incubation (h)</td>
<td>Glucose production&lt;sup&gt;a&lt;/sup&gt; (nmoles/10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
<td>Glucose production&lt;sup&gt;a&lt;/sup&gt; (as % of control)</td>
</tr>
<tr>
<td>1</td>
<td>222 ± 5&lt;sup&gt;a&lt;/sup&gt; (n=7)</td>
<td>-</td>
</tr>
<tr>
<td>CONTROL MEDIUM</td>
<td>2</td>
<td>335 ± 31&lt;sup&gt;a&lt;/sup&gt; (n=8)</td>
</tr>
<tr>
<td>3</td>
<td>339 ± 32&lt;sup&gt;a&lt;/sup&gt; (n=8)</td>
<td>-</td>
</tr>
</tbody>
</table>

1  | 281 ± 23<sup>a</sup> (n=8) | 127 | 227 ± 17<sup>a</sup> (n=9) | 102 | 306 ± 27<sup>a</sup> (n=8) | 86 |
| MEDIUM + | 2 | 382 ± 31<sup>a</sup> (n=8) | 114 | 330 ± 37<sup>a</sup> (n=10) | 113 | 607 ± 78<sup>a</sup> (n=7) | 101 |
| 10<sup>-5</sup>M HORMONE | 3 | 412 ± 29<sup>a</sup> (n=8) | 122 | **429 ± 34<sup>a</sup> (n=10) | 131 | 573 ± 73<sup>a</sup> (n=7) | 96 |

n = total number of incubations from 2 cell preparations (see text for details).

<sup>a</sup> Glucose production is shown as mean ± SEM

* Significantly higher than control at this time point, p < 0.05

** Significantly higher than control at this time point, p < 0.025

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
TABLE 3.13

Effect of adrenaline on glucose production in hepatocytes freshly isolated from term fetal rats of gestationally diabetic dams

<table>
<thead>
<tr>
<th>Concentration of Adrenaline (M)</th>
<th>Glucose Production Rate&lt;sup&gt;a&lt;/sup&gt; (n mole/10&lt;sup&gt;6&lt;/sup&gt; cells/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>732 ± 66 (n=82)</td>
</tr>
<tr>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>732 ± 119 (n=24)</td>
</tr>
<tr>
<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>794 ± 121 (n=25)</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>763 ± 106 (n=24)</td>
</tr>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>746 ± 83 (n=45)</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>770 ± 89 (n=44)</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>628 ± 131 (n=19)</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>664 ± 119 (n=19)</td>
</tr>
<tr>
<td>10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>628 ± 131 (n=19)</td>
</tr>
</tbody>
</table>

Incubation was for 30 min.

n = total number of incubations from 4 or 5 cell preparations (see text for details).

<sup>a</sup> Glucose production rate is shown as mean ± SEM.

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
the range used in concentration-response experiments with hepatocytes isolated from fetuses of normal maternal rats (10^{-10} M - 10^{-6} M), and extending to concentrations 10, 100 and 1000-fold higher, were investigated (Table 3.13 and Fig. 3.10). Concentrations of 10^{-5} - 10^{-3} M adrenaline produced a slight, non-significant, decrease in the glucose production rate.

**Glycogen breakdown**

The glycogen content of hepatocytes immediately after isolation from term fetal rats of gestationally diabetic dams was approximately 30% higher than the content of cells freshly isolated from fetuses of normal dams (Table 3.14 vs. Table 3.6). However, after 30 min incubation the glycogen content of hepatocytes from the offspring of diabetic mothers had dropped to approximately the same as, or slightly less than, that in identically-treated cells from the young of normal mothers.

Adrenaline at concentrations from 10^{-10} M - 10^{-3} M has no statistically significant effect on glycogen breakdown in hepatocytes freshly isolated from term fetuses of gestationally diabetic dams (Table 3.14 and Fig. 3.11).
### TABLE 3.14

**Effect of adrenaline on glycogen breakdown in hepatocytes freshly isolated from term fetal rats of gestationally diabetic dams**

<table>
<thead>
<tr>
<th>Concentration of Adrenaline (M)</th>
<th>Before Incubation</th>
<th>After Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10^-10</td>
</tr>
<tr>
<td>Glycogen Content(^a) (µg/10^6 cells)</td>
<td>649 ± 44 (n=50)</td>
<td>377 ± 27 (n=48)</td>
</tr>
<tr>
<td>Glycogen Breakdown Rate(^b) (µg/10^6 cells/h)</td>
<td>-</td>
<td>544</td>
</tr>
</tbody>
</table>

Incubation was for 30 min.

n = total number of incubations from 4 or 5 cell preparations, except 10^-5 - 10^-3 M, when only one cell preparation was used (see text for details).

\(^a\) Glycogen content is shown as mean ± SEM.

\(^b\) Glycogen breakdown rate was calculated from the mean glycogen content before and after incubation.

Statistical evaluation was carried out on the mean of the replicate values for separate preparations.
FIGURE 3.10  
Effect of adrenaline on glucose production in hepatocytes freshly isolated from term fetal rats of normal and gestationally diabetic dams.

Hepatocytes were isolated from day 22 d. v. fetal rats of normal (O—O) or gestationally diabetic (●—●) dams. Incubation was for 30 minutes, in the absence of any adrenergic agent, or in the presence of adrenaline as shown (see text for details).

FIGURE 3.11  
Effect of adrenaline on glycogen breakdown in hepatocytes freshly isolated from term fetal rats of normal and gestationally diabetic dams.

Legend as for figure 3.10.
3.4 DISCUSSION

3.4.1 OVERVIEW

The experiments reported in this chapter involved measuring the in vitro production of glucose in hepatocytes isolated from adult or perinatal rats. The method for isolation of hepatocytes from rats during the perinatal period (described in 3.2.4) produced viable, hormone-responsive cells. Adult hepatocytes produced by a liver perfusion technique (see 3.2.3) were also shown to produce glucose at a rate which was susceptible to hormonal influence. Although hepatocyte suspensions always showed high viability (never less than 95% when freshly isolated), and 5mM glucose was present in the media throughout the preparation period to prevent premature glycogen degradation, a large inter-preparation variation in the initial glycogen content and control glucose production rate was noted for cells isolated from fetal or neonatal animals at any one age. This variability may be partially attributable to differences in the precise age of the animals, resulting from variations in the exact time of conception, as the control glucose production rate varied markedly with age as anticipated (see e.g. Table 3.1). However, the magnitude of the variation, and the observation of similar, albeit slightly lesser, variability in glucose production by adult hepatocytes, excludes this from being the exclusive cause.

Rothermel et al. (1984) observed variations in control glucose production rates in adult male hepatocytes. Schülze et al. (1984) noted a similar variation in basal glucose production rates and also in glycogen content of term fetal hepatocytes prepared on different occasions, and suggested seasonal variation as a possible factor; this does not appear likely to be of particular significance in the work reported here, as even cell preparations produced on consecutive days frequently showed marked differences. Whatever the cause of variation in initial glycogen content and control glucose production rates, this resulted in large standard errors of mean values, thus reducing the possibility of attaining statistical significance for the effects of the agents investigated.
3.4.2 FINDINGS IN ADULT HEPATOCYTES

Control Glucose Production

Relatively little has been published concerning the response of glucose production in fetal or neonatal hepatocytes to the effects of adrenergic agonists (see 3.4.3), and therefore to validate the incubation and assay techniques used, some concentration response experiments were carried out using adult cells (Table 3.1 and Table 3.3).

Hepatocytes isolated from adult male rats showed a mean control production rate of 1434 nmoles glucose per $10^6$ cells per hour; unstimulated hepatocytes from adult females produced glucose at a rate of 757 nmoles per $10^6$ cells per hour.

Effects of Adrenergic Agents

In accordance with published observations, discussed in Chapter 1 (section 1.3.2), glucose production in both adult male and female hepatocytes was stimulated by adrenaline in the concentration range $10^{-8} - 10^{-6}M$, whereas only the glucose production rate in cells isolated from female adult rats was increased by the $\beta$-adrenergic agonist isoprenaline in the concentration range of $10^{-10} - 10^{-6}M$. These findings therefore support the current view that $\beta$-adrenoceptors are of functional importance in the adult female rat liver, but not in the male (Birnbaum & Fain, 1977; Studer & Borle, 1982). A more thorough investigation of the sex differences in adrenergic-responsiveness of adult rat hepatocytes was outside the scope of the present study.

3.4.3 FINDINGS IN PERINATAL HEPATOCYTES

Control Glucose Production and Glycogen Breakdown

The only literature reports of the determination of glycogen degradation and glucose production in freshly isolated fetal hepatocytes, and their stimulation by catecholamines, have come from Dargel's group in the German Democratic
Republic (Hühn et al., 1983; Schulze et al., 1984). The initial glycogen content of term fetal hepatocytes reported by this group was 1.17 μmol per 10^6 cells (Huhn et al., 1983), which is less than 50% of the mean value of 2.84 μmol per 10^6 cells found in the present study. This difference might be partly due to strain differences, but is probably largely due to the degradation of glycogen during hepatocyte isolation using the technique developed in Dargel's laboratory. In this method, based on incubation of liver material in the presence of trypsin, no glucose was included in the preparative media, so that glycogen breakdown might be expected to commence during hepatocyte isolation. Also, the dam was anaesthetised prior to obtaining fetuses, a procedure known to induce the loss of glycogen (DiMarco et al., 1976; Furner et al., 1972). The lower initial glycogen content of hepatocytes provides an explanation of the considerably lower glycogen breakdown and glucose release rates observed by Dargel's group compared with those documented here:

<table>
<thead>
<tr>
<th>Glycogen breakdown</th>
<th>Glucose production</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μmole glucose/10^6 cells/h)</td>
<td></td>
</tr>
<tr>
<td>Hühn et al. (1983)</td>
<td>0.23</td>
</tr>
<tr>
<td>Schulze et al. (1984)</td>
<td>0.47 - 0.63</td>
</tr>
<tr>
<td>Present study</td>
<td>1.11 - 2.22</td>
</tr>
</tbody>
</table>

The glucose production rates reported in this work are obtained by measurement of the glucose concentration in the medium in which hepatocytes have been suspended during incubation. However, this concentration is actually the net result of two intracellular processes - glucose production and glucose utilisation. The production of glucose in hepatocytes might be from breakdown of stored glycogen, but may also result from de novo synthesis from gluconeogenic precursors. In experiments studying glucose production by adult hepatocytes, animals are often starved prior to preparation of cells to deplete the cellular glycogen; it is then assumed that any glucose produced is exclusively via the gluconeogenic pathway. This technique is not suitable for perinatal animals, and
therefore in the work reported in this chapter gluconeogenesis and glycogenolysis were measured explicitly in hepatocytes from day 22 post coitum animals, although time limitations restricted the determinations made at other ages to total glucose production alone.

Glycogen breakdown rates in hepatocytes isolated from term fetal rats were more than sufficient to account for the glucose released into the incubation medium; the proportion of degraded glycogen which was released as glucose was unaffected by the presence of any agent stimulating the rate of glycogen breakdown (e.g. Table 3.1 & Table 3.6).

The absence of detectable gluconeogenic activity in freshly isolated term fetal hepatocytes might be predicted from the observed delay in the onset of gluconeogenesis in the immediately post partum rat liver (Ballard, 1971a; Medina et al., 1980).

**Concentration-Response Experiments**

Concentration-response curves were typically sigmoidal (Figures 3.1, 3.2, 3.3, 3.4 & 3.6). At higher concentrations of some agents, a decrease in the response occurred; this phenomenon is probably attributable to toxic effects of the compounds, although no reduction was seen in viability as determined by trypan blue exclusion.

The response to catecholamines of glucose production pathways in hepatocytes freshly isolated from term fetal rats observed in this series of experiments concurs with the findings of Hühn et al. (1983). However, the magnitude of the response was somewhat lower in the current work than that reported by Hühn et al. These workers observed stimulation of glycogen breakdown and glucose release by $10^{-6}$M phenylephrine to 133% and 147% of the control level,
respectively. In the present work, this concentration of α-adrenergic agonist promoted glycogen breakdown and glucose release to 111% and 115% of control, respectively; $10^{-4}\text{M}$ phenylephrine (the maximally effective concentration) produced increases in these pathways of 37% and 27%, respectively. $5 \times 10^{-6}\text{M}$ isoprenaline was reported by Hühn et al. to stimulate glycogenolysis and glucose release to 183% and 202% of the control values, respectively. The highest concentration of isoprenaline investigated in the current work was $10^{-5}\text{M}$, which caused an increase to 116% and 133% of control in glycogen breakdown and glucose release, respectively. For both of these processes, the concentration of isoprenaline eliciting the maximum response in the current study was below $10^{-6}\text{M}$.

The response to α- and β-adrenergic agonists found in the current study may be lower than those reported by Hühn et al. for a number of reasons. Strain differences in hepatic adrenoceptor populations could cause variability in catecholamine responsiveness between strains. Also, the lower control glycogen breakdown and glucose production rates observed by Hühn et al. compared with this study, as discussed earlier in this section, might account for the higher susceptibility of these pathways to hormonal stimulation. The difference in the incubation periods in the two studies (30 min for the current work and one hour for the study by Hühn et al.) might also be expected to result in different responses to adrenergic agents in the two investigations; as reported in section 3.3.3, the stimulation of glucose production by adrenaline was greater in the 30-60 min incubation period than the 0-30 min period.

When considering the findings from the concentration response experiments (see section 3.3.1), the effects of any of the adrenergic agents on glucose production in vitro may be evaluated with regard to two parameters: the greatest stimulation above the control rate elicited by an agent and the concentration of that agent required to produce a certain level of stimulation. Table 3.5 shows the
maximal stimulation (% increase above control) and the $EC_{50}$ (concentration producing 50% of the maximal stimulation) for each of the four adrenergic agonists used, at each of the ages studied. From these data, the perinatal ages at which the hepatocytes were isolated may be ranked to indicate age-dependent changes in sensitivity to the four agents. Below, the ages are ordered by descending responsiveness (descending maximal stimulation, ascending $EC_{50}$ values):

**MAXIMAL STIMULATION:**

<table>
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<tr>
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<tbody>
<tr>
<td>Adrenaline</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td></td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>&gt;</td>
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</table>

$EC_{50}^*$:

<table>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Adrenaline</td>
<td>&lt;</td>
<td>&lt;</td>
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<td>&lt;</td>
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<tr>
<td>Noradrenaline</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Isoprenaline</td>
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<td>&lt;</td>
</tr>
<tr>
<td>Phenylephrine</td>
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Changes with age in three factors might produce changes in the responsiveness of cellular processes to external agents; these factors are receptor number, receptor affinity and post-receptor events. In the situation where saturating concentrations of drug are present, an increase in receptor number will result in an increase in the maximal stimulation, provided that post-receptor events are not limiting, but will also increase the $EC_{50}$ for the drug. An increase of affinity in this situation would not affect the maximal response, but would reduce the $EC_{50}$. Changes in post-receptor events would influence the maximal response obtained, but would not affect the $EC_{50}$.
The concentration-response experiments reported here showed an increase in the maximal stimulation of glucose production in hepatocytes in vitro by adrenergic agents with increasing perinatal age, with the exception of adrenaline for which very little variation in the maximal response with age was seen. The ranking of EC$_{50}$ values suggests maximal sensitivity to the effects of adrenergic agents at term (or immediately before term for isoprenaline), with reduced sensitivity to any of the agents investigated occurring by day 2 post partum.

Factors which might produce the nadir in EC$_{50}$ value at term include a larger receptor population, high affinity for the agent under investigation, and/or a more effective linkage with, and execution of, post-receptor pathways. From the current work it is not possible to ascertain the relative importance of these factors; however the pattern of variation in hepatic adrenoceptor number through the perinatal period reported in Chapter 2 suggests that the size of the adrenoceptor population is an important parameter. As receptor numbers do not show a steady increase throughout the period studied, the increase in maximal stimulation with increasing age must be additionally attributable to changes in post-receptor events.

A well established event which occurs immediately after occupation of a β-adrenoceptor by an agonist is activation of the enzyme adenyl cyclase (Sutherland & Robison, 1969). Changes in the basal or hormone-stimulated activity of this enzyme in rat liver during development are well documented (Bitensky et al., 1970; Butcher & Potter, 1972; Christoffersen et al., 1973; Christoffersen & Øye, 1974). Changes in the coupling of β-adrenoceptors and adenyl cyclase with age have been shown in other tissues (Smith, 1984), and Kalish et al. (1977) have proposed that changes in hepatic cell membranes during development might produce changes in the catalytic sub-unit of adenyl cyclase. The hepatic activity of the enzyme phosphodiesterase, which hydrolyses the cyclic AMP formed as a result of adenyl cyclase activity, has also been observed to change in the perinatal rat.
(Christoffersen et al., 1973). As the cellular events which were seen in the current work to be influenced by adrenergic activation all depend on the activities of enzymes of carbohydrate metabolism, changes in such activities during development (Beaudry et al., 1977; Vernon & Walker, 1968; Burch et al., 1963) may also be at least partly responsible for the observed differences in response to adrenergic agonists of hepatocytes prepared from animals at different perinatal ages.

The concentration-response curves for the glucose production rate in hepatocytes isolated from term fetal rats and incubated in the presence of each of the catecholamines investigated are shown in Figure 3.5. The parallel shift of the curve when hepatocytes are exposed to agents with differing affinities for α- and β-adrenoceptors indicates the nature of the receptor mediating the stimulation of glucose production by these agents. The shift of the curve showing the response to increasing phenylephrine concentrations to the right of that for isoprenaline, together with an almost 400-fold higher EC$_{50}$ for phenylephrine compared with isoprenaline, demonstrates clearly that the effect of catecholamines on glucose production in term fetal rat hepatocytes is a β-adrenoceptor mediated phenomenon (Ahlquist, 1948). Similarly, the parallel shift to the right of the noradrenaline response curve compared with the adrenaline response curve, and the approximately ten-fold higher EC$_{50}$ for noradrenaline than for adrenaline, demonstrates that the β-adrenoceptor involved is of the β$_2$-subtype (Lands et al., 1967).

As for the effects on glucose production, comparison of the concentration-response curves (Fig. 3.6) produced by the adrenergic agonists studied, and of their EC$_{50}$ values (Table 3.7) confirm catecholamine effects on glycogen breakdown to be mediated primarily via β$_2$-adrenoceptors (cf. 3.3.1.).
Response Blocking Experiments

As previously discussed, hepatic carbohydrate metabolism in the adult male rat is believed to be predominantly, if not exclusively, affected by adrenergic agents via α-adrenoceptors, whilst in the adult female and immature animals, β-adrenoceptors have a substantial involvement; the work presented in this chapter supports such a concept. The findings from both radioligand receptor binding studies on hepatic membranes and concentration-response experiments with isolated hepatocytes suggested a predominance of β$_2$-adrenoceptors, and response blocking experiments were devised in order to confirm this finding.

The effects of propranolol, a non-subtype selective antagonist at the β-adrenoceptor, and the selective agents atenolol (β$_1$) and ICI 118 551 (β$_2$), on stimulation of glucose production in hepatocytes freshly isolated from near-term fetal rats by adrenaline provide further evidence that the predominant adrenoceptor activated in producing this response is of the β$_2$ subtype.

This effect of the α-antagonist phentolamine, in promoting glucose production, was unexpected. This agent elicited a 50% increase in the glucose production rate compared with the control rate, and when it was present in the incubation medium together with adrenaline, the very high rate of glucose production observed appeared to suggest an additive stimulatory effect of these two agents (Table 3.8). Phentolamine, a non-subtype selective α-adrenergic antagonist (Goodhardt et al., 1982), might produce an increase in glucose production by blocking the effect of adrenergic agonists at α$_2$-adrenoceptors; activation of this α-adrenoceptor subtype is associated with a decrease in adenyl cyclase activity (see section 1.3.1) and thus a reduction in glucose production. The relative size of the α$_2$-adrenoceptor population of the perinatal rat liver is disputed: Butlen et al. (1980) reported that hepatic α-adrenoceptors in fetal and
neonatal rats are mostly of the $\alpha_1$ subtype, whilst McMillan et al. (1983) showed figures in their work suggesting that $\alpha_2$-adrenoceptors were at least two-fold more numerous than $\alpha_1$-adrenoceptors throughout the perinatal period. However, even if substantial numbers of $\alpha_2$-adrenoceptors are present in the liver of near-term fetal rats, blocking of these receptors by phentolamine would affect only glucose production influenced by their potential activation due to the presence of $\alpha$-adrenergic agonists. In the preparation used, extensive washing was used to remove any endogenous agents, so that hepatocytes were exposed only to compounds included in the incubation mixture. It is therefore difficult to explain the stimulatory effect of phentolamine alone on glucose production in terms of a decreased occupation of $\alpha_2$-adrenoceptors by adrenergic agonists.

Although no explanation of the observed effects of phentolamine on in vitro hepatic glucose production is currently available, the reproducibility of the effect, and the observations of the effects of this compound in an $\alpha$-adrenoceptor radioligand binding assay (see section 2.3.2) suggest that this is a genuine finding. Other workers have found that the effects of adrenaline on tyrosine aminotransferase induction in fetal hepatocytes are potentiated by phentolamine, although the effects of the $\alpha$-antagonist alone have not been investigated (G. Sparmann, Institut fur Biochemie, Leipzig, GDR, personal communication).

**Time Course Experiments**

Having established an optimal concentration of adrenaline for stimulation of in vitro glucose production, the time course of this stimulatory effect on glycogen breakdown and glucose production in term fetal hepatocytes was studied over two hours (Table 3.10 & Table 3.11; Figure 3.8 & Figure 3.9). The abilities of adrenaline and noradrenaline to increase glucose production during a three hour incubation were also compared with the effect of an identical concentration of glucagon (Table 3.12).
A general trend of decreasing glucose production rates (and decreasing glycogen breakdown rates when these were assessed) in successive time periods was observed. This might be expected if glucose production is the result of glycogen breakdown, as Abdullah et al. (1963) showed that the rate of glycogen breakdown is proportional to the molecular weight of the glycogen being degraded; thus, as the mass of glycogen remaining decreases, so will the rate of glucose production. The proportion of degraded glycogen which was released as glucose into the medium also decreased with time during the two hour time course (Table 3.11).

In the current work, 10^{-5}M glucagon did not produce a significant increase in the glucose production rate after one, two or three hours of incubation (Table 3.12). In hepatocytes isolated from adult rats, glucagon at concentrations as low as 5 \times 10^{-11}M have been shown to stimulate glucose production (Assimacopoulos-Jeannet et al., 1982). Huhn et al. (1983) reported stimulation to 124% and 115% of the control rate of glucose production rate and glycogenolysis, respectively, in term fetal hepatocytes exposed to 2.9 \times 10^{-7}M glucagon over three hours. It is possible that the glucagon concentration chosen in the current work was supra-optimal, however other workers have also observed insensitivity of the fetal rat liver to the effects of glucagon (Blazquez et al., 1976; Snell & Walker, 1978; Vinicor et al., 1976).

**Experiments using the Offspring of Diabetic Rats**

The elevation in the control glucose production rate in hepatocytes isolated from term fetal rats of diabetic mothers is the result of the higher rate of glycogen breakdown in these cells compared with those obtained from the offspring of normal dams (Table 3.13 & Table 3.14; Figure 3.10 & Figure 3.11). Glycogen-breakdown rates in hepatocytes from fetuses of diabetic rats were 180-240% of those seen in hepatocytes from normal fetuses. This additional glycogenolysis is
more than sufficient to account for the increase in glucose production seen, and in fact the diabetic condition led to a decrease in the proportion of degraded glycogen released as glucose. The high rate of glycogen breakdown in hepatocytes from the young of diabetic rats results from the high hepatic glycogen content of these animals (Cuezva & Patel, 1980a; Snell 1982b), and thus the high initial glycogen content observed in the isolated hepatocytes used in this study. Fetal hyperinsulinaemia is exaggerated in the offspring of gestationally diabetic rats (Cuezva & Patel, 1980b; Cuezva et al., 1982a), and this, together with fetal hyperglycaemia (both of which result from maternal hyperglycaemia) are responsible for this increase in hepatic glycogen content in the infants of diabetic mothers.

The observations of the effects of maternal diabetes on control glucose metabolism in hepatocytes freshly isolated from term fetal rats were therefore as predicted from known facts. The finding of an apparently absent, or at least severely attenuated, response of glycogenolysis and glucose production to the stimulatory effects of adrenaline in hepatocytes from the fetuses of gestationally diabetic dams has not, however, been previously reported. Dighe et al. (1984) has reported a decrease in \[^{3}H\] prazosin binding sites (presumably representing \(\alpha_1\)-adrenoceptors) in the livers of adult rats with streptozotocin-induced diabetes, but no reports of effects on \(\beta\)-adrenoceptors, or on any receptors in the offspring of affected animals, have been made.

A prolonged period of postnatal hypoglycaemia has been observed in neonatal rats born to diabetic dams (Cuezva et al., 1982b; Snell, 1982b). Snell suggested that this resulted from a delay in the initiation of glycogen breakdown secondary to the hyperinsulinaemic condition of these animals. Attenuation of the postnatal increase
in plasma glucagon has also been proposed as a cause of the delay in glycogenolysis (Cuezva & Patel, 1983). The findings reported in this chapter provide another possible explanation of the observation: diminution of adrenergic responsiveness may reduce adrenergic hormone stimulation of glycogen breakdown, resulting in a delay in recovery of plasma glucose levels postnatally.

3.4.4 SIGNIFICANCE OF FINDINGS

The work reported in this Chapter clearly demonstrates that glucose production and glycogen breakdown in hepatocytes freshly isolated from rats during the perinatal period are sensitive to the stimulatory effect of adrenergic agents. Comparison of concentration-response curves for adrenergic agonists and blocking of adrenergic effects with adrenoceptor type and subtype-specific compounds confirm that adrenergic effects on carbohydrate metabolism in the perinatal rat liver, in contrast to the adult, are predominantly $\beta_2$-mediated.
CHAPTER 4

ADRENERGIC EFFECTS ON CARBOHYDRATE METABOLISM IN THE PERINATAL RAT IN VIVO
4.1 INTRODUCTION

4.1.1 ADVANTAGES AND DISADVANTAGES OF IN VIVO EXPERIMENTS

The use of intact animals to investigate the adrenergic responsiveness of perinatal glucose production pathways overcomes many of the limitations of in vitro techniques. Thus, both intra-hepatic and inter-organ relationships, lost when experiments are carried out in isolated cells, are still active when work is conducted in vivo. Provided that suitable controls are used, definite conclusions regarding the physiological role of the adrenergic system in the newly born rat may be drawn from in vivo experiments, whereas in vitro work can show only the potential significance of this system.

However, findings made from in vivo experiments require very careful interpretation. Although a particular response may be observed in an animal, the organ(s) and biochemical pathway(s) affected to produce such a response cannot be easily established. If in vivo administration of an adrenergic agent is seen to produce a change in plasma glucose concentration, there is no evidence that this is due to adrenergic modulation of hepatic glucose production pathways. In addition, the dose of any administered substance is crucial; if plasma levels in excess of those normally present in vivo are obtained, then changes observed may be due to pharmacological rather than physiological effects.

4.1.2 AGENTS USED AND TIME POINTS STUDIED

In this series of experiments, the effects of adrenaline, the α-adrenergic antagonist phentolamine, and the β-adrenergic antagonist propranolol were examined by their intraperitoneal administration immediately after caesarian delivery of term fetal rats.
If activation of hepatic adrenoceptors immediately after birth stimulates liver glucose production \textit{in vivo}, then administration of adrenaline would be expected to produce an elevation of plasma glucose concentration. If increased glucose production by adrenaline is the result of stimulation of hepatic glycogen breakdown, then a relatively greater loss of glycogen in adrenaline-treated neonates should be seen than in control animals. As plasma glucose levels in untreated neonates have been reported to reach a nadir at one to two hours \textit{post partum} (Cake et al., 1971; Cuezva et al., 1982a; Dawkins, 1963a; Girard et al., 1973a; Snell & Walker, 1973b), this time period should be the most sensitive to the effects of agents increasing plasma glucose concentration. Therefore, in experiments investigating the effects of \textit{in vivo} administration of adrenaline, plasma glucose and lactate concentrations and liver glycogen content were determined two hours after delivery.

If activation of $\alpha$- or $\beta$-adrenoceptors is an essential event in the increase in hepatic glucose production in the postnatal period, then blocking of these receptors with phentolamine or propranolol, respectively, would be expected to prevent the elevation of plasma glucose resulting from increased hepatic glucose production. Recovery from postnatal hypoglycemia in the rat is usually seen to be complete by three or four hours \textit{post partum} (Blazquez et al., 1974; Butcher & Potter, 1972; Cuezva et al., 1982a; Fernandez et al., 1983; Girard et al., 1973a; Kervran et al., 1976); therefore the effects of an agent which interferes with the pathways of glucose production responsible for the return to normoglycemia should be most easily demonstrated at this time. Three hours after delivery was therefore chosen as the optimum time point to investigate the effects of phentolamine and propranolol on plasma glucose and lactate and liver glycogen levels in the neonatal rat \textit{in vivo}.
Because of the importance of the dose of agent administered in an in vivo experiment (see section 4.1.1), the potential elevation of plasma catecholamine levels by intraperitoneally administered adrenaline was carefully considered when choosing the dose range to be investigated. The plasma concentration of catecholamines at the time of caesarian delivery of term rat fetuses has been reported to be around 7 ng/ml, dropping rapidly to around 1 ng/ml at one hour post partum (Cuezva et al., 1982a). Assuming a volume of distribution of 4 ml, and if there was complete absorption and no metabolism or excretion of exogenously administered adrenaline, then doses in the range of 4 to 400 ng would produce increases in the plasma catecholamine concentration at one hour post partum of approximately 2 to 100-fold. Initial experiments therefore involved administration of doses of adrenaline in this range in an attempt to establish a threshold dose producing an elevation of plasma glucose concentration. Doses of adrenaline used in later experiments, and the reasons for using such doses, will be discussed in section 4.4.2. The doses of antagonist used were based on adrenaline doses observed to produce effects, and will be considered in the discussion of this series of experiments (section 4.4.3).

4.1.3 AIMS OF THE EXPERIMENTS

Experimental findings reported in Chapters 2 and 3 provide evidence for a changing population of β-adrenoceptors on rat liver membranes during the perinatal period, and for the responsiveness of glucose production in hepatocytes freshly isolated from perinatal rats to the stimulatory effects of adrenergic agonists. However, although this establishes that β-adrenoceptors are present, and are coupled to post-receptor events affecting carbohydrate metabolism within the hepatocyte, it provides no insight into the possible physiological significance of hepatic adrenoceptor activation in the newly born rat. In vivo experiments are essential to investigate this question.
In these experiments the effects of an adrenergic agonist, adrenaline, and two adrenergic antagonists, phentolamine and propranolol, on glucose metabolism in vivo in the immediately postnatal period were investigated. If adrenaline can be shown to produce changes in glucose metabolic pathways, then this indicates that adrenoceptor activation might influence postnatal carbohydrate metabolism. More significantly, if an antagonist at the adrenoceptor is observed to prevent the recovery in the plasma glucose level usually seen in the healthy neonatal rat from around two hours post partum (Cake et al., 1971; Dawkins, 1963a; Fernandez et al., 1983; Snell & Walker, 1973b), it may be argued that activation of adrenoceptors is an essential factor in the physiological return to normoglycemia in the postnatal period in the rat.
4.2 MATERIALS & METHODS

4.2.1 MATERIALS

(+)Adrenaline, amyloglucosidase (7 iu/mg as a suspension in 3.2M (NH₄)₂SO₄) and type II oyster glycogen were obtained from Sigma Chemical Company Ltd., Poole, Dorset. Glacial acetic acid was from May & Baker Ltd., Dagenham, Essex. DL-Propranolol HCl was a kind gift from ICI, Alderley Edge, Cheshire; prazosin was generously donated by Pfizer Ltd., Sandwich, Kent. Phentolamine mesylate was supplied by Ciba Laboratories, Horsham, West Sussex. All other reagents were from BDH Chemicals Ltd., Poole, Dorset.

4.2.2 ANIMALS

Rats of a random-bred Wistar albino strain, obtained from the University of Surrey Animal Unit, were used. Each male was housed overnight with 5-10 females, and pregnancy was established by palpation 10 to 20 days after mating. The day following overnight housing with the male was taken as day 1 post coitum. Unanaesthetized maternal rats were killed by cervical dislocation. All animals were given food and water ad libitum.

Term fetal rats were obtained by caesarian section at a time between 9 and 10.30 a.m. on day 22 post coitum. Umbilical cords were broken by a sharp tug close to the fetal trunk. This procedure, which mimics that observed in the natural situation, prevented the blood loss seen if the cords were cut, and avoided time-consuming suturing. Following delivery, all fetuses of a litter were weighed together, and a mean weight per fetus was calculated. Fetuses were then randomly assigned to one of three groups. Animals in the first and second group were given an intraperitoneal injection of 5 μl of 20 μg/ml ascorbic acid in 0.9% NaCl solution, using a Hamilton microsyringe (Hamilton Bonaduz AG, Bonaduz,
Switzerland) held in a clamp. Animals of the first group were killed immediately, those of the second group were placed on tissue dampened with water at 37°C in a plastic box, which was then placed in a water bath at 37°C. Animals of the third group were given an intraperitoneal injection of 5 µl of 0.9% NaCl solution containing 20 µg/ml ascorbic acid and either an adrenergic agonist or antagonist and maintained identically to the second group described above. All injections were completed within ten minutes of delivery.

Effects of adrenergic stimulation

In experiments to investigate the effects of administration of adrenaline to newly-delivered rats, doses in the range 4 ng-10 µg of adrenaline per fetus were injected. When adrenaline was given to the animals of the third group, these fetuses and the control fetuses of the second group were killed two hours after the i.p. injection.

Effects of adrenergic antagonism

In experiments to assess the effects of administration of adrenergic antagonists to newly-delivered rats, a dose of 5 or 10 µg of the β-adrenergic antagonist propranolol, or 6.4 or 12.8 µg of the α-adrenergic antagonist phentolamine, was administered per fetus. 6.4 and 12.8 µg of phentolamine were calculated to be the molar equivalents of 5 and 10 µg of propranolol, respectively. When an antagonist compound was given, the animals of the third group (which received this compound) and the control animals of the second group were killed three hours after the i.p. injection.

Procedure

Unanaesthetized fetal animals were killed by decapitation. Blood was collected from the axial vessels of the trunk into fluoridated capillary tubes
(Microvette CB 300, Sarstedt, Leicester). The tubes were immediately centrifuged at 1800 g for 10 minutes at 4°C (Beckman J6B or IEC Centra-3R centrifuge). Plasma was aspirated using a pasteur pipette, and stored in a polypropylene tube for glucose and lactate assays; if the assays were not to be carried out within 24 hours, samples were kept at -20°C. Immediately after killing fetal animals and obtaining blood, livers were rapidly excised and frozen in liquid N₂. If the glycogen assay could not be carried out immediately, livers were stored at -20°C.

4.2.3 ASSAYS

**Plasma glucose assay**

Plasma glucose was measured directly using the hexokinase - glucose-6-phosphate dehydrogenase method on a Cobas-Bio centrifugal autoanalyser (Roche, Welwyn Garden City, Herts). No deproteinisation was necessary. Reagent kits (Glucose 'rapid', Roche Diagnostics, Welwyn Garden City, Herts., or 'Centrifichem' glucose assay kit, Baker Instruments, Allentown, P.A., U.S.A.) were used according to manufacturers' instructions. Appropriate glucose standards in distilled water (in the range 1-10 mM) were included for each set of determinations.

**Plasma lactate assay**

Plasma lactate was measured using the lactate dehydrogenase - glutamate pyruvate-transaminase method on a Cobas-Bio centrifugal autoanalyser (Roche, Welwyn Garden City, Herts). No deproteinisation was needed. A reagent kit (U.V. lactate kit, Boehringer Mannheim, Lewes, East Sussex) was used according to the manufacturer's instructions, which did not require the inclusion of standards.

**Liver glycogen assay**

Frozen livers (weighing about 300 mg) were rapidly weighed, placed in 1 ml of ice-cold 0.6M HClO₄ and immediately homogenised using a Potter-Elvehjem type
homogeniser. The tube and pestle were rinsed with two x 1 ml of 0.6M HClO₄. The homogenate was diluted 1:20 in 0.2M sodium acetate buffer, pH 4.8. Two 0.2 ml aliquots of the diluted homogenate were placed in 7.5 ml glass tubes and mixed with 1.0 ml of 0.2M acetate buffer, pH 4.8; one aliquot was mixed with buffer alone and one with buffer containing 74 iu/ml of amyloglucosidase. The tubes were shaken at 30 strokes/min in a water bath at 37°C for 4 hours, and were vortex mixed approximately half way through this time. Distilled water blanks and 0.2 ml aliquots of glycogen standards (0.1 - 1.0 mg/ml oyster glycogen in distilled water) were treated identically. At the end of the incubation, all tubes were centrifuged at 2,000 g for 10 minutes at room temperature (Beckman J6B or IEC Centra-3R centrifuge) to remove any sediment. Glucose in the supernatant was measured using the Cobas-Bio autoanalyser (see above). Significant levels of free glucose were found in the homogenates, and therefore glucose values obtained in the absence of amyloglucosidase were subtracted from those obtained in the presence of the enzyme in order to calculate the glycogen-derived glucose level. The glycogen content of the livers was then calculated from the standard curve; 97-100% of the glycogen present in standards was hydrolysed to glucose by this method.
4.3 RESULTS

4.3.1 PLASMA GLUCOSE

Control levels

The plasma glucose concentrations in control animals immediately postnatally and at two and three hours post partum are shown in Table 4.1. These animals were term fetal rats, given an intra-peritoneal injection of 5 µl of a 0.9% NaCl solution containing 20 µg/ml ascorbic acid immediately after delivery by caesarian section and maintained without feeding at 37°C. The plasma glucose level decreased significantly to 65% of the initial value by two hours post partum, and recovered slightly by three hours post partum (Fig. 4.1).

Effect of adrenaline

Administration of various doses of adrenaline immediately after delivery resulted in a dose-related increase in the plasma glucose concentration at two hours post partum when doses of 20 ng or higher were given (Table 4.2). The elevation of plasma glucose was statistically significant only following administration of 2 µg or 5 µg of adrenaline, but it would appear that an increase did occur at other doses, although inter-animal variation in plasma glucose levels prevented this being of statistical significance. A peak in the stimulatory effect of adrenaline on plasma glucose concentration was seen with a dose of 2 µg, which produced a plasma glucose level 166% of the level in control animals (Fig. 4.2).

Effect of adrenergic antagonists

Table 4.3 presents the plasma glucose concentrations three hours after surgical delivery of term fetal rats; the control level is shown together with the levels obtained when an adrenergic antagonist had been administered immediately post partum. Both the α-adrenergic antagonist phentolamine, and the β-adrenergic
TABLE 4.1

Control levels of carbohydrate metabolites in vivo during the first three hours post partum

<table>
<thead>
<tr>
<th>Time post partum (h)</th>
<th>Plasma$^a$ glucose (mM)</th>
<th>Plasma$^a$ lactate (mM)</th>
<th>Liver$^a$ glycogen (mg/g liver wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.49 ± 0.12 (n = 55)</td>
<td>8.03 ± 0.50 (n = 39)</td>
<td>105.6 ± 4.2 (n = 51)</td>
</tr>
<tr>
<td>2</td>
<td>*3.03 ± 0.19 (n = 28)</td>
<td>*3.13 ± 0.44 (n = 21)</td>
<td>102.0 ± 4.6 (n = 25)</td>
</tr>
<tr>
<td>3</td>
<td>*3.17 ± 0.05 (n = 46)</td>
<td>*2.99 ± 0.39 (n = 12)</td>
<td>b*80.6 ± 3.7 (n = 42)</td>
</tr>
</tbody>
</table>

5 μl of 0.9% NaCl solution containing 0.1 μg ascorbic acid was administered i.p. to all animals immediately after delivery.

n = total number of fetuses from 3-14 litters (values obtained for individual fetuses, see text for details).

*a Values are shown as mean ± SEM

* Significantly lower than at zero time, p < 0.001

b Significantly lower than at 2 hours post partum, p < 0.001
Day 22 p.c. fetal rats were delivered by caesarian section, and maintained at 37°C without feeding until sacrificed. Plasma glucose concentration (○—○), plasma lactate concentration (■—■) and liver glycogen content (▲—▲) were determined for individual animals. (see text for details).
TABLE 4.2

**Effect of adrenaline administered in vivo on levels of carbohydrate metabolites at two hours post partum**

<table>
<thead>
<tr>
<th>Dose of adrenaline (µg)</th>
<th>Plasma glucose (mM)</th>
<th>Plasma lactate (mM)</th>
<th>Liver glycogen (mg/g liver wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.03 ± 0.19 (n = 28)</td>
<td>3.13 ± 0.44 (n = 21)</td>
<td>102.0 ± 4.6 (n = 25)</td>
</tr>
<tr>
<td>.004</td>
<td>2.99 ± 0.22 (n = 5)</td>
<td>3.33 ± 0.48 (n = 3)</td>
<td>96.7 ± 9.6 (n = 5)</td>
</tr>
<tr>
<td>.02</td>
<td>3.16 ± 0.29 (n = 4)</td>
<td>3.28 ± 0.57 (n = 5)</td>
<td>104.8 ± 9.7 (n = 4)</td>
</tr>
<tr>
<td>.04</td>
<td>3.04 ± 0.18 (n = 5)</td>
<td>2.51 ± 0.23 (n = 4)</td>
<td>112.6 ± 5.9 (n = 5)</td>
</tr>
<tr>
<td>.4</td>
<td>3.17 ± 0.28 (n = 5)</td>
<td>3.09 ± 0.37 (n = 5)</td>
<td>97.9 ± 16.4 (n = 5)</td>
</tr>
<tr>
<td>1</td>
<td>4.24 ± 1.10 (n = 4)</td>
<td>5.27 ± 1.38 (n = 4)</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td><strong>5.04 ± 0.33 (n = 4)</strong></td>
<td><strong>5.82 ± 0.69 (n = 4)</strong></td>
<td>91.3 ± 8.3 (n = 5)</td>
</tr>
<tr>
<td>3</td>
<td>3.76 ± 0.31 (n = 5)</td>
<td>4.10 ± 0.59 (n = 5)</td>
<td>94.1 ± 7.2 (n = 5)</td>
</tr>
<tr>
<td>5</td>
<td>*3.79 ± 0.18 (n = 5)</td>
<td><strong>11.16 ± 0.68 (n = 4)</strong></td>
<td>92.7 ± 6.0 (n = 4)</td>
</tr>
<tr>
<td>10</td>
<td>3.58 ± 0.47 (n = 5)</td>
<td>ND</td>
<td><strong>59.3 ± 6.3 (n = 4)</strong></td>
</tr>
</tbody>
</table>

5 µl of 0.9% NaCl solution containing 0.1 µg ascorbic acid and adrenaline as indicated was administered i.p. immediately after delivery.

n = total number of fetuses from 1-7 litters (values obtained for individual fetuses, see text for details).

a Values are shown as mean ± SEM

ND Not determined

* Significantly different from control, p < 0.01

** Significantly different from control, p < 0.001
Adrenaline was administered i.p. immediately after surgical delivery of day 22 p.c. fetal rats, which were maintained at 37°C without feeding until sacrificed. Plasma glucose concentration (O---O), plasma lactate concentration (■---■) and liver glycogen content (▲---▲) were determined for individual animals (see text for details).
TABLE 4.3

Effect of adrenergic antagonists administered in vivo on levels of carbohydrate metabolites at three hours post partum

<table>
<thead>
<tr>
<th>Dose and drug</th>
<th>Plasma a glucose (mM)</th>
<th>Plasma a lactate (mM)</th>
<th>Liver a glycogen (mg/g liver wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.17 ± 0.05 (n = 46)</td>
<td>2.99 ± 0.39 (n = 12)</td>
<td>80.6 ± 3.7 (n = 42)</td>
</tr>
<tr>
<td>5 µg propranolol</td>
<td>2.78 ± 0.22 (n = 12)</td>
<td>**8.61 ± 1.23 (n = 9)</td>
<td>68.5 ± 10.8 (n = 11)</td>
</tr>
<tr>
<td>10 µg propranolol</td>
<td>**2.41 ± 0.15 (n = 15)</td>
<td>**7.32 ± 0.59 (n = 8)</td>
<td>63.6 ± 6.7 (n = 17)</td>
</tr>
<tr>
<td>6.4 µg phentolamine</td>
<td>*2.87 ± 0.11 (n = 11)</td>
<td>ND (n = 16)</td>
<td>62.7 ± 6.1</td>
</tr>
<tr>
<td>12.8 µg phentolamine</td>
<td>2.59 ± 0.22 (n = 9)</td>
<td>ND (n = 9)</td>
<td>83.5 ± 5.6</td>
</tr>
</tbody>
</table>

5 µl of 0.9% NaCl solution containing 0.1 µg ascorbic acid and an adrenergic antagonist as indicated was administered i.p. immediately after delivery.

n = total number of fetuses from 2-11 litters (values obtained for individual fetuses, see text for details).

a Values are shown as mean ± SEM

ND Not determined

* Significantly different from control, p < 0.025

** Significantly different from control, p < 0.001
FIGURE 4.3  Effect of adrenergic antagonists administered in vivo on carbohydrate metabolites at 3 hours post partum.

(a) Plasma Glucose Concentration

(b) Plasma Lactate Concentration

(c) Liver Glycogen Content

See text for details of treatment
antagonist propranolol produced a significant decrease in the plasma glucose concentration determined at three hours post partum. For both compounds, a more marked effect was seen with the higher dose, and a molar equivalent dose of propranolol had a greater effect than phentolamine (Table 4.3 and Fig. 4.3).

4.3.2 PLASMA LACTATE

Control levels

Plasma lactate concentrations in term fetuses given i.p. injections of 5 µl of a 0.9% NaCl solution containing 0.1 µg of ascorbic acid immediately after surgical delivery decreased significantly from immediately postnatally to two hours post partum, and showed a further slight decrease in the following hour (Table 4.1 & Fig. 4.1).

Effect of adrenaline

Adrenaline administered i.p. immediately after caesarian delivery produced an increase in the plasma lactate concentration at two hours post partum when doses in the µg range were given (Table 4.2). This elevation of plasma lactate levels was of statistical significance only when 2 or 5 µg adrenaline were given, although it appeared to be only the inter-animal variation which prevented doses of 1 and 3 µg achieving statistically significant effects. The maximal effect of adrenaline on plasma lactate concentration was at 5 µg, when a level 360% of the control value was obtained.

Effect of adrenergic antagonists

Due to a freezer failure, plasma samples from all animals receiving phentolamine, and some animals receiving propranolol, were lost before their lactate content could be determined. In Table 4.3, therefore, plasma lactate concentrations at three hours post partum are shown only for control animals and
those receiving propranolol for which lactate levels were found. The \( \beta \)-adrenergic antagonist propranolol, administered i.p. immediately after surgical delivery of term rat fetuses, stimulated the plasma lactate concentration three hours later whether 5 \( \mu \)g or 10 \( \mu \)g was given; with both doses the increase in plasma lactate level was statistically significant. The plasma lactate level obtained was 290% or 250% of the control level with the lower or higher dose of propranolol, respectively.

### 4.3.3 LIVER GLYCOGEN

**Control levels**

The amounts of glycogen per unit weight of liver immediately after and at two and three hours after delivery of term fetuses in the control group are shown in Table 4.1. The liver glycogen content in animals receiving no drug treatment did not decrease significantly from the level at delivery until three hours post partum, when the amount of hepatic glycogen present was reduced to 75% of the starting level (Fig. 4.1).

**Effect of adrenaline**

Intraperitoneal administration of adrenaline at doses in the \( \mu \)g range to newly-delivered term fetal rats produced an increase in postnatal glycogenolysis (Table 4.2). The reduction in liver glycogen content at two hours post partum achieved statistical significance only when 10 \( \mu \)g of adrenaline was injected, but this is probably due to the small number of fetuses receiving each dose of adrenaline, and the inter-animal variation in initial liver glycogen content. 10 \( \mu \)g of adrenaline reduced the amount of hepatic glycogen at two hours post partum to 60% of the amount present in control fetuses at this time point.
Effect of adrenergic antagonists

Table 4.3 shows the liver glycogen content at three hours post partum of animals given saline with or without an adrenergic antagonist. Surprisingly, both doses of the β-adrenergic antagonist propranolol appeared to stimulate glycogen loss by three hours post partum (although this result was not statistically significant); the lower dose of the α-adrenergic antagonist phentolamine had a similar (non-significant) stimulatory effect, whilst the higher dose had no apparent effect on liver glycogen content.
4.4 DISCUSSION

4.4.1 CONTROL VALUES

In the experiments reported in this chapter, the mean plasma glucose concentration in neonatal rats given control injections decreased from 4.5 mM immediately after delivery to 3.0 mM two hours later, and increased to 3.2 mM by the third postnatal hour (Table 4.1). Plasma glucose levels reported in the literature range from 2.6 to 9.0 mM immediately after delivery, from 1.1 to 2.9 mM at two hours post partum, and from 1.5 to 5.0 mM at three hours post partum (Benito et al., 1980; Butcher & Potter, 1972; Cake et al., 1971; Cuezva et al., 1980b, 1982a; Fernandez et al., 1983; Girard et al., 1973a; Jansen et al., 1984; Phillipe & Kitzmiller, 1981; Snell & Walker, 1973b; Sodoyez-Goffaux et al., 1979a). The plasma glucose levels and the changes in these levels seen in control animals in the first three hours postnatally were therefore of similar magnitude to those reported by other workers.

The mean plasma lactate concentration in control rats maintained at 37°C without feeding following surgical delivery dropped from 8.0 mM to 3.1 mM in the first two hours post partum and remained at a similar level during the following hour (Table 4.1). Reports of plasma lactate levels in newly delivered rats found in the literature range from 7.2 to 13.5 mM, with values of 2.0 to 3.2 mM and 1.8 to 3.0 mM at two and three hours post partum, respectively (Cuezva et al., 1980b; Fernandez et al., 1983; Girard et al., 1973a; Haymond et al., 1972; Jansen et al., 1984; Snell & Walker, 1973b). The control plasma lactate concentrations found in the current work at zero time and two and three hours post partum are therefore in agreement with levels reported for this time period by other workers.

Mean liver glycogen content immediately after delivery was 106 mg/g liver wt; this showed very little change in the first two hours post partum in control
animals, dropping only to 102 mg/g, but decreased significantly to 81 mg/g by three hours post partum (Table 4.1). The initial hepatic glycogen content determined in these experiments was of a similar order to values reported for cesarian-delivered animals in the literature, which range from 78 to 99 mg/g liver wt. (Benito et al., 1980; Butcher & Potter, 1972; Cuezva et al., 1980b, 1982a; Fernandez et al., 1983; Girard et al., 1972, 1973a; Jansen et al., 1984; Snell & Walker, 1973b). There was no significant loss of liver glycogen until the third postnatal hour in control neonates in these experiments (Table 4.1). A delay before the onset of postnatal glycogenolysis has been observed by a number of other workers, as discussed in Chapter 1, section 1.1.2. The decrease to 76% of the initial level of liver glycogen by three hours post partum in the current work is comparable with published glycogen breakdown levels over this time period, varying from 54 to 76% (Benito et al., 1980; Cuezva et al., 1982a; Girard et al., 1972; Fernandez et al., 1983; Snell & Walker, 1973b).

4.4.2 EFFECT OF ADRENALINE

400 ng of adrenaline administered i.p., although calculated to potentially increase plasma catecholamine concentration by 100-fold at two hours post partum (see section 4.1.2), had no significant effect on the parameters studied at this time. The lack of effect may be a reflection of incomplete absorption and/or rapid clearance of administered adrenaline. Determination of plasma catecholamine levels in control neonates and those receiving various doses of adrenaline would have been of great help in interpreting the observations made, but this was precluded by the complexity and/or expense of available assay techniques (Ben-Jonathan & Porter, 1976; Cuezva & Patel, 1982; Eriksson & Persson, 1982; Peuler & Johnson, 1977).

Doses of adrenaline of 1 µg appeared to stimulate net glucose and lactate production, whilst 2 µg produced a detectable increase in glycogenolysis; however,
the effect of adrenaline reached statistical significance only at 2 μg for glucose and lactate concentrations and 10 μg for glycogen content. The peak effect of adrenaline in increasing plasma glucose concentration was seen when 2 μg were administered, whilst the stimulation of plasma lactate was maximal at 5 μg (the highest dose for which samples were assayed), and promotion of glycogen breakdown increased with increasing dose up to the highest dose given (10 μg).

There have been very few reports of the effects on carbohydrate metabolism of adrenergic agonists given in vivo to rats during the perinatal period. Infusion of a 0.1% solution of the β-adrenergic agonist salbutamol into the saphenous vein of a gravid rat on day 21 post coitum produced a two-fold increase in blood glucose concentration in the fetuses, with a five minute latency and a peak 15 minutes after commencement of the infusion (Hauguel et al., 1982). This finding suggests metabolism of adrenergic compounds in the rat is rapid, possibly accounting for the high dose of adrenaline required to achieve an effect. In a study by Goldwater and Stetten (1974), a dose of adrenaline of 40 μg per 100 g body weight was given as two subcutaneous injections to a gravid rat at day 18.5 post coitum, and fetuses were taken 1 or 3 hours later. A marked decrease in fetal glycogen levels, to only 10-15% of control levels was seen. Yeung and Oliver (1968) administered 5 or 10 μg of adrenaline intra-peritoneally to fetal rats in utero. They observed that when fetuses were killed five hours after this treatment, hepatic phosphoenolpyruvate carboxykinase was present at detectable levels from as early as day 16 post coitum.

The observation that doses of adrenaline having a threshold and a peak effect on the three parameters of carbohydrate metabolism determined differ, is due to the fact that plasma levels of glucose and lactate reflect the net result of their production and utilisation. Hems (1977a) noted that in adult rats, liver glycogenolysis was seen with plasma adrenaline concentrations of 10⁻⁸M and above.
However, it was also seen that the lowest effective concentration of adrenaline produced no increase in the glucose released by the liver, and it was proposed that glycogen breakdown induced by adrenaline leads to the production of hexose phosphates for use in catabolic pathways such as glycolysis. Adrenaline treatment of perfused rat heart has been shown to induce not only glycogenolysis but increased uptake of glucose and formation of lactate (Clark & Patten, 1984); if the adrenaline administered to newly delivered rats in the current study had similar effects on the liver, it might produce apparently anomalous changes in the three parameters determined in this work.

4.4.3 EFFECT OF ANTAGONISTS

The doses of adrenergic antagonists given were equivalent for the two compounds, on a molar basis, being 5 or 10 μg of the β-adrenergic antagonist propranolol and 6.4 or 12.8 μg of the α-adrenergic antagonist phentolamine. The lower and higher doses of the antagonists were the molar equivalents of 3 and 6 μg adrenaline, respectively, and would therefore be expected to have some effect on carbohydrate metabolites if adrenergic stimulation is modifying the pathways of carbohydrate metabolism. As might be predicted from the experimental findings reported in Chapters 2 and 3, the β-adrenergic antagonist propranolol administered in vivo at birth produced a decrease in the plasma glucose concentration compared with control at three hours post partum (Table 4.3). However, the α-adrenergic antagonist phentolamine was also effective in reducing the physiological increase in plasma glucose concentration postnatally (Table 4.3). The studies reported in Chapter 3 provide evidence that the pathway for adrenergic modulation of hepatic carbohydrate metabolism in the perinatal rat is β-mediated. However, it was also shown that an α-adrenergic agonist, phenylephrine, stimulated glucose production by hepatocytes in vitro, albeit at higher concentrations than the β-adrenergic agonist isoprenaline (Fig. 3.3 vs. Fig. 3.4). Although the amounts of the two
antagonists administered to neonatal rats in vivo were identical on a molar basis, differences in absorption and/or clearance of the two components may have resulted in the liver being exposed to very different concentrations of the agents. As subtype selectivity of any adrenergic agent is a relative phenomenon, effects of phentolamine might be the result of blocking of β-adrenoceptors by this agent, if present in sufficiently high concentration.

As plasma lactate concentration was increased by adrenergic stimulation, it might be expected that adrenergic antagonism would decrease the lactate level. In fact, as seen in Table 4.3, the β-adrenergic antagonist propranolol produced an increase in the mean plasma lactate concentration. As plasma lactate levels reflect its production and its utilisation, an increase in the former or decrease in the latter, or a combination of both of these, would produce an increase in plasma lactate concentration. This will be discussed further after the effects of adrenergic antagonists on liver glycogen content has been considered.

As propranol and phentolamine both produce decreases in plasma glucose concentration compared with the control level, they might be expected to reduce the breakdown of liver glycogen, which is one source of plasma glucose. However, both doses of propranolol and the higher dose of phentolamine produced increased glycogenolysis compared with control, although this effect was not statistically significant (Table 4.3).

A possible explanation for the observed effects of administration of propranolol is that, in addition to glycogen breakdown, glucose may be produced postnatally by hepatic gluconeogenesis, the potential for which develops rapidly after birth (Girard et al., 1975; Snell & Walker, 1973b). Thus, if the decrease in plasma glucose levels induced by adrenergic antagonists is a result of inhibition of
gluconeogenesis, this might explain the apparent discrepancy between changes in plasma glucose and liver glycogen levels in Table 4.3. As discussed earlier, plasma lactate levels result from the balance of production and utilisation of this metabolite in metabolic pathways; lactate levels might be expected to increase with increasing glycogenolysis providing the substrate (glucose) for its production but to decrease with increasing gluconeogenesis which utilises this molecule as a substrate. The finding that treatment with an adrenergic antagonist produces a much greater proportional increase in plasma lactate concentration than in hepatic glycogenolysis is strong support for an inhibitory action of propranolol on gluconeogenesis. A possible mechanism for inhibition of the gluconeogenic pathway by an adrenergic antagonist is suggested by Yeung and Oliver (1968) who observed premature induction of the key gluconeogenic enzyme, hepatic phosphoenolpyruvate carboxykinase, in rat fetuses receiving adrenaline in vivo; interference with the normal postnatal appearance of this enzyme would prevent the development of gluconeogenic capacity in the liver.

Girard et al. (1973b) investigated the effect of administration of 10 μg of phentolamine or propranolol subcutaneously to day 21.5 post coitum fetal rats immediately after caesarian delivery on liver glycogen and blood glucose levels at six hours post partum. They found that the α-adrenergic antagonist caused some reduction in glycogen breakdown and in plasma glucose concentration, whilst the β-adrenergic antagonist had no detectable effect. The reason for the discrepancies between the current findings and those reported by Girard’s group are unclear.

4.4.4 SIGNIFICANCE OF FINDINGS

In the experiments reported in this Chapter, it was shown that adrenaline administered in vivo to newly-delivered rats produced changes in parameters of carbohydrate metabolism two hours later. It would therefore seem that an
adrenergic system is present and functional in the neonatal rat. The observation that inhibition of the adrenergic pathway interferes with the mechanisms of recovery from the postnatal hypoglycaemic phase suggest that activation of adrenoceptors is a necessary factor in the return to normoglycaemia seen in the healthy neonatal rat.
CHAPTER 5

GENERAL DISCUSSION
5.1 HORMONAL CONTROL OF CARBOHYDRATE METABOLISM IN THE
PERINATAL RAT

5.1.1 CURRENT VIEW

If the pathological consequences of prolonged hypoglycemia are to be avoided, a newborn rat must rapidly acquire the ability to produce glucose following the cessation of its supply via the placenta and umbilical blood supply. As described in Chapter 1 (Section 1.1.2), it is well established that, even in the absence of exogenous nutrients, normoglycemia is regained within hours of birth in healthy neonates. Glucose production occurs in the liver following changes in the activities of a number of enzymes of carbohydrate metabolism, and known postnatal changes in some of these have already been discussed in Chapter 1 (section 1.1.2). The rapidity with which glucose production is initiated, together with the observation that when gestation was experimentally prolonged, increases in the activities of gluconeogenic enzymes and commencement of glycogen breakdown were seen in utero despite the absence of a drop in blood glucose concentration (Portha et al., 1976, 1978a), suggest that postnatal hypoglycemia per se is not a sufficient stimulus for induction of glucose production.

A number of hormones show well documented changes in their plasma levels during the perinatal period (see section 1.2 for details) and as such may be considered candidates for regulation of postnatal carbohydrate metabolism. The most prevalent view at present is that the pancreatic hormones insulin and glucagon are the major hormonal factors controlling onset of glucose production in the neonatal liver Girard et al. (1973a) described postnatal changes in plasma concentrations of pancreatic hormones which were of a magnitude and direction, and at a time, such that they considered them to be 'prime regulators' of metabolic changes in the neonatal rat. The postnatal increase in plasma glucagon concentration, decrease in plasma insulin concentration, or rapid fall in the
insulin:glucagon ratio have all been proposed to be critical in triggering the onset of glucose production. Both glycogenolysis and gluconeogenesis have been suggested to be initiated by changes in the levels of these pancreatic hormones. Increased glucagon secretion and tissue responsiveness to the hormone around the time of birth has been suggested to stimulate hepatic glycogenolysis (Blazquez et al., 1972). Secretion of glucagon was proposed to result in an activation of hepatic glycogen phosphorylase and thereby glycogen breakdown by one laboratory (Girard et al., 1972), whilst another (Bourbon, 1981) has suggested that insulin has the primary physiological role in the regulation of phosphorylase activity in perinatal liver. Hanson et al. (1975) suggested that the decrease in insulin level and increase in glucagon level at birth produced increases in hepatic cyclic AMP concentrations and thus stimulated synthesis of phosphoenolpyruvate carboxykinase, a key enzyme of gluconeogenesis. This would support the suggestion that glucagon is a major factor in the postnatal induction of gluconeogenesis, with insulin having an antagonistic role (Girard et al., 1976). However, it is doubtful whether the increased synthesis of this enzyme after birth can occur rapidly enough to account for the onset of glucose production by the liver postnatally. A marked increase in the cyclic AMP level in rat liver has been noted after birth (Blazquez et al., 1974; Butcher & Potter, 1972; Novak et al., 1972). DiMarco and coworkers suggested that the relative concentrations of insulin and glucagon would regulate the level of cyclic AMP in the neonatal liver, and thereby the induction of a number of enzymes, including some critical for glucose production (DiMarco & Oliver, 1978; DiMarco et al., 1978). Glucagon may also stimulate flux through the gluconeogenic pathway by means of inhibition of pyruvate kinase, possibly by phosphorylation (Riou et al., 1976), and this would be a rapid regulatory mechanism.

Various experimental observations support the importance of the pancreatic hormones in the regulation of postnatal carbohydrate metabolism. When glucagon was included in the medium for fetal rat liver cultures, this resulted in induction of
phosphoenolpyruvate carboxykinase (Mandelli et al., 1973). A number of workers have investigated the effect of administering glucagon in vivo to newly delivered animals. Exogenous glucagon has been shown to prevent the period of hypoglycemia normally seen postnatally (Cake et al., 1971; Snell & Walker, 1973c, 1978). Administration of glucagon to a fetal rat has been shown to induce gluconeogenic enzymes (Blazquez et al., 1972), and to a neonatal rat to stimulate gluconeogenesis (Cake et al., 1971; Girard et al., 1976; Snell & Walker, 1978) and glycogenolysis (Snell & Walker, 1973c). Exogenous insulin has been shown to reduce gluconeogenesis in the neonate (Girard et al., 1976) and anti-insulin serum given postnatally promoted glycogen breakdown and abolished postnatal hypoglycemia (Snell & Walker, 1978).

However, there are also a number of findings which throw some doubt on the concept of changes in the levels of insulin and glucagon being the only, or indeed even the major, factor in the control of postnatal glucose production.

Although it has been discussed earlier that postnatal decrease in plasma glucose concentration per se is probably not a sufficient stimulus for initiation of glucose production, it would seem reasonable that any hormonal system responsible for regulation of the plasma glucose level would be responsive to that level. In the adult, sensitivity of the pancreatic A and B cells to acute glucose concentrations ensures appropriate secretion of glucagon and insulin, respectively, to maintain normoglycemia under normal physiological conditions. There are numerous reports, however, that the pancreas of the rat during the perinatal period does not show such glucose sensitivity. Asplund et al. (1975) have shown a lack of glucagon secretion in response to acute hypoglycemia, whilst Ferre et al. (1977) reported that the pancreatic A cells in the neonate are insensitive to acute changes in the plasma glucose concentration. Heinze & Steinke (1971) found that pancreatic islets isolated from neonatal rats released no insulin in response to glucose in the bathing
medium at a concentration invoking release from adult islets, until day 2 or 3 post partum. In vivo, the mechanism for insulin release from the pancreas in response to a raised plasma glucose concentration has been observed to reach full maturity only at 3 weeks post partum (Blazquez et al., 1975).

A second series of observations which raises questions on a critical role for glucagon during the postnatal period are those regarding its binding to hepatic receptors, and the responsiveness of the liver to its presence. Blazquez et al. (1976) suggested a greatly reduced sensitivity of the perinatal liver, compared with the adult, to the effects of glucagon. This interpretation was based on the paradoxically high liver glycogen content of fetal liver in relation to plasma glucagon concentration (although this might also be a result of the very high fetal plasma insulin concentration), together with the relatively poor responsiveness of hepatic adenyl cyclase to glucagon in the perinatal period. Vinicor et al. (1976) also observed a relative insensitivity of perinatal hepatic adenyl cyclase to glucagon. The most likely reason for the poor response of perinatal liver to glucagon is the reduced binding of this hormone to hepatic receptors in immature animals; maximal binding capacity of glucagon in term fetal liver has been reported to be 20-25% of that in adult liver (Blazquez et al., 1976; Pingoud et al., 1982; Vinicor et al., 1976).

Two further studies are of interest when considering the possible significance of pancreatic hormones in the regulation of postnatal carbohydrate metabolism. In the first study, it was observed that mannoheptulose, an inhibitor of insulin secretion, did not prevent the inhibition of glycogenolysis resulting from administration of glucose to neonatal rats (Martin et al., 1981). The authors argued that this was evidence that a postnatal decrease in insulin was not an essential factor in the initiation of glycogenolysis, and that as such the proposal that the insulin:glucagon ratio was the critical parameter in postnatal carbohydrate
metabolism could not be valid. The second study involved measurement of a range of hormones and metabolites during the perinatal period, in neonates from both normal and gestationally diabetic dams (Cuezva et al., 1982a). A critical finding in this work was that although in the offspring of diabetic mothers, the normal postnatal increase in plasma glucagon was not observed, an increase in hepatic cyclic AMP was still found in these neonates. As it is generally considered that the observed increase in hepatic cyclic AMP is the mechanism for enzyme activation and induction which in turn leads to the postnatal initiation of glucose production, and this study showed that this increase can occur without an increase in plasma glucagon, this is strong evidence for a factor, or factors, other than glucagon being of major regulatory significance for postnatal carbohydrate metabolism.

In searching for alternative or additional factors to the pancreatic hormones which might be involved in the regulation of glucose metabolism in the neonatal rat liver, it is worthwhile considering the timing of events during the postnatal period. As described in detail in Chapter 1 (Section 1.2), plasma insulin decreases and glucagon increases rapidly in the newborn rat, such that the insulin:glucagon ratio reaches a minimum just 30 to 60 minutes after birth (DiMarco et al., 1978; Girard et al., 1973a). However, as also discussed in Chapter 1 (Section 1.1), postnatal glycogenolysis does not appear to be initiated until around two hours post partum, and recovery from hypoglycemia is usually seen from around this time. Thus, it would seem that the events postulated to be triggered by the changes in plasma concentrations of insulin and glucagon take place some time after the maximum changes in their plasma levels. Plasma catecholamine levels are also known to undergo acute postnatal changes, although as discussed in Section 1.2.5 there is a relative paucity of information regarding the timing and magnitude of such changes. It would appear however, that catecholamines are very likely to be involved in postnatal initiation of glucose production; the evidence for this is discussed in the following section.
5.1.2 EVIDENCE FOR ADRENERGIC MODULATION

Evidence from the literature

If it is to be argued that adrenergic activation is a critical factor in invoking the required metabolic changes to allow recovery from postnatal hypoglycemia, it must be shown that the observations cited to lend doubt to the predominance of insulin and/or glucagon levels in regulation of carbohydrate metabolism do not apply to catecholamines.

It was stated in section 5.1.1 that if a hormone system were to be an efficient regulator of plasma glucose concentration, then glucose levels should affect hormone release. Phillipe & Kitzmiller (1981) showed that fetal plasma adrenaline and noradrenaline levels were both inversely correlated with plasma glucose concentrations, including during a period of insulin-induced hypoglycemia.

Relatively little work has been published concerning liver responsiveness to catecholamines during the perinatal period. The observation by DiMarco et al. (1976) that the increase in liver catecholamine levels in late gestation and during birth is paralleled by an increase in hepatic cyclic AMP appears to show that adenyl cyclase is fully responsive to adrenergic stimulation during the perinatal period. This group has also shown that the increase in hepatic cyclic AMP in response to exogenous adrenaline reaches a peak at day 2 post partum, whilst that to glucagon does not show maximum responsiveness until day 10 post partum. The finding of liver responsiveness to adrenaline during the perinatal period comparable with that seen in the adult is of little surprise when the binding of catecholamines to liver membranes is considered. In the only published study of the β-adrenoceptor (generally considered to be of greater significance for carbohydrate metabolism than the α-adrenoceptor in the perinatal rat), the maximal binding capacity around term was actually higher (by about two-fold) than that found in the liver of mature animals in the same study (McMillian et al., 1983).
When the timing of changes in the plasma concentrations of catecholamines in the immediately postnatal period is considered, it is clear that such changes might easily be modulating carbohydrate metabolism and producing the well-documented temporal changes in the neonate. Both Cuezva et al. (1982a) and Jansen et al. (1984) noted a secondary increase in plasma catecholamine concentrations in the time shortly following birth (see section 1.2.5 for details). In both studies a total plasma catecholamine concentration of around 3 ng/ml was seen at two hours post partum, which is considerably higher than that (10^{-8}M) which Hems (1977a) found was the threshold for stimulation of glycogenolysis in adult rat liver. In Cuezva's study (Cuezva et al., 1982a) control neonates were studied together with neonates from untreated and insulin-treated diabetic dams. Although the absolute timing of the changes differed in these three groups of animals, in each group the secondary increase in plasma catecholamine concentration followed the nadir in plasma glucose concentration, and the increase in catecholamine levels was associated with a decrease in liver glycogen content and an increase in plasma glucose concentration.

Experiments in which administration of adrenergic agonists and antagonists to newborn rats in vivo have been shown to interfere with the normal physiological regulation of carbohydrate metabolism in the neonate have already been discussed in Chapter 4 (sections 4.4.2 and 4.4.3). However, the observation by Cake & Oliver (1969) that administration of glucose or ergotamine tartrate both inhibit the postnatal increase in hepatic cyclic AMP concentration is worth noting as further evidence for adrenergic modulation of cyclic AMP levels, and thereby carbohydrate metabolism, in the perinatal rat.

Evidence from this study

Radioligand-receptor binding studies, described in Chapter 2, provided evidence for a substantial β-adrenoceptor population in the liver of term fetal rat,
there being approximately 50% of the number of receptors found in adult liver (30 fmols/mg protein cf. 60 fmols/mg protein: Guellaen et al., 1978; Munnich et al., 1981). The observation of a peak in the number of β-adrenoceptors at term is consistent with the concept of adrenergic regulation of postnatal glucose production, as maximum sensitivity to adrenergic agents would be required at term if the effects of such agents were of physiological significance. Proof of the presence of β-adrenoceptors able to bind adrenergic ligands is necessary, but not conclusive, evidence for involvement of the β-adrenergic system in modulation of perinatal carbohydrate metabolism. Investigation of the sensitivity of hepatic adenyl cyclase to adrenergic activation during the perinatal period would have provided additional interesting insight into the functional competence of the β-adrenoceptors (but time limitations precluded such studies).

A series of experiments investigating the stimulation of glycogen breakdown and gluconeogenesis in, and glucose release from, freshly isolated hepatocytes was carried out to establish the existence of functional adrenergic pathways able to influence carbohydrate metabolism in perinatal rat liver (Chapter 3). The findings made in these experiments established that adrenergic activation stimulates glucose production by both late fetal and early neonatal hepatocytes. The observed potency order of agonists in promoting glucose production and antagonists in docking the stimulation of glucose production by adrenaline gave very strong support to our view that the β-adrenoceptor is of greater importance in adrenergic effects on glucose metabolism in perinatal liver than the α-adrenoceptor. The concentration-response experiments reported gave clear evidence for maximum sensitivity to the effects of adrenergic agonists at term; this is possibly a reflection of the peak in hepatic β-adrenoceptor number that we also showed at this time, although post-receptor events might also influence responsiveness. The work carried out in vitro therefore produced results entirely compatible with the hypothesis that catecholamines are of major physiological significance in initiating the known postnatal changes in pathways of glucose metabolism.
The experiments reported in Chapter 4 provide conclusive evidence that the adrenergic system not only could, but actually does, play a vital role in the recovery from hypoglycemia normally observed in the first hours after birth of the laboratory rat. As these experiments were carried out in vivo, all humoral factors which might influence the pathways of carbohydrate metabolism would be present. Under these conditions, exogenous adrenaline was observed to reduce or even prevent the postnatal decrease in plasma glucose concentration seen in control animals, whilst the β-adrenergic antagonists propranolol (and to a lesser extent the α-adrenergic antagonist phentolamine) inhibited the return to normoglycemia which starts from two hours post partum.

5.1.3 POSSIBLE MECHANISMS OF ADRENERGIC EFFECTS

The enzymes involved in metabolism of carbohydrates, and their regulation, were described in Chapter 1 (section 1.1), as were the known adrenergic effects on hepatic carbohydrate metabolism in the perinatal rat (section 1.3.3).

Stimulation of glycogen breakdown by catecholamines in the neonatal rat is generally thought to result from conversion of glycogen phosphorylase b to a as a result of phosphorylation by cyclic AMP-dependent protein kinase; this view is supported by the observation of activation of phosphorylase by adrenaline or isoprenaline, but not by relatively high concentrations of the α-adrenergic agonist, phenylephrine (Sherline et al., 1974). An alternative mechanism is suggested however, by the demonstration in a number of studies that term fetal liver contains a high proportion of lysosomal glycogen (Devos & Hers, 1980; Iwamasa et al., 1975; Kotoulas & Phillips, 1970). Breakdown of glycogen in lysosomal structures is mediated via acid α-glucosidase rather than glycogen phosphorylase. No work appears to have been reported regarding possible effects of catecholamines on such enzymes, but Kotoulas & Phillips (1971) have shown that exogenous adrenaline stimulates breakdown of lysosomal glycogen in neonatal liver in vivo.
Stimulation of the gluconeogenic path by catecholamines might occur at a number of points: inhibition of the glycolytic enzymes pyruvate kinase and phosphofructokinase; or induction and/or activation of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, appear to be the most likely mechanisms of regulation and the first two are known to be of importance in the adult (see Chapter 1, section 1.3.2). The experimental observations reported in Chapters 2, 3 and 4 provide strong evidence for a functional \( \beta \)-adrenergic pathway in perinatal liver. The plasma levels of catecholamines during the postnatal period reported in the literature are of a magnitude such that \( \beta \)-adrenoceptors would be activated \textit{in vivo}, and such an activation is well documented to result in activation of adenylyl cyclase, production of cyclic AMP and the classical 'cascade' of protein phosphorylation reactions.

Evidence that protein phosphorylation reactions induced by activation of the \( \beta \)-adrenergic pathway could produce appropriate changes in the activity of gluconeogenic enzymes comes from a range of studies. Blair \textit{et al.} (1979b) observed inhibition of pyruvate kinase, with a reduction in lactate production, when hepatocytes isolated from juvenile (100-150g) rats were exposed to 10 \( \mu \)M adrenaline or isoprenaline; phenylephrine at a similar concentration had no such effect. These workers also concluded that the inhibition of pyruvate kinase by cyclic AMP in perfused adult liver was a result of phosphorylation of the enzyme by cyclic AMP-dependent protein kinase (Blair \textit{et al.}, 1976). Ljungstrom \textit{et al.} (1974) confirmed that phosphorylation of pyruvate kinase by cyclic AMP-dependent protein kinase produced a decrease in the enzyme's activity; this work was performed using the isolated, purified enzyme. Inactivation of pyruvate kinase and an associated increase in gluconeogenesis were produced in isolated adult hepatocytes by either adrenaline or cyclic AMP (Feliu \textit{et al.}, 1976). Exposure of isolated adult hepatocytes to cyclic AMP has also been shown to inhibit flux through phosphofructokinase, and to enhance flux through the opposing
gluconeogenic enzyme fructose 1,6-bisphosphatase (Castano et al., 1979; Clark et al., 1974). The lack of observations made using tissue from perinatal animals makes firm conclusions regarding adrenergic effects on the enzymes pyruvate kinase and phosphofructokinase in the immediately postnatal period impossible, but it does seem highly likely that such inhibition of these enzymes would occur and thus result in increased gluconeogenic flux. Presumably because of the known dramatic changes which take place in phosphoenolpyruvate carboxykinase (PEPCK) around the time of birth (see section 1.1.2), this enzyme has been studied in rather more detail than other enzymes of carbohydrate metabolic pathways during the perinatal period. Jost & Picon (1970) described activation of PEPCK in fetal liver by adrenaline and noradrenaline, and Yeung & Oliver (1968) found that 5 or 10 µg of adrenaline injected in utero intra peritoneally would induce the appearance of PEPCK within five hours from as early as day 16 post coitum. Adrenaline, the β-adrenergic agonist isoprenaline, or cyclic AMP were each able to stimulate the activity of PEPCK in fetal liver explants, but these experiments used long organ culture periods (Wicks, 1969, 1971) known to change adult liver adrenoceptor characteristics (see Chapter 1, section 1.3.3).

In addition to any direct effects which catecholamines might have in producing the dramatic changes in carbohydrate metabolism occurring around the time of birth, indirect effects of the catecholamines mediated via their ability to modulate pancreatic secretion of glucagon and insulin might also be of significance. Insulin secretion is stimulated via β-adrenoceptor activation, and inhibited via α-adrenoceptor activation (Gray et al., 1980; Hornbrook, 1970; Porte & Robertson, 1973; Woods & Porte, 1974). There is some evidence that stimulation of glucagon secretion by catecholamines is due to β-adrenoceptor activation (Woods & Porte, 1974). 10 µg of noradrenaline administered subcutaneously to term fetuses reduced the plasma level of insulin and increased the plasma level of glucagon (Girard et al., 1974); catecholamine release around parturition would
therefore reinforce the observed changes in the plasma levels of the pancreatic hormones.

Figure 5.1 is a schematic representation of potential mechanisms by which catecholamines might modulate carbohydrate metabolism in the perinatal rat.
Catecholamine stimulation of hepatic glucose production following birth: possible mechanisms

**Major Pathway**

**Catecholamines**

- Hepatic adrenoceptor

**Gluconeogenic Precursors**

- Pyruvate

**Phosphoenolpyruvate**

**Fructose-1,6-Bisphosphate**

**Fructose-6-Phosphate**

**Glucose-6-Phosphate**

**Glucose**

**Minor Pathway**

**Catecholamines**

- Hepatic adrenoceptor

**Protein kinase activity**

**cyclic AMP levels**

**Adenyl cyclase activity**

**Induction**

**F-1, 6-BP**

**F-2,6-BP**

**GP**

**GS**

**PbK**

**GS**

**PI turnover**

**Cytosolic Ca²⁺ levels**

F-1, 6-BP: Fructose-1,6-bisphosphate; F-2,6-BP: Fructose-2,6-bisphosphate;
GP: Glycogen phosphorylase; GS: Glycogen synthase; PbK: Phosphorylase b kinase;
PC: Pyruvate carboxylase; PEPCK: Phosphoenolpyruvate carboxykinase;
PFK: Phosphofructokinase; PK: Pyruvate kinase

⊕ indicates stimulation of enzyme activity; ☐ indicates inhibition of enzyme activity
5.2 FINDINGS IN OTHER SPECIES

Variations are known to exist between different species in the exact pathways and control of hepatic carbohydrate metabolism; Pogson et al. (1983) demonstrated a number of species differences using isolated hepatocytes and Dawkins (1966) commented on the heterogeneity of fetal and neonatal hepatic carbohydrate metabolism when a number of animal species are studied. This, together with the observation that there is wide inter-species variation in the degree of adrenal medulla development during the perinatal period of (Comline & Silver, 1966), makes it inadvisable to extrapolate findings made in the laboratory rat to other species too freely.

5.2.1 EXPERIMENTAL ANIMALS

The balance of evidence from the literature would appear to support a potential role for catecholamine-stimulated initiation of glucose production via a β-adrenergic mechanism in the rabbit at birth. In the late fetal rabbit the adrenal medulla contains adrenaline which is released in response to stimuli, such as anoxia, which would be present at parturition (Roffi, 1972). Using hepatocytes isolated from adult rabbits, Yorek et al. (1980, 1981) have demonstrated that adrenaline, isoprenaline, phenylephrine and cyclic AMP all stimulate gluconeogenesis by inhibiting pyruvate kinase; the use of adrenoceptor-selective adrenergic agonists and antagonists lead this group to conclude that this effect was mediated via activation of β-adrenoceptors. Increased glycogenolysis, resulting from stimulation of glycogen phosphorylase activity, is also seen in isolated rabbit hepatocytes in response to adrenergic agonists, and has been shown to be β₂-mediated (Arinze & Kawai, 1979, 1983; Arinze et al., 1983). Selective stimulation and blockage of α- and β-adrenoceptors in a perfused rabbit liver system provided evidence that adrenergic stimulation of glycogenolysis in the rabbit was also β-mediated, from as early as seven weeks of age (Rufo et al., 1981). Radioligand binding studies demonstrated a predominance of β-adrenoceptors, mainly of the β₂
subtype, in adult rabbit liver (Kawai & Arinze, 1983). Finally, in a detailed study of developmental changes in the hepatic adrenergic system of the rabbit, Kawai et al. (1985) demonstrated a reduction in the coupling of the β-adrenoceptor to adenyl cyclase at 4-6 hours post partum compared with at birth, with a subsequent increase in coupling up to 24 hours after delivery. Such a change in the β-adrenergic pathway in the hours following birth is suggestive of a functional role for this pathway in the immediately postnatal period in the rabbit.

Studies of carbohydrate metabolism in newborn lambs have produced some conflicting findings. Warnes et al. (1977a) found no gluconeogenesis from lactate in sheep in utero, despite the presence of significant phosphoenolpyruvate carboxykinase activity; this group suggested (Warnes et al., 1977b) that the very rapid postnatal onset of gluconeogenesis is the result of oxygenation, not increase in the activity of any enzyme or enzymes, a view previously put forward for the rat (Philippidis & Ballard, 1970). Parameswaren & Arinze (1981), by contrast, suggested that the rapid postnatal increase in mitochondrial phosphoenolpyruvate carboxykinase seen in sheep liver was vital for the initiation of gluconeogenesis. Initiation of glycogen breakdown was reported to be induced by catecholamines secreted at birth in the sheep (Sperling et al., 1984); however, while there is strong evidence for β-adrenergic mediated effects in neonatal rat, the effect in sheep was believed by these authors to be due to α-adrenoceptor activation.

Work by Arinze's group has provided evidence for an involvement of activation of the β-adrenergic pathway in postnatal changes in carbohydrate metabolism in the guinea-pig liver. In hepatocytes isolated from adult guinea pigs, stimulation of glycogen phosphorylase and thereby of glycogen breakdown by adrenergic agonists was shown to be due to activation of the adrenergic pathway via binding to β2-adrenoceptors (Arinze & Kawai, 1979, 1983; Arinze et al., 1983). Receptor labelling, studied using guinea pig liver membranes, demonstrated an age-
dependent increase in the size of the β-adrenoceptor population postnatally (Kawai & Arinze, 1981).

5.2.2 **CLINICAL OBSERVATIONS**

Studies of perinatal changes in pathways of carbohydrate metabolism and of the hormonal control of these pathways have largely been carried out using experimental animals. However, a great number of relevant observations have been made in human subjects, and Adam (1971) has reviewed the control of glucose metabolism in the human fetus and neonate. A newborn human baby shows a number of metabolic characteristics in common with neonatal rats. In the first 24 hours after birth, cord blood from human infants showed increasing concentrations of glucagon and free fatty acids, decreasing insulin levels, and catecholamine concentrations decreasing from very high levels at birth with some indication of a secondary increase soon after delivery (Blazquez et al., 1974; Lagercrantz & Bistolleti, 1977; Leonetti et al., 1980; Sperling et al., 1974). Gluconeogenesis, absent from the human liver in utero, is active a matter of hours after birth in healthy neonates (Frazer et al., 1981; Kalhan et al., 1980). As in many animal species, there is a period of postnatal hypoglycemia in the human neonate which is corrected even in the absence of suckling; prolongation of this hypoglycemic phase might occur if the mechanisms involved in initiating glucose production postnatally where disturbed in some way, and this could have tragic consequences. The potentially damaging effects of hypoglycemia in an infant are well documented (Beard et al., 1971; Cryer, 1983). Severe hypoglycemia in newborn babies is known to have serious consequences, which may include permanent neurological damage (Haworth and McRae, 1965; Pagliara et al., 1973).

It is of great interest to establish the possible role of catecholamines in regulation of perinatal hepatic carbohydrate metabolism in the human, so that appropriate steps might be taken to correct exceptionally prolonged or pronounced
postnatal hypoglycemia. Little direct evidence is available as to the role, if any, of the high catecholamine levels seen at birth, although it has been suggested that the adrenergic system does have a 'functional role' in the neonate (Lagercrantz & Bistolleti, 1977), and that fetal catecholamine secretion during delivery may be an 'important adaptive mechanism' (Padbury et al., 1982). Some interesting observations have arisen from the use of β-sympathomimetic drugs in the control of premature labour. Metabolic effects have been observed to result from such treatment (Lunell et al., 1977, 1978; Wager et al., 1981), and potentiation of postnatal hypoglycemia has been reported in some cases (Epstein et al., 1979), which it has been suggested might be due to β-adrenergic mediated depletion of fetal hepatic glycogen stores (Unbehaun, 1974). However, other case reports have shown no such effect (Andersson et al., 1977) and it has been suggested that tolerance to the drug treatment would develop rapidly (Wager et al., 1981) and adverse effects to the fetus were unlikely (Lunell et al., 1977). This series of findings would seem to suggest that, although administration of β-sympathomimetic drugs to pregnant women is unlikely to produce any harmful changes in perinatal hepatic carbohydrate metabolism, an active adrenergic pathway for modulation of glucose production after birth does exist in the human fetus and neonate.
5.3 CONCLUSIONS

The present study has included characterisation of the developmental changes of hepatic β-adrenoceptors during the days immediately before and after birth. To complete this picture, similar studies of the α-adrenoceptor population are required, as previous studies showed major discrepancies, as discussed in Chapter 2 (section 2.4.6). Recent work on the interconversion of adrenoceptor subtypes in incubated or cultured hepatocytes (see Chapter 1, section 1.3.1) reveals the plasticity of receptor organisation in the liver. Since the perinatal rat liver has been clearly shown in this study to be characterised by a β-specific adrenergic responsiveness, which is in contrast to the established adrenergic responsiveness of the adult, the timing, control and physiological role of this switch to an α-adrenoceptor mediated adrenergic pathway are intriguing questions. This temporal change in the predominant hepatic adrenergic receptor population might involve a redistribution in the proportions of the adrenoceptor population consisting of the different types, or may be the result of maturation of the intracellular post-receptor signalling mechanisms.

Evaluation of the roles of the adenyl cyclase-cyclic AMP and the phosphatidyl inositol-calcium signalling mechanisms as possible 'second messengers' in the effects of catecholamines on glucose production observed in the present work both in vitro (Chapter 3) and in vivo (Chapter 4) would be of great help in elucidating the developmental changes taking place in the perinatal rat liver. Such studies might also clarify the unanticipated but intriguing observations made with the α-adrenergic antagonist phentolamine, both in the radioligand binding studies (Chapter 2) and the experiments in which glucose production rates in isolated hepatocytes were determined (Chapter 3). Complex interrelationships between occupation of various adrenoceptor subtypes and subsequent effects on alternative intracellular signalling systems might underlie the apparently paradoxical effects observed.
In the present study, the effects of catecholamines on glucose production in hepatocytes isolated from rats at various times in the perinatal period were characterised in terms of minimum concentration to elicit an effect, maximal effect achieved and sensitivity of the glucose production pathway to stimulation by catecholamines; this is the first time such findings have been reported. To gain greater insight into these observed effects, it would be very informative to determine the flux through enzymes of glucose metabolism in isolated perinatal hepatocytes in the absence or presence of concentrations of catecholamines known to stimulate glucose production.

A physiological role for the adrenergic system in postnatal initiation of hepatic glucose production in the laboratory rat has been demonstrated by the experiments reported in this thesis. The paucity of relevant human data makes extrapolation of the observations in the rat to the human neonate unreasonable, and similar studies to those described here should be carried out using post mortem human fetal and neonatal liver tissue before any conclusions may be drawn regarding the relevance of the findings of this study to the clinical situation.
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