STUDIES ON THE METABOLISM AND TOXICITY OF PHENFORMIN

by

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Department of Biochemistry,
University of Surrey,
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For Kate
'Tis strange — but true; for truth is always strange;

Stranger than fiction

Lord George Gordon Byron

How often have I said to you that when you have eliminated the impossible, whatever remains, however improbable, must be the truth?

Sir Arthur Conan Doyle
Phenformin (N\(^1\)-phenethylbiguanide) is an oral hypoglycaemic agent which has been in use in the U.K. since 1959 for the treatment of some forms of diabetes. In recent years there have been reports of lactic acidosis associated with phenformin therapy, particularly in patients with renal or hepatic diseases.

Metabolic and pharmacological studies in the rat and guinea-pig have shown that a number of factors are involved in the differences in response to phenformin in these species. Following a low oral dose of \([2'\,-^{14}C]\)phenformin rats excreted the radioactivity more rapidly than guinea-pigs and metabolised the drug more extensively. The rat eliminated almost 88% of a 7 mg/kg dose in the urine and faeces in 24h and the urine contained almost entirely 4-hydroxyphenformin (free and conjugated with glucuronic acid) which had no effects on blood lactate or glucose concentrations in this species. The guinea-pig excreted only 57% of a 25 mg/kg dose and 50% of the urinary radioactivity (18.5% of the dose) was unchanged phenformin.

The rat actively eliminated a large proportion (26% in 6h) of a 20 mg/kg intraduodenal dose of \([2'\,-^{14}C]\)phenformin in the bile, most of the radioactivity being attributable to the parent compound. In the guinea-pig biliary excretion was initially rapid but declined so that only 6% of the same dose was eliminated in 6h. The possibility that active biliary excretion of phenformin is inhibited by the pharmacological effects of the drug in the guinea-pig is discussed.

After oral administration of \([2'\,-^{14}C]\)phenformin guinea-pig urine contained no 4-hydroxyphenformin but small amounts were detected after intraperitoneal administration. After both routes of administration guinea-pig urine contained a novel metabolite and its glucuronide. After oral administration a second novel metabolite was detected in
guinea-pig faeces. These metabolites have not been completely characterised but in both cases the biguanide portion of the metabolites had been modified. The urinary metabolite is probably the product of aliphatic hydroxylation rather than aromatic hydroxylation. The identification and pharmacological properties of these metabolites remain to be determined.

Preliminary in vitro studies using isolated rat and guinea-pig hepatocytes showed a faster rate of metabolism of [2'-14C]phenformin in rat cells. Rat cells produced significant amounts of 4-hydroxyphenformin and its glucuronide but guinea-pig cells did not.

In the rat the time taken to eliminate the dose and the extent to which phenformin was excreted unchanged in the urine increased with larger doses of the drug. After a high oral dose (100 mg/kg) 24% of the urinary radioactivity (12.5% of the dose) was attributable to unchanged phenformin.

Pharmacological studies showed that the rise in blood lactate concentrations and fall in blood glucose concentrations associated with phenformin were dose related in the rat. The relationship between this observation and the increased elimination of unchanged drug in rat urine after high doses is discussed in terms of a first-pass effect for phenformin in the rat. The dose-response data indicated a critical dose in rats in the region of 120 mg/kg intraperitoneally, at which point marked increases in the pharmacological effects of the drug were seen. Normal and fasted guinea-pigs were more susceptible than rats to both pharmacological actions of phenformin. 4-Hydroxyphenformin produced hyperlactataemia in guinea-pigs without affecting blood glucose levels.

The possibility that the guinea-pig may produce a biologically
active metabolite has not been resolved, but the differences in pharmacological response to phenformin in the rat and guinea-pig have been explained by the results in the thesis, which show a slower rate of metabolism and excretion of the drug in the guinea-pig, together with the established biochemical differences in the mechanisms of carbohydrate regulation in these species.

Sodium dichloroacetate was effective in reducing phenformin-associated hyperlactataemia in rat and guinea-pig and prevented the increase in blood lactate levels caused by 4-hydroxyphenformin in guinea-pigs.
ACKNOWLEDGEMENTS

I would like to thank all those who have helped in the preparation of this thesis, and am particularly grateful to the following:-

Professor D.V. Parke for offering me the opportunity to work in his department and for his interest in the progress of the research.

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CHAPTER 1

INTRODUCTION
1.1 HISTORICAL REVIEW

The development of biguanides as a treatment of diabetes stemmed from the reports that parathyroidectomy produced hypoglycaemia which was accompanied by an increase in blood levels of guanidine (Underhill et al., 1914; Paton et al., 1917). This led to the suggestion that guanidine might have potential as a hypoglycaemic agent (Watanabe, 1918), and to a number of pharmacological and metabolic studies using guanidine.

Unfortunately, guanidine itself (see FIG. 1.1) is a highly toxic substance, its lethal dose in mice being 4-5 mg/kg (Sterne, 1969), and so attempts were made to modify the molecule to obtain a less toxic material which retained the blood sugar-lowering capability. Early investigators concluded that for the hypoglycaemic effect, it was necessary to avoid substitution at both ends of the guanidine moiety. Of the so-called "mono-guanidines" which were synthesised, only iso-amyl-guanidine (Galegin) showed even a slight possibility of being effective in the treatment of diabetes (Mehnert and Sadow, 1969).

The diguanidines (see FIG.1.1), however, were quite effective as hypoglycaemic agents and less toxic than the monoguanidine derivatives. The first of these, Synthalin A, was introduced in 1926, and an improved version two years later (Frank et al., 1926, 1928). Although these compounds were used for a number of years reports of their toxicity, primarily gastrointestinal reactivity, were prevalent. There were also reports of other toxic side-effects which were not assessed accurately since pharmacological methods of testing were not highly developed at this time. The long methylene chain breaks releasing fragments of guanidine which exert a toxic effect upon the liver and
kidneys. The recording of a number of digestive, hepatic and renal complications led to discontinuance of this therapy. In fact the reports of complications have been questioned by many of the leading diabetologists of the Synthalin era (Mehnert and Sadow, 1969), and inadequate monitoring of patients along with a poor understanding of the true diabetic state in each of the diabetic classes treated undoubtedly contributed to the ultimate failure of the diguanidines.

In 1929 Slotta et al. reported the preparation of a number of alkyl biguanides and found that they had hypoglycaemic activity as well as an apparently lower toxicity than the guanidines. Although the alkyl biguanides seemed to be useful, a combination of factors essentially closed down research in this area for nearly 25 years; namely the advent of insulin, increasing reports of guanidine toxicity and the assumption that since guanidines were highly hepatotoxic, biguanides were also hepatotoxic.

Ambiguity in nomenclature was evident at this time. The Synthalins are diguanidines with two discrete 3-nitrogen groupings, whereas the biguanides contain one 5-nitrogen moiety (see FIG.1.1).

The hypoglycaemic effects of the biguanides were not re-examined until 1957, when Ungar reported the hypoglycaemic effect of α-phenethylbiguanide (Ungar et al., 1957). Since that time a number of biguanides have been prepared and evaluated as hypoglycaemic agents. The relative effectiveness of various substituted biguanide compounds is discussed in SECTION 1.2.
\[ \text{guanidine} \]
\[ \text{guanylguanidine} \] (biguanide)
\[ \text{the diguanidines:} \]
\[ n = 10, \text{Synthalin A} \]
\[ n = 12, \text{Synthalin B} \]
\[ \beta\text{-phenethylbiguanide} \] (phenformin)

**FIG. 1.1** Structure of guanidine derivatives
A number of biguanides have been synthesised and pharmacologically tested in animals (Hesse and Taubman, 1929; Ungar et al., 1957; Shapiro et al., 1959a,b,c,d). These possess the general formula (1). Biguanide itself has about the same blood sugar-lowering effect as simple guanidine (2).

\[
\begin{align*}
\text{NH}_2 & \quad \text{C} \quad \text{NH}_2 \\
\text{NH} & \\
\end{align*}
\]

Substitution on \( N^1 \) produces a number of effective preparations but the choice of substituents is very limited. Aralkyl substitution is also very effective when the aliphatic portion consists of one or two carbons. Substitution on the alkyl chain by hydroxy, methoxy or mercapto groups gives inactive compounds.

Di-substitution on \( N^1 \) appears to produce effective compounds only if one of the groups is methyl, and is most effective when the other group is methyl or benzyl.

According to Shapiro et al. (1959a,b,c,d) substitution on \( N^5 \) only forms effective compounds if the total of the C atoms of the substituents does not exceed five and only three of the four radicals are substituted. In contrast, Osterloh (1960) has reported that tetra-substitution with
butyl radicals produces an effective hypoglycaemic compound, and N\textsuperscript{1}-butyl-N\textsubscript{5},N\textsubscript{5}-dimethylbiguanide has also been shown to be active.

The relative effectiveness of various substituted biguanides is summarised in TABLE 1.1.

The structure of the three biguanide derivatives in clinical use at the present time are shown below, (3-5).

\[
\text{PHENFORMIN (3) } \quad N^1\text{-phenethylbiguanide}
\]

\[
\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{NH-CNHNH}_2\quad \text{BUFOMIN (4)} \quad N^1\text{-butylbiguanide}
\]

\[
\text{CH}_3\text{NH}_N\text{NH}_N\text{N-C-NH}_{\text{CH}}\text{NH}_2\quad \text{METFORMIN (5)} \quad N^1,N^1\text{-dimethylbiguanide}
\]

**Reactions of the biguanides**

Although the structures of biguanides are conventionally represented as shown above, the biguanide moiety may also be represented by a number of tautomeric forms, which in turn may also exist in several resonant forms (Elpern, 1968) some of which are shown in FIG.1.2.

In addition it has been suggested by Shapiro et al. (1959b) that the effective biguanide derivatives under physiological conditions exist in ring form as monobasic, conjugated hydrophilic cations. This ring structure (6) is readily formed by biguanides under a number of conditions.
<table>
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<th>Substituents on N¹</th>
<th>Substituents on N⁵</th>
<th>hypoglycaemic activity</th>
</tr>
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<tr>
<td>H, H</td>
<td>H, H</td>
<td>biguanide - about same activity as guanidine</td>
</tr>
<tr>
<td>alkyl, H</td>
<td>H, H</td>
<td>C₄-C₅ most effective, e.g. buformin; greater than C₁₀ ineffective - branching reduces activity</td>
</tr>
<tr>
<td>ary1, H</td>
<td>H, H</td>
<td>relatively inactive</td>
</tr>
<tr>
<td>alkyl,alkyl</td>
<td>H, H</td>
<td>generally lose activity unless one group is methyl - most active when other group is benzyl or methyl (metformin)</td>
</tr>
<tr>
<td>hetero-ring with N</td>
<td>H, H</td>
<td>relatively inactive</td>
</tr>
<tr>
<td>aralkyl,H</td>
<td>H, H</td>
<td>most effective when alkyl group contains one or two carbons, e.g. phenethyl(phenformin) and p-chlorobenzyl, and no substituents.</td>
</tr>
<tr>
<td>alkyl, H</td>
<td>alkyl, H</td>
<td>effective if only one group is larger than methyl.</td>
</tr>
<tr>
<td>alkyl,alkyl</td>
<td>alkyl, H</td>
<td>reportedly ineffective unless total of C atoms in substituents does not exceed 5¹ but this rule not confirmed; seems to require no more than one substituent to be larger than methyl. Thus N¹-propyl- and N¹-butyl-N⁵, N⁵-dimethylbiguanides are effective.</td>
</tr>
<tr>
<td>alkyl,alkyl</td>
<td>alkyl,alkyl</td>
<td>generally believed to be inactive, however tetrabutylbiguanide has some activity²</td>
</tr>
</tbody>
</table>

(1) Shapiro et al. (1959b); (2) Osterloh (1960); (3) other references - Hesse and Taubmann (1929); Ungar et al. (1957); Shapiro et al. (1959a,c,d); Elpern (1968); Bändner (1969).
Wickramasinghe and Shaw (1972) have reported the thermal instability of phenformin hydrochloride to gas chromatographic conditions. Analysis by mass spectrometry of the single GC peak seen at 225°C showed signals consistent with the s-triazine structure (7) shown below.
FIG. 1.2  Resonant and tautomeric forms of the biguanide moiety

FIG. 1.3  Acylation reaction of phenformin with acetic anhydrides to form triazine derivatives.
1.3 BIGUANIDES IN THE TREATMENT OF DIABETES MELLITUS

Clinical experience with the biguanides has shown that the drugs can lower blood sugar extrapancreatically and reduce excessive, delayed, post-prandial insulin release. Biguanides cannot increase endogenous insulin release, suppress lipolysis or promote lipogenesis. Thus in the insulin-dependent diabetic total insulin replacement is impossible since the lowering of blood sugar is almost certainly less important than the suppression of the excessive lipolysis which can lead to ketoacidosis.

The overweight, ketoacidosis-resistant diabetic is usually an over-secretor of insulin in response to hyperglycaemia. Excessive body weight and hyperinsulinaemia seem to have a tendency to go hand-in-hand. In this case the use of biguanides to lower blood sugar without calling upon more endogenous insulin permits a reduction in the hyperinsulinaemic response and avoids lipogenesis without further suppressing lipolysis.

Accordingly, because of the gradual and persistent reduction in body weight and serum cholesterol levels produced by the drugs (Miller, 1960; Brown and Gabert, 1962; McKendry, 1962; Patel and Stowers, 1964; Alterman and Lopez-Gomez, 1968; Craig et al., 1968; Gershberg et al., 1968; Meinert and Schwartz, 1968) the biguanides are considered by many to be the drug of choice in the stable adult-onset, ketoacidosis-resistant diabetic who has a strong tendency towards becoming overweight.

Biguanides have also been used successfully in the treatment of some unstable adult-onset diabetics (Krall et al., 1958) particularly in cases of insulin resistance (Roush et al., 1961; Gold, 1962).
The combination of a biguanide with a sulphonylurea (usually phenformin and chlorpropamide) has been found effective in maintaining diabetic control in patients over 50 years of age particularly in view of the high incidence of secondary sulphonylurea failures, where the pancreatic β-cells become less capable of regenerating insulin in response to repeated doses of the drug. There are numerous reports of full recovery of control of diabetes by combined therapy in patients who responded poorly to either drug alone (Bradley, 1959; Bloom et al., 1961; Clarke et al., 1965; Gibbs, 1966). Combined therapy provides maximal hypoglycaemic effect and tends to avoid side-effects as dosage of each drug is reduced, but the risk of hypoglycaemia is increased.

1.4 PHARMACOLOGY AND MODE OF ACTION OF THE HYPOGLYCAEMIC BIGUANIDES

Despite a large amount of data on the effects of biguanides both in vitro and in vivo, the mechanism by which hypoglycaemia is produced has not yet been fully elucidated. A very important aspect is the relationship between the mode of action of the biguanides and lactate metabolism. There is no doubt, from clinical and experimental evidence, that lactate metabolism is influenced by the biguanides, and that in special circumstances, when lactate metabolism is disturbed, the drugs can exert a toxic action. This problem is discussed in more detail in SECTION 1.5.

Hypoglycaemic effects

The hypoglycaemic dose of all biguanides differs considerably from one drug to another, and there is a widely varying response to the same drug in different species. The nutritional state of the animals also has considerable bearing on the hypoglycaemic activity (Ungar, 1961)
and in the same species may vary by 100% of the lowest dosage depending upon the individual susceptibility.

Hypoglycaemia appears in non-diabetic animals only after administration of very large doses, far higher than therapeutic doses and even as high as 50% of the lethal dose. In non-diabetic humans a number of authors have reported insensitivity to doses of phenformin which cause a pronounced reduction of blood sugar in diabetics (Fajans et al., 1960; Madison and Ungar, 1960). This specific antidiabetic action has also been reported with metformin (Rambert et al., 1961) and buformin (Osterloh, 1960; Söl and Creutzfeldt, 1960). The biguanides can lower blood sugar in experimentally-induced diabetic animals. Although there have been conflicting reports on this effect by various authors these are almost certainly due to the difficulty in defining the exact nature of the diabetic state produced. In streptozotocin-induced diabetic rats the blood glucose concentration was significantly reduced by doses of phenformin (30 to 100 mg/kg, intraperitoneally) which were virtually inactive in normal rats (Polacek and Ouart, 1974). Beckmann (1961) succeeded in maintaining the blood sugar at a normal level in alloxan-induced diabetic rats with 100-150 mg/kg of buformin orally, the glucose concentration dropping from 366 to 60 mg/100 ml in 3 days. Similar effects in alloxan diabetic rats given 700 mg/kg of metformin orally have been reported by Sterne (1969).

Experiments in animals after surgical pancreatectomy have produced conflicting results, probably due to differing experimental procedures and varying degrees of trauma. In the partially eviscerated guinea-pig from which the pancreas, stomach and intestines were removed and in which
the liver was excluded from the circulation, phenformin produced a marked hypoglycaemia even in the presence of a dextrose infusion (Nielsen et al., 1958). On the other hand Köhler and Lippmann (1963) found that blood sugar in pancreatectomised dogs was not affected by phenformin. Metformin (50 mg/kg, orally) administered to pancreatectomised dogs caused a reduction in blood glucose according to Hesse and Taubmann (1929), but Sterne (1969) was unable to reproduce these findings and concluded that the presence of some pancreatic islets was essential for the biguanides to act effectively. In view of the varying degrees of surgical trauma and the difficulty of ensuring a total pancreatectomy in some species (Sterne, 1969) the results of these experiments are difficult to interpret.

**Toxic effects**

These have been carefully studied, partly because the biguanides are given for prolonged periods and partly because earlier oral antidiabetic agents such as the diguanidines had created adverse impressions.

The toxic dose varies from species to species and, as death generally results from hypoglycaemia, there is considerable variation depending on the nutritional state and individual susceptibility of the animal. The Rhesus monkey and the guinea-pig are both exquisitely sensitive to the hypoglycaemic effects of phenformin, while the rat and mouse are resistant. A comparison of the LD$_{50}$ values of phenformin and metformin in a number of laboratory animal species is shown in TABLE 1.2. Equally extensive data on the acute toxicity of buformin does
<table>
<thead>
<tr>
<th>Species</th>
<th>Phenformin LD$_{50}$ (mg/kg)</th>
<th>Metformin LD$_{50}$ (mg/kg)</th>
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</thead>
<tbody>
<tr>
<td>Guinea-pig</td>
<td>26.5</td>
<td>150</td>
</tr>
<tr>
<td>Rat</td>
<td>38</td>
<td>500</td>
</tr>
<tr>
<td>Oral (p/o)</td>
<td>190</td>
<td>300-500 approx.</td>
</tr>
<tr>
<td>Subcutaneous (s/c)</td>
<td>650</td>
<td>1,000 approx.</td>
</tr>
<tr>
<td>Rat (Fasted)</td>
<td>88</td>
<td>150</td>
</tr>
<tr>
<td>Mouse</td>
<td>150</td>
<td>225</td>
</tr>
<tr>
<td>s/c</td>
<td>235</td>
<td>800</td>
</tr>
<tr>
<td>p/o</td>
<td></td>
<td>3,500 approx.</td>
</tr>
<tr>
<td>Rabbit</td>
<td>14.5</td>
<td>63 approx.</td>
</tr>
<tr>
<td>Intravenous (i/v)</td>
<td>800</td>
<td>12-15 approx.</td>
</tr>
<tr>
<td>Cat</td>
<td>150</td>
<td>13-5-15 approx.</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>350</td>
<td>13-5-15 approx.</td>
</tr>
</tbody>
</table>

* taken from Ungar et al. (1957), Ungar et al. (1960), Sterne (1961, 1969).
not appear to have been published.

Investigation of chronic toxicity of phenformin in laboratory animals has been studied, although the problem of fatal hypoglycaemia at large doses has caused some difficulties. In rats given 100 mg/kg orally each day for 6 months no harmful effects were seen on growth, blood sugar, nitrogen, cholesterol, protein, or electrolytes. Organ weights and histology in the treated group were indistinguishable from control animals. In the same study guinea-pigs given 10 or 20 mg/kg orally for 6 months showed no harmful effects (Ungar, cited by Sterne, 1969), and no untoward effect on blood chemistry other than a fall in blood sugar. Some minor necrosis of hepatic cells and histological changes in the renal tubules was reported in guinea-pigs after administration of 25 mg/kg sub-cutaneously (Creutzfeldt and Moench, 1958).

Chronic toxicology studies with metformin in the rat (125 mg/kg, orally) and rabbit (100 mg/kg, orally) given the drug daily for one year, failed to detect any abnormalities in blood chemistry or tissue histology (Sterne, 1969). After 5 months treatment with buformin (50 mg/kg, daily) in the rat, a number of hepatic lesions including some sclerosis developed and fatty deposits were found in the renal epithelium (Georgii, 1960). The extent of the lesions, particularly fibrosis and glycogen depletion, was more pronounced in rats with experimentally induced cirrhosis.

In the human diabetic in the absence of other illnesses and at therapeutic doses, the biguanides are non-toxic (Mehnert and Sadow, 1969; Hermann, 1973). Recently, Graeber et al. (1976) described the occurrence of severe pancreatitis in an elderly diabetic patient receiving phenformin (50 mg orally, twice a day). The implication of phenformin as the aetiological
agent was based primarily on the absence of other causes of pancreatitis at laparotomy, and the fact that the patient had not suffered a recurrence of the condition since termination of biguanide therapy. In previously published cases cessation of the drug terminated relapsing bouts of pancreatitis (Wilde, 1972; Levitan, 1973; Goodley et al., 1973).

In the presence of other complicating disorders biguanides can give rise to lactic acidosis. The causal connection between biguanide therapy and the development of lactic acidosis in diabetic patients is a controversial issue at present. Although a slight elevation of blood lactate is common in phenformin treated diabetics (Craig et al., 1960; Fajans et al., 1960; Nattrass et al., 1977) a significant increase is unlikely to occur except in cases where some other factor is present, such as poor renal function (Cohen et al., 1973; Ball et al., 1974; Heuclin et al., 1975; Conlay and Loewenstein, 1975; Searle and Siperstein, 1975; Assan et al., 1976; Thimme et al., 1976; Williamson, M., 1976; Wise et al., 1976), liver disease (Cohen et al., 1973; Assan et al., 1975; Heuclin et al., 1975; Assan et al., 1976), reduced circulatory efficiency, which in turn would affect renal function (Assan et al., 1975; Dembo et al., 1975; Assan et al., 1976; Thimme et al., 1976; Wise et al., 1976), severe infections (Dembo et al., 1975; Heuclin et al., 1975; Romankiewicz, 1975; Thimme et al., 1976; Williamson, M., 1976) and acute alcoholosis (Johnson and Waterhouse, 1968; Heuclin et al., 1975; Assan et al., 1976). Lactic acidosis also occurs in diabetic patients treated with metformin (Lebacq and Tirzmalis, 1972; Hayat, 1973; Heuclin et al., 1975; Bismuth et al., 1976; Assan et al., 1977) and buformin (Clavadetscher et al., 1976; Thimme et al., 1976). Although the incidence of lactic acidosis with
metformin and buformin is lower than that in phenformin treated patients, the existence of the same predisposing conditions has been reported (Clavadetscher et al., 1976; Thimme et al., 1976; Assan et al., 1977).

While there have been some reports of lactic acidosis in diabetic patients receiving biguanides in whom no known causes of lactate accumulation were detected (Blumenthal and Streeten, 1975; Fulop and Hoberman, 1976) the weight of evidence is strongly in favour of regarding the biguanides as having a contributory rather than a causal role. This evidence is discussed in more detail in SECTION 1.5.

The effect of phenformin on diabetic patients has been studied under strict balance conditions, (Geldermans et al., 1975). In all five patients there was a lowering of blood glucose and decreased glycosuria. Other metabolic effects included a positive nitrogen, phosphorus and calcium balance and a slight increase in body weight, possibly due to a positive caloric balance mainly caused by the diminished glycosuria. No change in the basal metabolic rate or respiratory quotient was seen, but phenformin treatment did result in a drop in cholesterol and total lipid levels in the blood. A slight loss of appetite was reported by most of the patients, and a strong feeling of anorexia accompanied by vomiting in one subject. No toxic effects were observed in the 15 days of therapy, nor in the subsequent period of observation.

Metabolic effects and mode of action

Although the mechanism of action of the biguanides is not yet clear, a number of theories have been proposed to explain the hypoglycaemic action based on laboratory studies in enzyme systems, isolated animal tissue and several species of laboratory animals, as well as clinical
The earliest theory of biguanide action proposed that the fall in blood sugar arose from an inhibition of tissue respiration with an increase in peripheral anaerobic glycolysis producing lactic acid (Steiner and Williams, 1958). Other workers have attributed the hypoglycaemic and hyperlactataemic effects of the biguanides to an inhibitory action on gluconeogenesis (Tyberghein and Williams, 1957; Patrick, 1966; Meyer et al., 1967; Altschuld and Kruger, 1968; Jangaard et al., 1968; Toews et al., 1970; Haeckel and Haeckel, 1972; Lloyd et al., 1975). It has also been suggested that intestinal absorption of sugars may be inhibited by the biguanides, which have been shown to accumulate in the intestine (Biro et al., 1961; Arvanitakis et al., 1973).

Sterne (1969) has proposed that the activity of the drugs in the body is related in some way to the presence of insulin, even though the biguanides have been shown to exert a hypoglycaemic effect in pancreatectomised animals (Nielsen et al., 1958; Steiner and Williams, 1959a; Sterne 1969).

Other workers have proposed that the metabolic effects of the biguanides can be explained by the effects of the drugs on fatty acid oxidation (Stone and Brown, 1968; Muntoni et al., 1970; Muntoni, 1974).

Attempts to explain the different effects of the biguanides, particularly the enhanced hypoglycaemic activity in the diabetic subject compared to the non-diabetic, have produced a number of apparently conflicting results. Animal experiments have further complicated the interpretation of these findings because of the considerable species
differences in pharmacological susceptibility to the drugs.

In this section, a brief consideration of the experimental and clinical evidence relating to the mechanism of action of the biguanides is presented, along with an attempt to evaluate these findings in order to arrive at a comprehensive view of the metabolic effects of the compounds.

(a) Effects on carbohydrate metabolism

Tyberghein and Williams (1957) showed that phenformin administered to guinea-pigs (20 mg/kg) caused a lowering of blood glucose, depletion of liver glycogen and a significant rise in blood lactate levels. Incubation of isolated rat hemidiaphragm preparations with phenformin resulted in an increased uptake of glucose which was accounted for by lactic acid production, not by glucose oxidation (Williams et al., 1957). The glycogen content of the muscle decreased in the presence of phenformin.

Steiner and Williams (1958) studied the effect of several biguanides on the respiration of rat and guinea-pig isolated diaphragm and liver slices. Phenformin inhibited oxygen consumption by the muscle preparations and increased glucose uptake and lactic acid production. Significant effects were only seen at high concentrations such as $1 \times 10^{-3}$M phenformin. In vivo administration of phenformin (40 mg/kg, subcutaneously) to rats caused a subsequent depression in oxygen uptake in liver slices in vitro. The authors also showed that phenformin (25 mg/kg, subcutaneously) in guinea-pigs resulted in hypoglycaemia 2-3 h after dosing, and elevated lactic acid and inorganic phosphate levels in the blood. These findings were similar to tissue anoxia reported following the administration of the respiratory inhibitors cyanide, fluoride and malonate (Handler, 1945).
Accordingly, Steiner and Williams studied rat and guinea-pig liver mitochondrial respiration and found a small degree of inhibition of succinate dehydrogenase and a larger inhibition of cytochrome oxidase with phenformin ($3 \times 10^{-4}$-$6.7 \times 10^{-3}$M).

The exact site of respiratory inhibition appeared to differ according to the tissue being studied. In rat epididymal fat preparations a number of enzymes of the tricarboxylic acid cycle were inhibited by $2.1 \times 10^{-3}$M phenformin (Wick et al., 1958), the point of inhibition being between succinate dehydrogenase and reduced cytochrome C. Similar results were obtained with rat kidney homogenates incubated with $2.1 \times 10^{-3}$M phenformin (Kruger et al., 1960).

These data led Steiner and Williams (1958) to suggest that the hypoglycaemia experienced after biguanide administration may be due to tissue anoxia, caused by inhibition of tissue respiration. They suggested that the primary inhibitory effect of the drugs on oxidative phosphorylation leads to a lowering of ATP levels which increases peripheral anaerobic glycolysis and decreases gluconeogenesis.

This explanation has lost favour but according to Cohen and Woods (1976) its implications regarding lactate metabolism are still widely accepted. The greatest criticism is that the hypothesis is based upon the results of experiments using high phenformin concentrations. The plasma level in humans following a therapeutic dose of 50 or 100mg normally does not exceed 0.1-0.2µg/ml, i.e., $0.5-1.0 \times 10^{-6}$M (Beckmann, 1968; Mehnert, 1969a; Karam et al., 1974; Sterling Winthrop Research Laboratories, personal communication, 1974; Alkalay et al., 1975; Matin et al., 1975). This represents the peak
(2-3h) level and is short lived, and while it is possible that certain organs may experience concentrations higher than that in plasma, there is no evidence in the literature to indicate that levels ever reach the $1.0 - 2.1 \times 10^{-3} \text{M}$ used in in vitro experiments. The tissue concentrations of phenformin (Hall et al., 1968), buformin (Beckmann, 1965) and metformin (Cohen and Costerousse, 1961) after administration to animals are discussed in SECTION 1.6.

As man is reportedly like the rat in metabolising and eliminating phenformin as the phenol (Beckmann, 1968) it would seem unlikely that particularly high concentrations of the unchanged biguanide occur in the tissues.

Therefore, in interpreting the in vitro actions such as glycogen depletion in rat liver slices (Steiner and Williams, 1958; Sölting, 1969) it should be remembered that the effects have only been observed with high concentrations of biguanides, and even the lower levels ($4 \times 10^{-4} \text{M}$ phenformin) used by Altschuld and Kruger (1968) to inhibit gluconeogenesis and oxidative phosphorylation in isolated, perfused guinea-pig liver are considerably greater than those measured in the plasma of patients after therapeutic doses.

No reduction in liver glycogen nor any indications of inhibition of oxidative phosphorylation was observed in patients treated effectively with biguanides (Sterne, 1969). Sölting (1969) found no evidence of glycogen depletion or reduced oxidative phosphorylation in rat liver perfused in vitro with biguanides at a dose level ($2.5 \times 10^{-5} \text{M}$) which does not greatly exceed the plasma concentration for therapeutic doses in man.
In the guinea-pig phenformin produced hypoglycaemia at a dose of 10mg/kg subcutaneously while 100mg/kg had no effect on blood sugar in the rat (Altschuld and Kruger, 1968). However, the absence of hypoglycaemia in the normal rat does not indicate a lack of effect on carbohydrate metabolism, since the production of $^{14}\text{CO}_2$ from [U-$^{14}$C]glucose was increased by doses of phenformin which did not lower blood sugar (Losert et al., 1971). The guinea-pig was also more sensitive than the rat to the hypoglycaemic actions of hypoglycin (methylene cyclopropylalanine) and cyclopropanecarboxylic acid (Sherratt, 1969). If the dose of biguanide was adjusted according to the sensitivity of the animal the guinea-pig responded similarly to the rat. Thus, while 25mg/kg phenformin produced marked hypoglycaemia, 2.5 mg/kg did not. This lower dose, however, did stimulate the production of $^{14}\text{CO}_2$ from [U-$^{14}$C]glucose in the guinea-pig (Losert et al., 1971). This suggests that there are two effects of biguanides in the non-diabetic: enhancement of glucose oxidation at lower doses, and hypoglycaemia associated with inhibition of oxidative phosphorylation, reduction of hepatic ATP levels and inhibition of gluconeogenesis at high doses of the drugs.

It may be that the hypoglycaemia produced in non-diabetic animals is a toxic effect which is produced by large doses of biguanides. The considerable difference between the hypoglycaemic dose in normal, non-fasted subjects and the dose which is therapeutically effective in appropriate forms of diabetes in man supports this proposal. It seems likely that in man, as well as in laboratory animals, the mechanism responsible for the antidiabetic action is different from the one causing the toxic effects.

Davies et al. (1971) showed that pretreatment with phenformin and
buformin increased glucose turnover and recycling in the normal rat and
dog without changes in glucose oxidation or liver glycogen concentration.
In contrast Losert et al. (1971) reported that the oxidation of [U-14C]
glucose to 14CO₂ in the normal rat was increased by phenformin, metformin
and buformin with a dose-effect ratio, and that each biguanide produced
an increase in blood lactic acid concentrations.

In the normal, non-diabetic human therapeutic doses (100-150 mg/day)
of phenformin produced an increase in the glucose turnover rate (Searle
and Cavalieri, 1968; Kreisberg, 1968b) accounted for by increased glucconeo-
genesis from lactate and other substrates (Kreisberg, 1968b; Searle et al.,
1969). Blood glucose levels were unchanged (Searle et al., 1966; Searle
and Cavalieri, 1968) while blood lactate levels were elevated (Searle and
Cavalieri, 1968; Kreisberg, 1968b). Glucose oxidation after phenformin
administration, determined as 14CO₂ production, was enhanced with [6-14C]
glucose as substrate (Searle et al., 1969) but was reduced when [1-14C]glucose
was used. A possible reason for this discrepancy is discussed later.

In the non-diabetic obese subject, whose resting glucose kinetics
were similar to those in normal man except that glucose recycling (i.e.
glucose to lactate to glucose) was increased, phenformin administration
appeared to exert a varying influence, perhaps depending upon the degree
of obesity (Muntoni, 1974). Kreisberg (1968a) reported an increased
recovery of[14C]lactate from [1-14C]glucose in phenformin-treated obese
patients, but later found that the drug also caused an increase in glucose
turnover due to glucose recycling, with a rise in both the rate and amount
of conversion of lactate to glucose (Kreisberg, 1968b; Kreisberg et al.,

In diabetic patients phenformin administration resulted in a fall in
blood glucose levels accompanied by an increased glucose turnover and $^{14}$CO$_2$ production from [U-$^{14}$C]glucose (Searle and Cavalieri, 1968). In a later study with a diabetic patient receiving chlorpropamide, alone or in addition to phenformin (50-150 mg/day), Searle and Siperstein (1975) showed that although lactate oxidation was increased under the influence of phenformin this increase was not in balance with the increase in anaerobic metabolism. They suggested that aerobic metabolism in the presence of phenformin did not keep pace with the increased level of anaerobic metabolism, and that this imbalance accounted for the hyperlactataemia associated with phenformin therapy. It was further suggested that the increased gluconeogenesis noted with phenformin treatment in both normal and diabetic subjects (Kreisberg, 1968b; Searle et al., 1969; Kreisberg et al., 1972; Searle and Siperstein, 1975), represents a compensatory mechanism. Kreisberg (1968b) has proposed that oxidative metabolism is inhibited by phenformin resulting in enhanced anaerobic glycolysis with no inhibition of gluconeogenesis.

The disagreement regarding the effect of phenformin on the aerobic metabolism of glucose may arise from the different radio-isotopes used in the various studies. This possibility has been dealt with in some detail by Muntoni (1974). The experiments in which the production of $^{14}$CO$_2$ was increased were carried out with [U-$^{14}$C]glucose (Searle and Cavalieri, 1968; Losert et al., 1971), [U-$^{14}$C]lactate (Searle and Siperstein, 1975) or [6-$^{14}$C]glucose (Searle et al., 1969). Those in which glucose oxidation was reduced or unchanged used [1-$^{14}$C]glucose (Kreisberg, 1968a, b; Kreisberg et al., 1970). The degree of glucose oxidation in the citric acid cycle is best estimated with [U-$^{14}$C]glucose where there is a higher probability that $^{14}$CO$_2$ will be formed directly.
from the entry of pyruvate into the cycle. It is well established that
the first atoms to appear in $\text{CO}_2$ are $C_3$ and $C_4$, followed by $C_2$ and $C_5$
and lastly $C_1$ and $C_6$ (Mahler and Cordes, 1971). As it is possible that
intermediates containing $^{14}$C may leave the cycle and enter synthetic
pathways (e.g. amino-acids and porphyrins) only $[\text{U}^{-14}\text{C}]$glucose (or
lactate) is sure to give rise to $^{14}\text{CO}_2$. The finding that there is a
smaller increase in $^{14}\text{CO}_2$ production from $[\text{6}^{-14}\text{C}]$glucose than from
$[\text{U}^{-14}\text{C}]$glucose (Searle et al., 1969) and that there is a reduction in
$[\text{1}^{-14}\text{C}]$glucose oxidation in phenformin treated subjects (Kreisberg, 1968b)
may be a result of the inhibition of the pentose phosphate pathway which
occurs with phenformin (Wick et al., 1958; Söling et al., 1967). As $C_1$
of glucose is oxidised to $\text{CO}_2$ by this pathway, inhibition by phenformin would
be expected to lower the $^{14}\text{CO}_2$ production from $[\text{1}^{-14}\text{C}]$glucose. Similarly,
as the direct oxidative pathway produces a higher proportion of triose-
phosphate compounds incorporating $C_4$, $C_5$ and $C_6$ than $C_1$ (which is oxidised
to $\text{CO}_2$) or $C_2$ and $C_3$ (which may be condensed onto other sugars) phenformin
may reduce the production from $[\text{6}^{-14}\text{C}]$glucose of triose-phosphates which
would subsequently be oxidised to give $^{14}\text{CO}_2$ (Muntoni, 1974).

Thus the overall effect of phenformin at therapeutic levels appears
to be to stimulate both anaerobic and aerobic metabolism of glucose and to
increase gluconeogenesis. The increase in blood lactate levels in the
presence of biguanides therefore, is not attributable to an inhibition of
oxidative phosphorylation, as the lactate concentration is also raised when
the increase in glucose oxidation is marked (Searle and Cavalieri, 1968;
Losert et al., 1971). However, Searle and Siperstein (1975) have found
that the increased oxidation of lactate does not keep pace with its
production, and the overall effect is a relative inhibition of oxidative
metabolism.
While these findings are at variance with much of the animal work previously mentioned, the large differences in the drug levels used in the in vitro and in vivo studies in animals and in the therapeutic management of human patients make comparison very difficult. Such comparisons also raise great difficulties because of species differences. However, Losert et al. (1971, 1972b) have shown that at very low doses of biguanides which do not produce hypoglycaemia in animals, the production of $^{14}$CO$_2$ from [U-14C]glucose and the blood lactate concentrations are increased.

(b) Effects on gluconeogenesis

Gluconeogenesis is the process responsible for the formation of glucose from various non-carbohydrate precursors, such as amino acids, lactic acid or glycerol, and plays an important role in the maintenance of the optimal concentration of the sugar in the blood. The available carbohydrate reserves which can be used to support body function are stored primarily in the liver, and can account for no more than 8 to 10 hours of glucose requirement by peripheral tissues such as the brain and the blood cells. Thus during starvation gluconeogenesis plays a key role in replenishing the blood glucose concentration (Hanson and Mehlman, 1976). This process is confined to the liver and, under some physiological conditions, the renal cortex.

The finding that administration of biguanides in animals produces an increase in blood levels of lactic acid, together with the belief that the compounds are concentrated in the liver, led to a number of investigations into the effects of the drugs on gluconeogenesis.
There is little evidence, however, to support the belief that the hypoglycaemic biguanides are substantially concentrated in hepatic tissue, and the statement that 'phenformin is concentrated in the liver' (Cohen and Woods, 1976) is not supported by the meagre data in the literature. This data is discussed in more detail in SECTION 1.6.

However, there is evidence to suggest that higher blood concentrations of the drugs do arise in certain diabetic patients with other serious illnesses such as impaired renal or hepatic function (see SECTION 1.5), and the effects on hepatic gluconeogenesis seen in animal studies using 'moderate' concentrations of the biguanides may be relevant in these conditions.

Administration to fasted guinea-pigs of phenformin (Tyberghein and Williams, 1957; Steiner and Williams, 1958; Altschuld and Kruger, 1968; Losert et al., 1972b) or buformin (Losert et al., 1972b) at a dose level of 20-25 mg/kg subcutaneously caused a reduction in blood glucose concentrations of 70-95% in 2-3h. Similar reductions were seen after 300 mg/kg doses of metformin in the guinea-pig (Losert et al., 1972b). Liver glycogen levels were almost totally depleted, even in non-fasted animals (Tyberghein and Williams, 1957), and hepatic ATP levels were reduced by some 40% (Altschuld and Kruger, 1968). An elevation of blood lactate concentrations was produced ranging from 70% (Tyberghein and Williams, 1957) to 800% (Steiner and Williams, 1958) after subcutaneous doses of 20-25 mg/kg phenformin, and was also seen after intraperitoneal injections of 12-15 mg/kg (Stork and Schmidt, 1970; Haeckel and Haeckel, 1972).

In the rat, however, no significant changes in blood glucose or
hepatic ATP concentrations were produced by phenformin even at a dose of 100 mg/kg subcutaneously (Altschuld and Kruger, 1968). Losert et al. (1972b) have shown that doses of buformin of greater than 50 mg/kg intraperitoneally were necessary to produce a fall in blood glucose levels in the rat.

Studies using guinea-pig liver slices showed that lactate output was increased and glycogen levels were diminished by phenformin (2.1 x 10^{-3} M), but no change in glucose output was produced (Tyberghein and Williams, 1957). Patrick (1966) has reported that rat kidney slices are more sensitive to inhibition of glucose production and ATP depletion by phenformin than rat liver slices; concentrations of 6.7 x 10^{-4} M phenformin in liver slices and 2.0 x 10^{-4} M phenformin in kidney slices were required to produce a 50-60% inhibition of gluconeogenesis from pyruvate. Buformin also diminishes gluconeogenesis from pyruvate in rat liver slices; concentrations of 10^{-4} M caused a 20% reduction, while 10^{-3} M reduced the rate to less than 50% of controls (Losert et al., 1972b).

According to Altschuld and Kruger (1968) phenformin (4.1 x 10^{-4} M) lowered ATP levels in the isolated, perfused guinea-pig liver by approximately 40% and reduced the uptake of glucose and its synthesis from lactate and glycerol, but had little effect in the rat liver even at the higher concentration of 2.1 x 10^{-3} M. This lack of effect has not been confirmed by subsequent workers. Toews et al. (1970) found that gluconeogenesis in the perfused rat liver was inhibited by phenformin with lactate, pyruvate or alanine as substrate, with an inhibition constant of 5.0 x 10^{-4} M. A differential effect of phenformin in the isolated, perfused livers of
normal-fed and fasted, non-diabetic rats and streptozotocin-induced diabetic rats has been reported (Connor, 1973). A dose-effect response in the inhibition of gluconeogenesis from pyruvate was seen in all three groups with complete inhibition occurring at $1.7 \times 10^{-3} \text{M}$ phenformin. Reducing the phenformin concentration resulted in an increase in gluconeogenesis until, at $5.0 \times 10^{-4} \text{M}$ the rate of glucose synthesis in the normal-fed, non-diabetic rat was similar to that in the control group. In the livers from 48h fasted or diabetic rats 80-85% inhibition of gluconeogenesis still occurred with $5.0 \times 10^{-4} \text{M}$. However, although an increase in gluconeogenesis both in diabetes and during fasting has been reported (Weber et al., 1965; Forbath and Hetenyi, 1966; Foster et al., 1966; Owen et al., 1976) it should be noted that the rate of glucose production in the livers from the three groups in the absence of phenformin was very similar.

The effects of both phenformin and metformin on gluconeogenesis from lactate in the isolated, perfused rat liver have been studied by Woods and Alberti (1973). At high drug concentrations ($2.0 \times 10^{-3} \text{M}$) lactate uptake and glucose output were completely inhibited by phenformin and partially inhibited by metformin. Inhibition still occurred at $10^{-5} \text{M}$ although to a lesser extent (phenformin, 45%; metformin, 30%). These results are of interest as they were obtained with concentrations of phenformin which might exist in the blood of man after therapeutic doses in situations where excretion or metabolism are impaired (see SECTION 1.5).

The inhibition of gluconeogenesis has been variously attributed to effects such as lowering of the ATP levels (ATP/ADP ratio) and inhibition of individual enzyme systems. A decrease in hepatic and renal ATP
concentrations (Patrick, 1966; Altschuld and Kruger, 1968) would be expected to lead to a decrease in gluconeogenesis; 6 moles of ATP are required for the production of one mole of glucose from lactate and ATP is required for the phosphorylation of glycerol which is the initial step in the synthesis of glucose from glycerol. In addition, decreased ATP concentrations lead to increased levels of ADP and AMP which may lead to inhibition of pyruvate carboxylase and fructose 1,6-diphosphatase respectively (Pontremoli and Grazi, 1968). The causal relationship between the reduction in the ATP/ADP ratio and the inhibition of hepatic gluconeogenesis in the presence of phenformin has been questioned by Haeckel and Haeckel (1972). In isolated, perfused guinea-pig livers studied 2h after administration of phenformin in vivo (12 mg/kg, intraperitoneally) the ATP/ADP ratio was halved while glucose formation from lactate was unaffected. Subsequent addition of phenformin to the perfusate (2 x 10^-5 M) led to a significant reduction of gluconeogenesis without further affecting the ATP/ADP ratio. As the 3-hydroxybutyrate/acetoacetate ratio was increased, both in the perfusate in vitro and in the blood after in vivo doses of phenformin (12 mg/kg, intraperitoneally), Haeckel and Haeckel (1972) suggested that phenformin had an effect on the mitochondrial redox state causing an increase in the NADH/NAD^+ ratio which raises the concentration of malate with respect to oxaloacetate, thus decreasing the availability of oxaloacetate for gluconeogenesis. Other workers have reported that the production of malate in isolated mitochondria is stimulated and the synthesis of both citrate and phosphoenolpyruvate is decreased when the NADH/NAD^+ ratio is elevated (Garber and Ballard, 1970; Williamson, J., 1976).

After determining the concentrations of the intermediates of the gluconeogenic sequence in the absence and presence of phenformin, Toews et al.
(1970) proposed that the drug exerted an inhibitory action at either the level of glyceraldehyde 3-phosphate dehydrogenase or 3-phosphoglycerate kinase, and that glutamate-alanine transaminase may also be inhibited.

Lloyd et al. (1975) have shown that the decrease in lactate consumption and glucose output by the isolated, perfused guinea-pig liver in the presence of phenformin is associated with a fall in the intracellular pH. A correlation between lactate uptake and intracellular pH was observed which may indicate that the rate of lactate uptake and metabolism is a major factor in the control of hepatic cell pH, and suggests that the low intracellular pH values in phenformin-perfused livers could be due to an inhibition of lactate uptake, either due to inhibition of lactate metabolism or to interference with lactate transport into the cell. Previously Lloyd et al. (1973) showed that above a hepatic intracellular pH of about 7.1 there was no obvious effect on lactate consumption, while at values below 7.1 lactate uptake was rapidly diminished and production of lactate occurred. Low intracellular pH may reduce lactate uptake by its influence on the gluconeogenesis enzyme pyruvate carboxylase (Cohen and Woods, 1976; Iles et al., 1977). The pH optimum of this enzyme lies between 7.4 and 8.0 (Pontremoli and Grazi, 1968). The total intracellular oxaloacetate concentration is reduced in isolated rat livers perfused with lactate under conditions simulating metabolic acidosis (pH 6.7-6.8), leading to a decrease in gluconeogenesis due to substrate limitation of phosphoenolpyruvate carboxykinase (Iles et al., 1977). Whether the decreased availability of oxaloacetate is a result of inhibition of pyruvate carboxylase, or a rise in the malate/oxaloacetate ratio due to an increase in the NADH/NAD⁺ ratio which accompanies the fall in intracellular pH is not clear (Iles et al., 1977). The significance of the effects of pH on gluconeogenesis in the development of lactic acidosis is discussed in SECTION 1.5.
The studies of the effects of phenformin on carbohydrate metabolism and gluconeogenesis in man have been discussed earlier. At therapeutic doses of phenformin gluconeogenesis is not inhibited and may even be stimulated (Kreisberg, 1968b; Searle et al., 1969; Kreisberg et al., 1972; Searle and Siperstein, 1975). This may be a result of a direct effect of phenformin on the gluconeogenic sequence, or may occur as a compensatory mechanism for the enhanced anaerobic utilisation of glucose which provides increased quantities of lactate as a substrate for gluconeogenesis.

This interpretation is not accepted by Muntoni (1974) who has stated that biguanides do not inhibit gluconeogenesis in normal liver but selectively inhibit gluconeogenesis induced by fasting, cortisol, obesity or diabetes. The author has proposed that this occurs by a single mechanism, namely inhibition of fatty acid oxidation. Such an action of biguanides on fatty acid oxidation would reduce the supply of acetyl-CoA necessary for the adequate functioning of the gluconeogenic enzyme pyruvate carboxylase (Utter and Keech, 1963; Utter and Scrutton, 1969). This mechanism, according to Muntoni (1974), functions in diabetes, obesity, hypercortisolism and in other pathological conditions characterised by a high free fatty-acid supply to the liver.

Despite the elaborate reasoning of Muntoni (1974) there is little evidence to suggest that a specific inhibition of gluconeogenesis occurs in the diabetic or obese human. The findings of Searle and Siperstein (1975) that phenformin administration to a diabetic patient increases the proportion of glucose derived from lactate, and of Kreisberg et al. (1970, 1972) that significant rises in the rate and amount of lactate conversion to glucose occur in the phenformin treated obese patient bring this theory into question.
The biguanides may interfere with the intestinal absorption of glucose and other sugars in man and in animal species. All three hypoglycaemic biguanides inhibited in vitro absorption of glucose and galactose in rings of hamster intestine with minimum inhibitory concentrations of $10^{-3}$ M phenformin, $2 \times 10^{-3}$ M buformin and $6 \times 10^{-3}$ M metformin (Caspary and Creutzfeldt, 1971). In the rat, in vivo administration of phenformin (5-150 mg/kg) inhibited the subsequent in vitro transport of glucose in everted intestinal sacs with a log dose-response relationship in the range studied (Kruger et al., 1970). Biro et al. (1961) reported a reduced intestinal absorption of glucose in the phenformin treated rat, while Czyzk et al. (1968) have shown the same effect in the dog after a single dose of buformin (10 mg/kg).

In contrast, some workers have failed to observe an inhibitory effect of biguanides on glucose absorption. Creutzfeldt et al. (1962) found that buformin (50-100 mg/kg) in the glucose-filled rat intestine in situ did not decrease glucose absorption, but when the same dose was given subcutaneously a slight, temporary decrease in absorption was observed. The authors suggested that gastric emptying, rather than intestinal absorption was affected by buformin. Similar findings were reported by Förster et al. (1965).

The effects of phenformin have been studied in man, using a segmental perfusion technique. The absorption of glucose was reduced by almost 50% two hours after ingestion of 100 mg phenformin, and this was associated with a depressed absorption of sodium and water and alterations in the structure of the mitochondria in the villus absorptive cells of the jejunum (Arvanitakis et al., 1973). Similar findings have been found
in rat intestinal preparations after phenformin administration, along with reduced ATP concentrations (Riecken et al., 1971).

These effects on intestinal absorption of glucose may explain the observations that phenformin increases the tolerance to orally administered glucose but has no effect on intravenous glucose tolerance in normal (Hollobaugh et al., 1970) and diabetic humans (Czyzyk et al., 1968; Riecken et al., 1971).

The extent to which this action of the biguanides may contribute to their therapeutic activity in diabetes is uncertain. Although phenformin may decrease the rate of glucose absorption in man, thereby contributing to an alteration in glucose tolerance curves, it does not alter total carbohydrate absorption (Olsen and Rasmussen, 1974). In addition, phenformin antagonises the hyperglycaemic effect of an intravenous infusion of adrenaline in normal anaesthetised rats (Polacek and Ouart, 1974) showing that the hypoglycaemic action of the drug is not totally related to intestinal absorption of glucose.

In view of the essential role of ATP present in the intestinal cell in the absorption of glucose it seems likely that the reduction in glucose transport caused by biguanides is a consequence of an inhibitory effect on oxidative phosphorylation and hence ATP generation in these cells. This may arise from the particularly high concentrations of the biguanides which are found in the intestines of animals (especially after oral doses) (Wick et al., 1960; Cohen and Costerousse, 1961; Hall et al., 1968) due to the basicity of the biguanide moiety.

Thus, there is evidence that biguanides can substantially reduce the rate of intestinal glucose absorption, but do not alter the total
carbohydrate absorption. A significant reduction of total absorption of carbohydrate would result in symptoms of starvation ketosis (Sterne, 1969) and osmotic diarrhoea (Arvanitakis et al., 1973) which do not occur clinically. The effect probably results from the inhibition of ATP generation in intestinal epithelial cells by high concentrations of the drugs which are found in the gut, and is unlikely to contribute to the specific antidiabetic activity of the biguanides.

(d) Effects on lipid metabolism

It is generally agreed that the biguanides do not share the ability of insulin to suppress lipolysis in diabetic patients (Mehnert and Sadow, 1969). This is evident from the inability of the drugs to control diabetes in truly insulin-deficient patients, where in spite of the hypoglycaemic action of the biguanides, ketosis can arise from the continual mobilisation of fatty acids in the absence of insulin. Phenformin (1.7 x 10^{-4} M) reduced the release of both glycerol and free fatty acids in isolated fat cells of rat, and decreased the lipolysis stimulated by glucagon, growth hormone and adrenocorticotropic hormone. This effect did not appear to involve insulin and occurred in the absence of glucose, suggesting that it is independent of the action of phenformin on glucose metabolism (Stone and Brown, 1968). In view of the high concentration of phenformin used to produce this antilipolytic action, and the clinical observations mentioned earlier, it seems doubtful that this effect is produced by therapeutic doses in man.

Lower concentrations of the biguanides have been shown to possess antilipogenic activity. Phenformin inhibited a number of steps in the biosynthesis of cholesterol and other lipids in vitro (McDonald and
Dalidowicz, 1962; Dalidowicz and McDonald, 1965; Dempsey, 1968). In isolated fat cells lipogenesis was inhibited by $5 \times 10^{-6}$M buformin (Ditschuneit et al., 1967) which is similar to the concentrations measured in human serum after single therapeutic doses (Beckmann and Hübner, 1965; Lintz et al., 1974).

After a single dose of phenformin (50 mg/kg, intraperitoneally) blood free fatty acid levels in the non-fasted rat showed a slight, temporary increase followed by a slight decrease to lower than resting levels (Muntoni et al., 1970). In the rat with free fatty acid levels elevated by hypothermia or noradrenaline phenformin caused a further increase in the levels or caused high concentrations to last longer. As phenformin has no known lipolytic action, Muntoni et al. (1970) proposed that the drug caused an inhibition of fatty acid oxidation in addition to its antilipolytic activity.

Phenformin provides an effective means of lowering the elevated serum lipids in maturity-onset diabetics (Schaefer, 1968) and has a beneficial effect in human obesity (Roginsky and Sandler, 1968), which is common in diabetic patients of middle age (Meinert and Schwartz, 1968). Obese diabetic patients and experimentally obese mice treated with phenformin showed a slow persistent loss of weight which could not be attributed to anorexia (Grodsky et al., 1963; Waxler and Leef, 1968). This loss of body weight in diabetic patients who were well controlled by phenformin therapy was associated with a decrease in serum cholesterol and triglycerides (Alterman and Lopez-Gomez, 1968; Mirsky, 1968; Schaefer, 1968). Lowering of circulating cholesterol was greatest in those patients with levels varying most from the normal range (Alterman and Lopez-Gomez, 1968). Total serum lipids were also significantly reduced in diabetic
patients with elevated levels, but not in normolipidaemic diabetics or non-diabetic subjects with high lipid levels (Schaefer, 1968).

The mechanism by which the biguanides exert an influence on lipid metabolism is not known. Muntoni (1974) has suggested that the biguanides may act on lipogenesis through an interaction with carnitine. The rate of long-chain fatty acid oxidation is controlled by their transport through the mitochondrial membrane, which is mediated by the formation of acyl-carnitine derivatives (Fritz, 1967). Carnitine also influences fatty acid synthesis. Carnitine and its long-chain acyl derivatives release the inhibition of acetyl-CoA carboxylase which is inhibited by high levels of acyl-CoA (Fritz, 1967). Thus carnitine has an important regulatory function in both synthesis and oxidation of long-chain fatty acids. Muntoni (1974) suggested that a block in the carnitine system would cause feedback inhibition of acetyl-CoA carboxylase by long-chain acyl-CoA derivatives thus reducing lipogenesis. This in turn would give rise to an increase in the NADPH/NADP⁺ ratio which would inhibit the pentose phosphate pathway, effects which are known to occur with the biguanides (Wick et al., 1958, Ditschuneit and Hoff, 1964; Söling et al., 1967). In addition, by reducing the effectiveness of the carnitine system for transporting fatty acids into the mitochondria the biguanides could inhibit fatty acid oxidation.

This indirect inhibition of acetyl-CoA carboxylase has not been demonstrated experimentally, but does account for both of the metabolic effects of the biguanides on lipid metabolism. Although this mechanism may explain, at least in part, the weight-reducing effect of phenformin in obese diabetic subjects the reason for the lack of influence in normolipidaemic diabetics and hyperlipidaemic non-diabetics reported by Schaefer
(1968) is not clear. The relationship between these effects on lipid metabolism and the antidiabetic therapeutic actions of the biguanides remains to be investigated.

(e) Biguanides and insulin

In whatever way the biguanides may lower blood sugar it is generally agreed that this effect is not primarily mediated by insulin. However, some degree of interaction between the two hypoglycaemic agents is indicated by a number of experimental results.

Biguanides do not increase the quantity of insulin released, or its rate of release from the pancreas (Boshell et al., 1968; Mehnert and Sadow, 1969). The compounds do have a beneficial secondary influence on the patterns of the late and excessive insulinogenic response of the pancreas to hyperglycaemia in many overweight, stable adult diabetics (Grodsky et al., 1963; Mehnert and Sadow, 1969). Phenformin therapy produces an improvement in carbohydrate tolerance and a concomitant decrease in circulating serum insulin levels (Boshell et al., 1968; Butterfield, 1968).

According to some authors (Butterfield, 1968; Sterne, 1969; Muntoni, 1974) the presence of insulin is necessary for at least some effects of biguanides, such as the increased peripheral uptake of glucose. The experiments of Butterfield (1968) on the effects of phenformin on peripheral glucose utilisation and insulin action in human obese and diabetic patients suggested that phenformin causes a clearance of insulin from the blood towards the insulin-responsive muscles enabling glucose to be used more efficiently. A number of in vitro experiments with isolated muscle preparations have demonstrated
this increase in glucose uptake in the presence of phenformin without addition of insulin (Tyberghein and Williams, 1957; Steiner and Williams, 1958). According to Muntoni (1974) this does not necessarily indicate an action independent of the hormone as insulin may be fixed to the muscle.

Similarly, the experiments in surgically pancreatectomised animals are not unequivocal, bearing in mind the extent of surgery and the possible influence of insulin bound to tissues and blood proteins.

However, the work of Butterfield (1968) does not explain the selective action of phenformin on serum unsulin levels. According to Roddam and Boshell (1966) and Boshell et al. (1968) the effect of phenformin on serum insulin only occurs significantly in patients with hyper-insulinaemia.

The relationship between the biguanides and insulin are far from well understood. This complex picture is to be expected in view of the multiple metabolic effects of both insulin and the drugs and although there is evidence for a beneficial interaction in the overweight diabetic, the significance in other groups of diabetics are not clear.
1.5 BIGUANIDES AND LACTIC ACIDOSIS

There is no doubt, from clinical and experimental evidence, that lactate metabolism is influenced by the biguanides. In ordinary therapeutic use the drugs are non-toxic substances (Mehnert and Sadow, 1969) but in special circumstances when lactate metabolism is disturbed, lactic acidosis can arise. The association between biguanide therapy (particularly with phenformin) and lactic acidosis has received a great deal of attention since the first report by Tranquada et al. (1963).

There has been considerable disagreement over the definition and classification of lactic acidosis. Many authors have defined the condition in terms of blood lactate exceeding certain stated limits such as 1.3 mM (Peretz et al., 1964), 2 mM (Oliva, 1970) and 7 mM (Tranquada, 1964). These definitions are necessarily arbitrary, and a more reliable course in the diagnosis of the condition may be to employ a definition which is based on the clinical state of the patient such as 'a state of metabolic acidosis associated with a high blood concentration of lactate' (Cohen and Woods, 1976) or 'an ill patient with clinical signs of an acidosis together with a blood lactate concentration persistently above 5 mM and an arterial pH of 7.25 or less' (Alberti and Nattrass, 1977).

In this type of lactic acidosis there tends to be no clear evidence of tissue anoxia, and diagnosis is often difficult (Alberti and Nattrass, 1977). Clinical features may include hyperventilation, vomiting, diarrhoea, abdominal pain and various degrees of reduced consciousness from drowsiness to coma, and may be accompanied by hyperketonaemia and arterial pH values as low as 6.62 (Ball et al., 1974; Dembo et al., 1975; Heucelin et al., 1975; Cohen and Woods, 1976; Fulop and Hoberman, 1976; Thimme et al., 1976;
Williamson, M., 1976). Similar features have been recorded in cases of metformin- and buformin- associated lactic acidosis (Clavadetscher et al., 1976; Thimme et al., 1976; Assan et al., 1977).

The available evidence clearly indicates that lactic acidosis does not usually occur in 'fit' patients (Cohen and Woods, 1976), but that diabetics on biguanide therapy may develop the condition when seriously ill from other causes (Oliva, 1970; Fulop and Hoberman, 1976; Alberti and Nattrass, 1977). The nature of these complicating illnesses and their involvement in the development of lactic acidosis in biguanide therapy will be discussed in a later section.

Incidence of biguanide-associated lactic acidosis

From the frequency of case reports in medical journals it appears that lactic acidosis occurs with phenformin therapy far more frequently than with metformin or buformin. Phenformin (Dibotin) was introduced into the U.K. in 1959 and since then it has been estimated that about 340,000 patient-years of treatment have been given. From the time of its introduction until February 1977, there have been 24 published cases in the U.K. of lactic acidosis associated with Dibotin (Sterling Winthrop Research Laboratories, personal communication, 1977). Comparison with the other biguanides is difficult as metformin and buformin are not widely used in the U.K. Metformin is prescribed predominantly in France, and buformin in Germany, and accurate assessment of the occurrence of the illness may be hampered by the different philosophies regarding reporting of adverse cases.

Although a number of cases of metformin associated (Lebacq and Tirzmalis, 1972; Hayat, 1973; Robert et al., 1973; Bismuth et al., 1976;
Assan et al., 1977) and buformin associated (Clavudetscher, 1976; Thimme et al., 1976) lactic acidosis have been reported, there can be no doubt that such cases are less frequent. The collection of data from cases of biguanide-induced lactic acidosis reported worldwide, shows that those associated with phenformin now number some 281, as compared with metformin-and buformin-associated cases which number 16 and 10 respectively (Sterling Winthrop Research Laboratories, personal communication, May 1977).

At present there is no widely accepted explanation for the higher incidence of lactic acidosis in phenformin treated diabetics. The possible explanation that the differences may lie in the relative usage of the three drugs is unlikely to provide the whole answer. In France, where 3 times as many patients receive metformin as phenformin, there is a higher incidence of lactic acidosis reported with phenformin (Isnard and Lavieuville, 1977). While less is known of buformin, the study by Thimme et al. (1976) in Berlin showed that lactic acidosis is more frequent with phenformin, although buformin is the more frequently prescribed drug.

As there is little evidence to suggest that the biguanides do not share a common mechanism of action, it is possible that the differences which exist regarding the frequency of adverse reactions, may be related to differences in pharmacokinetics (Bismuth et al., 1976; Assan et al., 1977). The existence of various contributory clinical conditions in cases of biguanide-induced lactic acidosis has received much attention in recent years (Assan et al., 1975; Heuclin et al., 1975; Romankiewicz, 1975; Cohen and Woods, 1976; Mehnert, 1976; Thimme et al., 1976; Wise et al., 1976; Alberti and Nattrass, 1977; Assan et al., 1977). The effects
of these complaints such as renal failure, hepatic and myocardial diseases, circulatory disorders, acute infections and alcoholosis on the normal pharmacokinetics of the biguanides, particularly in relation to the aetiology of lactic acidosis, will be discussed further in a later section.

**Control of lactate metabolism**

Lactic acid is formed as an end-product in glycolysis under anaerobic conditions. The reaction (1) occurs in the cytosol and is catalysed by the enzyme lactate dehydrogenase requiring NADH. The equilibrium normally lies in favour of lactic acid with a 7-15 to 1 ratio on a molar basis. This is reflected by blood concentrations in resting man of 0.44 - 1.8 mM lactate and 0.07 - 1.2 mM pyruvate (Harper, 1965). Lactate utilisation in anaerobic conditions depends upon reversal of the reaction either in the tissues where lactate is formed (predominantly muscle) or in other tissues (liver and kidneys).

\[
\text{pyruvate + NADH + H}^+ \xrightarrow{\text{dehydrogenase}} \text{lactate + NAD}^+ \quad (1)
\]

Rearrangement of (1) shows that the reaction can be pushed in the direction of lactate by an increase in the NADH concentration or a decrease in pH (Huckabee, 1961; Cohen and Woods, 1976). This is shown in equation (2).

\[
\frac{[\text{lactate}]}{[\text{pyruvate}]} = \alpha \frac{[\text{NADH}] [\text{H}^+]}{[\text{NAD}^+] [\text{H}^+]} \quad (2)
\]
The NADH/NAD\textsuperscript{+} ratio is linked to the state of phosphorylation of the cell through the substrate-level phosphorylation reactions involved in the interconversion of glyceraldehyde 3-phosphate and 3-phosphoglycerate.

\[
glyceraldehyde\ 3\text{-phosphate} + \text{NAD}^+ + \text{Pi} \underset{\text{(3)}}{\overset{\text{1,3-diphosphoglycerate} + \text{NADH} + \text{H}^+}{\longrightarrow}}
\]

\[
\text{1,3-diphosphoglycerate} + \text{ADP} \underset{\text{(4)}}{\overset{\text{3-phosphoglycerate} + \text{ATP}}{\longrightarrow}} 
\]

The implications of this relationship have been discussed in detail by Stubbs et al. (1972) and by Cohen and Woods (1976). Briefly, impaired phosphorylation of ADP and an increased NADH/NAD\textsuperscript{+} ratio can result from a block in the electron-transport chain, both producing an elevation of the lactate/pyruvate ratio.

However, the value of the lactate/pyruvate ratio in characterising lactic acidosis is questionable (Olsen, 1963; Alpert, 1965; Cohen and Woods, 1976) particularly as the ratio is determined in the blood rather than the various tissues. Since there are separate pools of NADH and NAD\textsuperscript{+} in the cytoplasm and mitochondria of the cell (Boxer and Devlin, 1961), the blood lactate/pyruvate ratio, which depends upon the cytoplasmic NADH-NAD\textsuperscript{+} system, may not reflect the NADH/NAD\textsuperscript{+} ratio within mitochondria which is the ultimate determinant of oxidative metabolism (Alpert, 1965). In addition, it is unlikely that all tissues will have similar lactate/pyruvate ratios. In blood itself, due to a difference in erythrocyte permeability to lactate and pyruvate, the intracellular lactate/pyruvate ratio is greater than the extracellular ratio (Huckabee, 1956) so that the presence of red cells may distort the lactate/pyruvate ratio relative to the tissues.
Lactate elimination may proceed either by direct oxidation of pyruvate by the citric acid cycle, or by conversion to glucose (gluconeogenesis) in the liver and kidneys.

The rate at which lactate is eliminated in the citric acid cycle depends upon the rate of metabolism of pyruvate to acetyl-CoA by the enzyme pyruvate dehydrogenase, which exists in an active (dephosphorylated) and an inactive (phosphorylated) form. The interconversion of these forms is catalysed by an ATP-requiring kinase and a phosphatase (Linn et al., 1969; Wieland et al., 1971). The reaction is inhibited by the products, acetyl-CoA and NADH and therefore the enzyme is maintained in an inactive state when the concentration of ATP and acetyl-CoA are kept high by oxidation of fuels such as fatty acids. Thus pyruvate dehydrogenase has an important regulatory function in lactate metabolism because its activity decides whether lactate is converted to acetyl-CoA or whether it is used to resynthesise glucose in the liver and kidneys.

For gluconeogenesis to proceed several special reactions are required to overcome the unfavourable energy transitions which prevent the total reversal of glycolysis. The irreversible stages of glycolysis are those controlled by pyruvate kinase, phosphofructokinase and hexokinase or glucokinase.

The interconversion of pyruvate and phosphoenolpyruvate through the combined actions of pyruvate carboxylase and phosphoenolpyruvate carboxykinase is presently believed to be the principal site of control of hepatic gluconeogenesis, as this stage controls the generation of hexose derivatives from lactate and amino acids. The stages involving fructose 1,6-diphosphatase and glucose 6-phosphatase control the conversion of hexose
derivatives to glucose and the regulation of blood glucose levels (Nordlie, 1976).

The control systems for pyruvate carboxylase and phosphoenolpyruvate carboxykinase in mammalian species are complicated and will only briefly be discussed. More detailed descriptions have recently been published (Cohen and Woods, 1976; Hanson and Mehlman, 1976).

While pyruvate carboxylase is generally believed to be located in mitochondria (Barritt et al., 1976) phosphoenolpyruvate carboxykinase exhibits a species dependent intracellular distribution (Nordlie and Lardy, 1963). In rat liver phosphoenolpyruvate carboxykinase is mainly cytosolic, while in guinea-pig, rabbit and human liver the enzyme is distributed to differing degrees between the cytosol and mitochondria. According to Lardy et al. (1965) the synthesis of phosphoenolpyruvate by the cytosolic enzyme requires the transport of four-carbon units from the mitochondria, as the mitochondrial membrane is relatively impermeable to oxaloacetate. Thus mitochondrial oxaloacetate is either converted to aspartate by glutamate-oxaloacetate transaminase, or reduced to malate by malate dehydrogenase and NADH; both of these four-carbon units are relatively easily transported into the cytosol and reconverted to phosphoenolpyruvate. The balance between malate and aspartate transport from the mitochondria in rat liver depends upon whether sufficient NADH is generated in the cytosol to meet the needs of glyceraldehyde 3-phosphate dehydrogenase (Williams, J., 1976). Thus with lactate as substrate, NADH is formed in the cytosol and transport of aspartate provides the four-carbon moiety for glucose synthesis (see FIG. 1.4a) whereas when pyruvate is the substrate malate transport provides both the carbon units and a mitochondrial reducing equivalent for gluconeogenesis (see FIG. 1.4b).
FIG. 1.4 Metabolic schemes for the transport of four-carbon units from mitochondria to the cytosol during gluconeogenesis in rat liver from (a) lactate and (b) pyruvate according to Williamson, J. (1976). For explanation of schemes see text.

Enzymes:— (1) pyruvate carboxylase; (2) phosphoenolpyruvate carboxykinase; (3) glutamate-oxaloacetate transaminase; (4) malate dehydrogenase; (5) glyceraldehyde 3-phosphate dehydrogenase; (6) lactate dehydrogenase.
Thus aminooxyacetate, a transaminase inhibitor, causes almost complete inhibition of gluconeogenesis from lactate in rat liver, but very little inhibition from pyruvate (Anderson et al., 1971). In guinea-pig liver aminooxyacetate causes a 50-60% inhibition of gluconeogenesis from lactate and a significant inhibition from pyruvate, indicating the involvement of the mitochondrial as well as the cytosolic phosphoenolpyruvate carboxykinase in gluconeogenesis in this species (Arinze et al., 1973). In man the carboxykinase enzyme is located in both the cytosol and the mitochondria which suggests that, as in the guinea-pig, lactate metabolism could be significantly impaired by inhibition of transamination.

Phosphoenolpyruvate carboxykinase in the cytosol is responsive to dietary and hormonal stimuli (Shrago et al., 1963; Nordlie et al., 1965; Hanson and Garber, 1972; Söling and Kleineke, 1976). A recent report (Elliott and Pogson, 1977) suggests that the mitochondrial enzyme in the guinea-pig is also increased during starvation. The enzyme activity is affected by a number of hormones and the response varies with different tissues; thus insulin has no effect on the renal enzyme, but decreases the activity in liver and adipose tissue (Shrago et al., 1963). When rats are made insulin-deficient by alloxan administration the activity of phosphoenolpyruvate carboxykinase rises sharply and insulin injection returns the activity to normal (Shrago et al., 1963; Foster et al., 1966). There is also evidence for a role of insulin as a mediator of the fasting response, perhaps by lowering intracellular cyclic AMP levels (Tilghman et al., 1976) but whether this occurs by a direct action on the liver or by blocking the release of another hormone such as glucagon is still not clear.
Glucagon and the catecholamines produce increases in phosphoenolpyruvate carboxykinase activity in the liver, effects which are mediated by cyclic AMP (Shrago et al., 1963; Reshef and Hanson, 1972) and involve de novo synthesis of the enzyme (Wicks et al., 1972). The renal enzyme activity increases during metabolic acidosis within a few hours (Flores and Alleyne, 1971), an effect which probably involves hormonal regulation (Iynedjian et al., 1975).

The activation of pyruvate carboxylase by acetyl-CoA is strongly pH dependent in vitro (Kleineke and Söling, 1971) and could have the greatest significance in the metabolic changes leading to lactic acidosis (Cohen and Woods, 1976). The concentration of acetyl-CoA required to activate the enzyme is much greater at acid pH (Scrutton and Utter, 1967; Kleineke and Söling, 1971; Scrutton and White, 1974), and in chicken liver the enzyme is virtually inactive below pH 7.0 (Scrutton and Utter, 1968). The intracellular pH in liver may, therefore, be an important regulating factor in the rate of gluconeogenesis from lactate, pyruvate and some amino acids.

Lloyd et al. (1973) have shown that lactate uptake in the perfused rat liver decreases when the intracellular pH falls below 7.1. The decrease in lactate consumption and glucose output observed in the presence of phenformin (5 x 10^-5 M - 5 x 10^-4 M) is associated with a fall in intracellular pH (Lloyd et al., 1975) and the rate of lactate uptake appeared to be a controlling factor in the maintenance of hepatic cell pH.

The liver cell pH during lactic acidosis has not been determined. Cohen and Woods (1976) have suggested that decreases of 0.2 - 0.3 units
may accompany such changes in extracellular pH as could be expected to occur in this condition, but point out that these values are derived from experiments in the normal cell and that larger decreases may occur in some conditions. Such a pH change would be equivalent to a doubling of the lactate/pyruvate ratio (see equation 2) but in cases of phenformin-associated lactic acidosis blood lactate/pyruvate ratios tend to be greater than this (see FIG. 1.6) and the arterial pH is usually lower than in non-phenformin-associated cases (Cohen and Woods, 1976). Thus it remains a possibility that hepatic cell pH is considerably decreased in phenformin-associated lactic acidosis.

When the intracellular pH in rat liver falls below 7.0 - 7.07 lactate uptake ceases and production of lactate may occur (Lloyd et al., 1973,1975). Cohen et al. (1967, 1971) suggested that the metabolism of lactate may be accompanied by intracellular production of hydroxyl ions which results in the consumption of intracellular protons. Thus interference with lactate consumption may lower cell pH, and by bringing the value below 7.1 further inhibit uptake. Cohen and Woods (1976) have suggested that a 'vicious circle' could arise where an inhibition of gluconeogenesis from lactate (e.g. by high levels of phenformin) causes a fall in the intracellular pH which in turn (perhaps by inhibiting the activation of pyruvate carboxylase by acetyl-CoA) further inhibits lactate uptake and metabolism. Such a mechanism could explain the rapid development of lactic acidosis once the process has started. The possible involvement of such a sequence in phenformin-treated diabetics suffering from lactic acidosis is discussed later.
Biguanides and lactate metabolism

(a) Blood lactate concentration

Biguanide administration can increase the blood lactate concentration in normal and diabetic animals and man, but as with the hypoglycaemic effect of the drugs (see SECTION 1.4) there is a considerable variation in response between different species and between diabetic and non-diabetic subjects. The results of studies on the effects of the biguanides on the blood lactate concentrations in animals and man are shown in TABLE 1.3.

These investigations have shown that hyperlactataemia is produced in the guinea-pig with lower doses of phenformin than in the rat, and that the diabetic rat is more susceptible to this effect than the normal rat. Fasted animals show a greater increase in blood lactate concentration after biguanide therapy than do non-fasted animals. The hyperlactataemic response appears to be dose dependent (Bramanti et al., 1971a; Losert et al., 1972b).

Experiments in guinea-pig (Tyberghein and Williams, 1957) and rat (Heuclin et al., 1975) have demonstrated that phenformin causes a much greater increase in blood lactate levels in nephrectomised animals than in normal animals. This finding is significant when considering the aetiology of phenformin-associated lactic acidosis, and will be discussed in a later section.

In non-diabetic human subjects only very small increases in blood lactate concentrations are seen with normal therapeutic doses of phenformin, even after fasting (Fajans et al., 1960; Lyngsoe et al., 1972). The changes in blood lactate levels in man after phenformin administration
<table>
<thead>
<tr>
<th>Biguanide</th>
<th>Dose/Route</th>
<th>Species</th>
<th>Condition of Subject</th>
<th>Time After (Last) Dose</th>
<th>Blood Lactate* (mg/100ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenformin</td>
<td>20 mg/kg, s/c</td>
<td>Guinea-pig</td>
<td>Normal-fed</td>
<td>1h</td>
<td>13.5 (10)</td>
<td>20.7 (10) Tüberghief-Williams</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal-fed</td>
<td>4h</td>
<td>15.8 (10)</td>
<td>27.3 (10) Tüberghief-Williams</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nephrectomised</td>
<td>4h</td>
<td>14.1 (6)</td>
<td>75.6 (6) Tüberghief-Williams</td>
</tr>
<tr>
<td>Phenformin</td>
<td>20-25 mg/kg, s/c</td>
<td>Guinea-pig</td>
<td>16-24h fasted</td>
<td>2-3h</td>
<td>12.0 (5)</td>
<td>71.0 (5) Steiner &amp; Williams</td>
</tr>
<tr>
<td>Phenformin</td>
<td>12.5 mg/kg, s/c</td>
<td>Guinea-pig</td>
<td>22h fasted</td>
<td>3h</td>
<td>18.6 (10)</td>
<td>27.4 (11) Ramachandran et al.</td>
</tr>
<tr>
<td>Phenformin</td>
<td></td>
<td>Guinea-pig</td>
<td>Normal-fed</td>
<td>4h</td>
<td>28.8 (800)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mg/kg, p/o</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>22.5 (6) Bramanti et al. (1971a)</td>
</tr>
<tr>
<td></td>
<td>25 mg/kg, p/o</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>33.0 (6) Bramanti et al. (1971a)</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg, p/o</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>48.3 (6) Bramanti et al. (1971a)</td>
</tr>
<tr>
<td></td>
<td>50 mg/kg, p/o</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>106.0 (6) Bramanti et al. (1971a)</td>
</tr>
<tr>
<td></td>
<td>75 mg/kg, p/o</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>110.0 (6) Bramanti et al. (1971a)</td>
</tr>
<tr>
<td>Phenformin</td>
<td>30 mg/kg, intraduodenally</td>
<td>Dog</td>
<td>18h fasted</td>
<td>4h</td>
<td>18.5 (8)</td>
<td>49.5 (8) Valette et al. (1975)</td>
</tr>
<tr>
<td>Phenformin</td>
<td>200 mg/kg, p/o</td>
<td>Rabbit</td>
<td>Normal-fed</td>
<td>3h</td>
<td>19 (7)</td>
<td>90 (5) Ramachandran et al. (1971)</td>
</tr>
<tr>
<td>Phenformin</td>
<td>40 mg/kg, s/c</td>
<td>Rat</td>
<td></td>
<td>3h</td>
<td>6.8 (11)</td>
<td>12.2 (11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Daily for: 4 days</td>
<td>22h fasted</td>
<td>7.5 (12)</td>
<td>16.0 (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 days</td>
<td></td>
<td>9.6 (17)</td>
<td>18.4 (20)</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg, s/c</td>
<td>Rat</td>
<td>22h fasted</td>
<td>3h</td>
<td>-</td>
<td>30.2 (4)</td>
</tr>
<tr>
<td></td>
<td>7 doses in 4 days</td>
<td></td>
<td>Alloxan diabetic</td>
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</table>

* Number of subjects in parentheses
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<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Route</th>
<th>Animal</th>
<th>Duration</th>
<th>Time Taken</th>
<th>Peak Value</th>
<th>Ranges or Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenformin</td>
<td>40 mg/kg, s/c daily for 4 days</td>
<td>Rat</td>
<td>Normal-fed</td>
<td>3h</td>
<td>13.6 (12)</td>
<td>23.4 (12)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Phenformin</td>
<td>75 mg/kg, i/p rat</td>
<td>Normal-fed</td>
<td>Alloxan diabetic</td>
<td>2.5h</td>
<td>12.2 (15)</td>
<td>24.5 (15)</td>
<td>Ruggles (1968)</td>
</tr>
<tr>
<td>Phenformin</td>
<td>16 mg/day, i/p rat</td>
<td>Normal-fed</td>
<td>Nephrectomised</td>
<td>0.25h</td>
<td>9.6 (8)</td>
<td>13.5 (12)</td>
<td>Heuclin (1975)</td>
</tr>
<tr>
<td>Buformin</td>
<td>50 mg/kg, i/p rat</td>
<td>16h fasted</td>
<td>0.75h</td>
<td>11.0 (9)</td>
<td>-</td>
<td>22.5 (9)</td>
<td>Losert (1972b)</td>
</tr>
<tr>
<td>Phenformin</td>
<td>200 mg/day man</td>
<td>Fasted</td>
<td>Normal</td>
<td>2-3h</td>
<td>6.2 (7)</td>
<td>6.6 (7)</td>
<td>Fajans (1960)</td>
</tr>
<tr>
<td>Phenformin</td>
<td>2-18 days at:</td>
<td>Fasted</td>
<td>Diabetic</td>
<td>Overnight</td>
<td>7.6 (6)</td>
<td>-</td>
<td>Craig et al. (1977)</td>
</tr>
<tr>
<td></td>
<td>100 mg/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125 mg/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>150 mg/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenformin</td>
<td>175 mg/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenformin</td>
<td>50 mg, twice daily man</td>
<td>Fasted</td>
<td>Diabetic</td>
<td>Mean value of 12 hourly measurements on last day of treatment</td>
<td>10.5 (6)</td>
<td>13.8 (6)</td>
<td>Nattrass (1977)</td>
</tr>
<tr>
<td>Metformin</td>
<td>500 mg, three times daily man</td>
<td>Fasted</td>
<td>Diabetic</td>
<td></td>
<td>8.3 (6)</td>
<td>10.8 (6)</td>
<td>Nattrass (1977)</td>
</tr>
<tr>
<td>Phenformin</td>
<td>100 mg/day for 3 days man</td>
<td>Normal-fed</td>
<td></td>
<td>Not specified</td>
<td>6.8 (6)</td>
<td>8.1 (7)</td>
<td>Lyngsoe (1972)</td>
</tr>
<tr>
<td>Phenformin</td>
<td>150 mg man</td>
<td>Fasted</td>
<td></td>
<td>2-3h</td>
<td>5.7 (20)</td>
<td>8.5 (20)</td>
<td>Kreisberg et al. (1977)</td>
</tr>
</tbody>
</table>
are greatly increased by ethanol ingestion (Johnson and Waterhouse, 1968), a fact which will be discussed further in a later section.

In lactic acidosis associated with phenformin therapy blood lactate concentrations reach much higher levels, often 15 - 20 times higher than those of untreated diabetics. The distribution of blood lactate concentrations in two hundred published cases of phenformin-associated lactic acidosis is shown in FIG. 1.5. Over 70% of the patients had blood lactate levels between 50 and 200 mg/100 ml (5.5 - 22.2 mM), while Ball et al. (1974) have shown that the normal value (taken from 308 diabetic patients) is 9.0 ± 3.6 mg/100 ml (1.0 ± 0.4 mM). In a study of eighty-two recorded cases of lactic acidosis, Cohen and Woods (1976) reported that blood lactate concentrations in patients treated with phenformin were slightly but significantly (P < 0.02) higher than those in cases not associated with biguanide therapy.

Similar blood lactate concentrations occur in metformin- and buformin-associated lactic acidosis. In the twelve of thirteen published cases of metformin-associated lactic acidosis in which blood lactate levels were determined, concentrations of 60-180 mg/100 ml (6.5 - 20.0 mM) were recorded, while in the ten published cases of buformin-associated lactic acidosis blood lactate levels ranged from 60 - 270 mg/100 ml (6.5-30 mM).

(b) Blood lactate/pyruvate ratio

The fact that the blood lactate/pyruvate ratio may not be an accurate indicator of the state of tissue oxidation has been discussed earlier. Nevertheless, the ratio is considered by some to be a useful clinical parameter related to tissue perfusion (Oliva, 1970; Harken, 1976).
FIG. 1.5 Distribution of blood lactate concentrations in two hundred published cases of lactic acidosis associated with phenformin therapy.

FIG. 1.6 Distribution of blood lactate/pyruvate ratio in forty-eight published cases of lactic acidosis associated with phenformin therapy.
The distribution of values of blood lactate/pyruvate ratios in forty-eight published cases of lactic acidosis associated with phenformin therapy is shown in FIG. 1.6. Some 80% of the patients had ratios higher than the normal range (7-15), and two-thirds were greater than 30. This is in agreement with the report of Cohen and Woods (1976) who found that the blood lactate/pyruvate ratios in patients treated with phenformin were significantly (P < 0.001) higher than in cases not associated with biguanide therapy; most of the values in the non-phenformin group were below 30.

The presence of contributory illnesses

Biguanide-associated lactic acidosis does not usually occur in diabetics who are otherwise healthy, and is almost invariably a complication of serious illnesses. Of the 281 published cases of lactic acidosis in patients receiving phenformin, less than 40 had no concomitant condition at the time of treatment (data extracted from literature survey provided by Sterling Winthrop Research Laboratories, 1977). Of the remaining patients most suffered from renal, cardiovascular, hepatic or infectious diseases. Similar conditions have been identified in cases of metformin-associated (Lebacq and Tirzmalis, 1972; Hayat, 1974; Heucin et al., 1975; Bismuth et al., 1976; Assan et al., 1977) and buformin-associated lactic acidosis (Clavadetscher et al., 1976; Thimme et al., 1976).

Renal failure appears to be the most frequent aetiological factor in precipitating biguanide-associated lactic acidosis. Metformin and buformin are normally excreted rapidly by the kidneys, and are not metabolised in man (Cohen and Costerousse, 1961; Pignard, 1962; Beckmann, 1968). Phenformin is partly metabolised to 4-hydroxyphenformin which may
be less pharmacologically active (although there is no published information on the effects of this compound in man). Thus both renal and hepatic insufficiency promote accumulation of phenformin. Increased circulating phenformin concentrations of 3-15 times normal have been measured in diabetic patients with lactic acidosis due to the presence of renal and liver diseases (Assan et al., 1975).

In subtotally nephrectomised rats administration of phenformin (16 mg/day) for 5-7 days produced a 7-10-fold increase in blood lactate concentration compared to untreated nephrectomised rats and phenformin-treated control rats, and reduced the rate of glucose production from lactate by some 60% (Heuclin et al., 1975). This blockage of gluconeogenesis is reversible; it disappears from perfused rat liver when the biguanide is removed from the circulating fluid (Cook et al., 1973b). In rats with chronic renal insufficiency phenformin excretion is reduced to 10% of the normal rate and the drug is still present in the plasma 24h after administration, whereas it is no longer detectable in the plasma of control animals after 1h (Heuclin et al., 1975).

It therefore appears that, while at therapeutic levels of biguanides lactate removal is unaffected or even stimulated (see SECTION 1.4), at higher concentrations, such as those achieved by suicidal self-poisoning (1 x 10⁻⁵ M, Karam et al., 1974) or in cases of renal insufficiency removal of lactate is impaired. Thus over-production and under-utilisation of lactic acid may produce the self-perpetuating series of events described earlier (SECTION 1.4) and lead to lactic acidosis.

Apart from an obvious association with impaired renal function, patients with cardiovascular complaints are predisposed to lactic acidosis because of
the poor tissue perfusion which favours the over-production of lactate (Assan et al., 1976).

Lactic acidosis can occur despite normal circulatory conditions in patients with dangerous infections, and such patients are particularly at risk in biguanide therapy (Thimme et al., 1976).

Ethanol ingestion causes a sharp rise in the hepatic NADH/NAD$^+$ ratio which in turn inhibits gluconeogenesis from lactate. In normal man there is a small increase in lactate, but in the presence of phenformin ethanol can lead to lactic acidosis (Johnson and Waterhouse, 1968; Cohen and Woods, 1976; Alberti and Nattrass, 1977).

These contraindications have led to the publication of lists of restrictions for the use of biguanides (Mehnert, 1976). Unfortunately, diabetic patients treated with the drugs tend to be in the older age-groups and may acquire some of these complaints during therapy, and constant monitoring is essential (Cohen et al., 1973; Assan et al., 1975, 1976, 1977; Cohen and Woods, 1976; Thimme et al., 1976; Alberti and Nattrass, 1977).

Dichloroacetate and lactic acidosis

The clinical treatment of lactic acidosis by methods such as dialysis, insulin therapy and alkalinisation is not always successful and the mortality rate in phenformin-associated lactic acidosis is 45-50% (compared with 80% mortality in other forms of lactic acidosis, Alberti and Nattrass, 1977). For this reason there is considerable interest in developing other forms of treatment such as dichloroacetic acid.
(a) Metabolic effects of dichloroacetate

Acetate and its halogenated derivatives are known to affect several aspects of metabolism. Acetate itself exerts an antilipolytic effect on adipose tissue but has no significant effect on blood sugar levels (Abramson and Arky, 1968) or may lead to hyperglycaemia by increasing citrate levels and inhibiting phosphofructokinase (Williamson, J., 1967). Hoffman and Paulshock have reported a number of acetate derivatives to be effective in lowering blood glucose in diabetic animals, including dichloroacetic acid (Hoffman and Paulshock, 1961).

Lorini and Ciman (1961) first showed that diisopropylammonium dichloroacetate, a mild vasodilator, reduced the hyperglycaemia of alloxan-induced diabetic rats without affecting normal rats. The active component of this compound is the acid moiety, dichloroacetic acid (Stacpoole and Felts, 1970; Eichner et al., 1974).

A reduction in blood glucose levels is seen in the diabetic rat after enteral (Eichner et al., 1974) and parenteral (Lorini and Ciman, 1961; Stacpoole and Felts, 1970, 1971; Blackshear et al., 1975) administration of dichloroacetate as the sodium or diisopropylammonium salt. Hypoglycaemia is produced by the compound in 24h-fasted rats (Blackshear et al., 1974) but not normal fed animals (Lorini and Ciman, 1961; Stacpoole and Felts, 1970; Eichner et al., 1974).

Experiments with rat diaphragm and heart muscle preparations show that dichloroacetate stimulates glucose oxidation which has been inhibited by alloxan-induced diabetes or by 3-hydroxybutyrate, acetate and palmitate (Stacpoole and Felts, 1970, 1971; McAllister et al., 1973) and inhibits the
oxidation of acetate, 3-hydroxybutyrate, palmitate and oleate (Stacpoole and Felts, 1970; McAllister et al., 1973; Whitehouse and Randle, 1973). Stacpoole and Felts (1970, 1971) have demonstrated that dichloroacetate returns to normal the elevated citrate and reduced glycerol 3-phosphate levels in heart and diaphragm muscle of diabetic rats, but has no effect in tissues from normal rats. In hearts from normal rats perfused with glucose and insulin dichloroacetate increases the concentrations of acetyl-CoA and acetyl carnitine and the conversion of pyruvate to acetyl-CoA (McAllister et al., 1973). It has therefore been suggested that dichloroacetate may directly activate pyruvate dehydrogenase which exists in an active (dephosphorylated) and an inactive (phosphorylated) form. Interconversion is catalysed by a specific ATP-requiring kinase and a phosphatase (Linn et al., 1969; Wieland et al., 1971). Dichloroacetate increases the activity of pyruvate dehydrogenase in rat hearts perfused with glucose or glucose and acetate, and inhibits the incorporation of $^{32}$P from $[\gamma-^{32}$P]ATP into the enzyme probably by inhibition of pyruvate dehydrogenase kinase (Whitehouse and Randle, 1973; Whitehouse et al., 1974).

Blackshear et al. (1974, 1975) have shown that the effects of dichloroacetate on glucose metabolism are similar in severely diabetic and fasted normal rat. Blood glucose, lactate and pyruvate levels are decreased, as are the hepatic concentrations of most gluconeogenic intermediates. Dichloroacetate also causes a decrease in the accumulation of lactate, pyruvate and alanine in the blood of functionally-hepatectomised diabetic animals and abolishes the rise in blood lactate levels caused by insulin administration. These effects could arise from an activation of extra-hepatic pyruvate dehydrogenase (Whitehouse et al., 1974) and suggest that dichloroacetate causes hypoglycaemia by decreasing the net release of gluconeogenic precursors from peripheral tissues (Blackshear et al., 1974, 1975; Whitehouse et al., 1974).
In the non-diabetic fasted rat dichloroacetate causes an increase in the blood concentration of acetoacetate and 3-hydroxybutyrate which is associated with a marked reduction of ketone-body uptake (Blackshear et al., 1974). However, in the diabetic rat dichloroacetate infusion decreases the blood concentration and peripheral uptake of the ketones, but no antilipolytic effect was demonstrated (blood glycerol levels remained constant). The impaired ketone-body metabolism may arise from competition for coenzyme A between activated pyruvate dehydrogenase and the enzymes of ketone-body oxidation (McAllister et al., 1973) or from the elevated tissue concentration of acetyl-CoA which would inhibit the formation of acetyl-CoA from ketones and thus ketone-body uptake (Blackshear et al., 1975). The decrease in blood levels of ketones in spite of the impaired peripheral uptake in the diabetic rat probably results from a fall in hepatic production (Blackshear et al., 1975).

In the non-ketotic, adult-onset diabetic sodium dichloroacetate given orally (0.8-4.0 g/day for 6 - 7 days) reduced plasma glucose levels by an average of some 10%, plasma triglycerides by 50% and plasma cholesterol concentrations by approximately 15% (Stacpoole et al., 1976). Lactate, alanine and free fatty acid concentrations were also decreased in all 5 patients studied, but insulin levels were unchanged. These findings are similar to those observed in the rat (Blackshear et al., 1974, 1975) and suggest that peripheral release of gluconeogenic precursors and fatty acids is inhibited by dichloroacetate, in the absence of an effect on insulin.

(b) Dichloroacetate and phenformin-associated lactic acidosis

In view of the ability of dichloroacetate to decrease blood lactate and glucose concentrations, the effects of the compound on phenformin-associated hyperlactataemia and hypoglycaemia have been studied. Man and
Alberti (1976) have shown that dichloroacetate given to rats simultaneously with phenformin (75 mg/kg, i.p.) every 12h over a period of 28 days can prevent the development of lactic acidosis. The reduced blood glucose levels seen after combined treatment with phenformin and dichloroacetate are not significantly different from those seen after administration of either compound alone. Phenformin administration considerably elevates blood lactate concentrations, while the levels in rats given phenformin and dichloroacetate together are as low as, or lower than those in saline-treated control animals. The increased hepatic concentrations of gluconeogenic precursors produced by phenformin are reversed by combined administration with dichloroacetate, but ketone-body levels are elevated.

As well as preventing the development of lactic acidosis, bolus injections of dichloroacetate (i.v.) reverse a moderate phenformin-induced lactic acidosis in the fasted, non-diabetic and non-ketotic diabetic rat, but hyperketonaemia may result (Alberti and Holloway, 1977).

In the dog dichloroacetate given as an infusion commencing before administration of phenformin prevents hyperlactataemia without affecting the hypoglycaemic action, but is less effective when infusion is started 3h after the phenformin injection (Loubatières et al., 1976).

There is considerable interest in the potential of dichloroacetate in combination with phenformin for use in the treatment of diabetes, but whether it will have a beneficial effect on lactic acidosis in man remains to be investigated.
1.6 THE METABOLIC FATE OF THE BIGUANIDES

Although there has been a great deal of research to determine the mechanism of action of biguanides, their metabolism and disposition has received relatively little study.

The concentrations of phenformin and its metabolites in blood and tissues have been estimated by fluorimetric (Hall et al., 1968) and colorimetric methods (Freedman et al., 1961; Tranquada, 1961; Assan et al., 1975) but the validity of these methods is not generally accepted (Mehnert, 1969a; Beckmann, 1971; Cohen and Woods, 1976). More recently a specific and sensitive g.l.c. method for the determination of phenformin in biological fluids has been developed (Sterling Winthrop Research Laboratories, personal communication, 1974; Alkalay et al., 1975; Matin et al., 1975) but it is not possible to determine the concentration of metabolites by this technique.

Because of these difficulties a number of metabolic studies have used radioactively-labelled phenformin (Hick et al., 1960, 1961; Beckman, 1967, 1968). However, in view of the ability of the biguanide moiety to alternate between several resonant and tautomeric structures (Shapiro, 1959; Elpern, 1968; see FIG. 1.2) the randomly-labelled$^{3}$Hphenformin used by Beckmann (1967, 1968) may be susceptible to exchange of label between the compound and water, particularly in acid solution.

Absorption and excretion of the biguanides

Determination of the unchanged phenformin in plasma of human patients by g.l.c. has shown that the concentration of the drug after oral administration of 50-150 mg/day reached peak levels of 100-200 ng/ml (0.5-1.0 x $10^{-6} M$), 2-4h after ingestion (Sterling Winthrop Research Laboratories, personal communication, 1974; Karam et al., 1974; Alkalay et al., 1975;
Matin et al., 1975). The composite level of phenformin and its metabolites (measured as plasma radioactivity) 2h after oral administration of 100 mg \[^3\text{H}\]phenformin was of the order, 220 ng/ml, $1.1 \times 10^{-6}\text{M}$ (Beckmann, 1967, 1968).

The pharmacokinetics of buformin and metformin have been the subject of little attention. After oral ingestion of 100 mg \[^{14}\text{C}\]buformin the peak plasma level of radioactivity at 1-2\text{h} was equivalent to $2.0-6.4 \times 10^{-6}\text{M}$ (Beckmann and Hübner, 1965; Lintz et al., 1974). Pignard (1962) has reported serum concentrations of metformin of $4 \times 10^{-5}\text{M}$ during oral therapy (1g/day) and the levels may be as high as $3.5-8.5 \times 10^{-4}\text{M}$ in patients with lactic acidosis (Assan et al., 1977) although the specificities of the colorimetric assays used for biguanide determinations are not generally accepted.

After intraperitoneal administration of phenformin to the rat (100 mg/kg) and guinea-pig (15 mg/kg) the livers of both species contained 18-35% of the radioactivity in the first 2-3h but this rapidly declined to 2-3% at 12h and less than 1% at 24h (Wick et al., 1960, 1961; Hall et al., 1968). This does not indicate a selective concentration of the active parent drug as almost all of the radioactivity in rat liver and more than half in guinea-pig liver was attributable to metabolic products (Hall et al., 1968). The stomach and intestines also contained large amounts of radioactivity after parenteral administration of phenformin, suggesting that the drug and/or its metabolites were secreted into the stomach and the gut, perhaps due to the extremely basic nature of the biguanide moiety. Bases such as aniline (Shore et al., 1957) and nicotine (Andersson et al., 1965) were secreted from the plasma into the stomach of rats. Biliary excretion of radioactivity into the lumen of the gut may also occur (Hall et al., 1968).
After oral administration of phenformin to rats most of the radioactivity was associated with the gastrointestinal tract and appeared to be trapped by the acidic stomach juices rather than associated with the tissue (Wick et al., 1960).

The excretion of radioactivity in urine and faeces after oral and intraperitoneal administration of phenformin is shown in TABLE 1.4. It can be seen that the data is inconclusive as there is considerable disagreement. The values reported by Wick et al. (1960) were obtained from one animal each and must be viewed with reservations. Although the major portion of the dose was rapidly eliminated within 24h the excretion in subsequent days was also significant. Beckmann (1967) found small amounts of radioactivity in the 48-72h urine and faeces after intraperitoneal administration of phenformin to rats, and the drug could still be detected by g.l.c. in the urine of human patients 60-100h after ingestion of 100 mg phenformin (Alkalay et al., 1975; Matin et al., 1975).

Beckmann (1967, 1968) reported that the rat completely metabolised phenformin, mainly to 4-hydroxyphenformin glucuronide, after an oral dose of 50 mg/kg [3H]phenformin (see FIG. 1.7). A small amount of the free phenol was also detected. Murphy and Wick (1968) also found only 4-hydroxyphenformin and its glucuronic acid conjugate in rat urine after oral administration of 100 mg/kg [14C-biguanide]phenformin but reported that these metabolites existed in equal quantities. Rat faeces contained mainly phenformin with small quantities of 4-hydroxyphenformin and its glucuronic acid conjugate (Beckmann, 1967). Biliary excretion in the rat accounted for some 5-12% of a 50 mg/kg of [3H]phenformin in 24h and the bile contained mainly phenformin. The metabolites 4-hydroxyphenformin and its glucuronide accounted for some 10-30% of the biliary radioactivity (Beckmann, 1967).
<table>
<thead>
<tr>
<th>Isotope</th>
<th>species(1)</th>
<th>dose (mg/kg)</th>
<th>route of administration</th>
<th>% of dose excreted</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0-24 h</td>
<td></td>
</tr>
<tr>
<td>[2'-14C]phenformin</td>
<td>rat (1)</td>
<td>100</td>
<td>oral</td>
<td>97.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat (1)</td>
<td>100</td>
<td>intraperitoneal</td>
<td>134.0(2)</td>
<td></td>
</tr>
<tr>
<td>[3H]phenformin</td>
<td>rat (5)</td>
<td>50</td>
<td>oral</td>
<td>27.5 55.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat (4)</td>
<td>50</td>
<td>intraperitoneal</td>
<td>67.8 12.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>guinea-pig (1)</td>
<td>50</td>
<td>oral</td>
<td>38.0 58.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rabbit (1)</td>
<td>50</td>
<td>oral</td>
<td>40.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mouse (10)</td>
<td>50</td>
<td>oral</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>man (4)</td>
<td>50 or 100 mg</td>
<td>oral</td>
<td>47.5</td>
<td></td>
</tr>
<tr>
<td>[14C-biguanide]phenformin</td>
<td>rat</td>
<td>100</td>
<td>oral</td>
<td>56.0(3) 45(3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Murphy and Wick (1968)</td>
</tr>
<tr>
<td>[14C-biguanide]phenformin</td>
<td>rat (4-10)</td>
<td>100</td>
<td>intraperitoneal</td>
<td>54.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>guinea-pig(4-10)</td>
<td>15</td>
<td>intraperitoneal</td>
<td>75.0</td>
<td></td>
</tr>
</tbody>
</table>

(1) Number of subjects in parentheses; (2) the reason for this high value was not given; (3) these values were obtained from the pooled urine and faeces from an unspecified number of animals.
The mouse, rabbit and guinea-pig excreted the same metabolites in urine as the rat after oral administration of \([^3\text{H}]\)phenformin but also eliminated some unchanged drug (Beckmann, 1967). The degree of metabolism varied with species and only traces of metabolic products were detected in guinea-pig urine.

Human urine contained phenformin and 4-hydroxyphenformin in a 2:1 ratio but no glucuronic acid conjugate was detected (Beckmann, 1967, 1968).

Buformin in rat and man after oral administration was excreted predominantly by the kidney, some 60-80% of the dose being eliminated in the urine in 24h (Beckmann, 1965; Losert et al., 1972a; Lintz et al., 1974). After intravenous administration only 60-90% of the dose in man was excreted in the urine and 10-40% in the faeces. The presence of the drug in the lumen of the gut may be associated with biliary excretion or secretion from the blood into the intestinal lumen (Lintz et al., 1974).

Rat and mouse metabolised buformin more or less quantitatively by introduction of a hydroxyl group to give \(\text{L-}(3\text{-hydroxybutyl})\) biguanide (see FIG. 1.7); man appeared to excrete only unchanged buformin (Beckmann, 1965, 1966, 1967, 1968).

In the rat and guinea-pig after oral administration of 10 mg/kg buformin all of the tissues examined contained levels of radioactivity significantly higher than that of the blood at 24h after dosing (Losert et al., 1972a). There was no evidence of selective concentration of radioactivity in the liver. Higher concentrations than those in liver were found in the gastrointestinal tract and skeletal muscle in rat and in the urinary bladder, gut and kidneys in the guinea-pig. The very high level of radioactivity in the bladder and kidneys of the guinea-pig may have reflected the slower rate of
elimination in this species. At 24h the rat had excreted some 85% of the dose compared with 69% in the guinea-pig (Losert et al., 1972a).

A study of the tissue distribution of radioactivity after oral, subcutaneous and intravenous administration of $[^{14}\text{C}}$-biguanide]metformin in the mouse showed that the radioactive label was rapidly associated with the stomach and gastrointestinal tract and was eliminated primarily in the urine (Cohen and Costerousse, 1961). After oral administration the liver did not show a significant accumulation of radioactivity and after the parenteral doses the accumulation was only transitory. The rapid appearance of $^{14}$C in the gut after parenteral administration did not appear to involve biliary excretion. The radioactivity in the urine was associated with unchanged metformin.

The metabolism of metformin has been studied in the rat (Beckmann, 1969) and in man (Pignard, 1962; Debry and Cherrier, 1965). Only unchanged metformin has been found in the urine, but when metformin was administered to diabetic patients only a small part of the dose was recovered in the urine and faeces. Debry and Cherrier (1965) suggested that this might indicate that metformin was partly metabolised in man, but in view of the relatively non-specific colorimetric technique used these findings are not generally accepted (Beckmann, 1967, 1968; Mehnert, 1969a).
1.7 THE OBJECTIVE OF THIS THESIS

A survey of the literature to date shows that the metabolic fate of phenformin has not been adequately studied (see SECTION 1.6). The most comprehensive study has been that of Beckmann (1968), who, perhaps unwisely, used $[^3H]$phenformin and whose results did not agree with those obtained using $[^{14}C]$phenformin (Hall et al., 1968; Murphy and Wick, 1968); see TABLE 1.4. In view of the current concern regarding the occurrence of lactic acidosis associated with the biguanides a more detailed knowledge of the absorption, excretion and metabolism of phenformin is desirable.

Although there has been a great deal of research into the problem of lactic acidosis it has been largely concerned with the clinical treatment of diabetic patients. Only recently have studies on the mechanism of biguanide-associated lactic acidosis been published (Heuclin et al., 1975; Lloyd et al., 1975; Assan et al., 1976; Man and Alberti, 1976; Alberti and Holloway, 1977). Although it is generally believed that the pharmacologically active molecule is the parent compound this has not been demonstrated experimentally. Conflicting reports have been published regarding the hypoglycaemic activity of 4-hydroxyphenformin; according to Wick et al. (1970) the phenol is half as effective as phenformin in the rat while Beckmann (1968) found the metabolite to be ineffective in the mouse. Cook et al. (1973a) have reported that the metabolism of phenformin to 4-hydroxyphenformin is the controlling factor in the differential hypoglycaemic response of rats and guinea-pigs to phenformin, but no attempt was made to justify the assumption that the metabolic products were 4-hydroxyphenformin and its glucuronic acid conjugate.

The relationship between the metabolism of phenformin and its pharmacological activity remains to be investigated, particularly with
a view to understanding the species difference in hypoglycaemic and hyperlactataemic response to the drug.

In these studies the absorption, excretion and metabolism of [2'-14C]phenformin were studied in the rat and guinea-pig with particular regard to the possible existence of pharmacologically active metabolites. The hypoglycaemic and hyperlactataemic activities of phenformin and 4-hydroxyphenformin were investigated in both species and the potential of dichloroacetic acid (and other compounds of interest) as a means of controlling phenformin-associated lactic acidosis was studied.
CHAPTER 2

METABOLISM OF PHENFORMIN IN RAT AND GUINEA-PIG
2.1 MATERIALS AND METHODS

Chemicals

Sodium $[^{14}\text{C} ]$ cyanide (specific radioactivity 50mCi/m mole) was obtained from the Radiochemical Centre, Amersham, Bucks. and tyramine hydrochloride from Sigma Chemical Co. Ltd., London.

Chemicals used in scintillation counting were 2,5-diphenyloxazole, Instagel and Cab-O-Sil thixotropic gel-powder (Packard Instruments Ltd., Reading, Berks.) and 1,4-di(2-methylstyryl)benzene (Koch Light Laboratories, Colnbrook, Bucks.). Toluene of low sulphur content used for scintillation counting was obtained from Fisons, Loughborough, Leics.

Saccharic acid 1,4-lactone was purchased from Sigma (London) and Ketodase, ox-liver $\beta$-glucuronidase from Warner-Chilcott Ltd., Eastleigh, Hants.

Except where otherwise specified, all other solvents and chemicals used were from common laboratory suppliers and were of reagent grade.

Phenformin hydrochloride was kindly donated by Sterling Winthrop Research Laboratories (Fawdon, Newcastle-upon-Tyne). Visualisation under u.v. light (254nm) after t.l.c. on silica gel $\text{GF}_{254}$ and alumina $\text{60F}_{254}$ in solvent systems A and G respectively revealed a single component which gave a positive colour reaction with a nitroprusside-ferricyanide spray reagent (see Chromatography).

Sodium pentobarbitone anaesthetic was obtained from Abbot Laboratories, Queenborough, Kent (Nembutal) or May and Baker Ltd., Dagenham, Essex
Urethane anaesthetic solution was prepared from the reagent grade material supplied by British Drug Houses Ltd., Poole, Dorset.

Materials

Polypropylene mini-vials (5ml) used in scintillation counting were obtained from G. D. Searle and Co. Ltd., High Wycombe, Bucks. All cannula tubing was supplied by Portex Ltd., Hythe, Kent. Neutral glass collection tubes (2ml) were obtained from Glass Wholesale Supplies Ltd., London and plastic cups with concave base (1ml auto-analyser cups) from Scientific Supplies Co. Ltd., London.

Synthesis of $[2'-^{14}\text{C}]$phenformin hydrochloride

Radioactively labelled phenformin was synthesised essentially according to the procedure described by Sterling Winthrop Research Laboratories (personal communication, 1974). Carbon-14 is introduced as sodium $[^{14}\text{C}]$cyanide as shown in FIG. 2.1.

\[
\text{C H}_0\text{C} + \text{Na}^{14}\text{CN} \rightarrow \text{C H}_2^{14}\text{CN} 
\]

\[
\text{C H}_2^{14}\text{CN} \rightarrow \text{C H}_2^{14}\text{C}-\text{NH}-\text{C}-\text{NH}_2\cdot\text{HCl} \rightarrow \text{C H}_2^{14}\text{C}-\text{NH}-\text{C}-\text{NH}_2\cdot\text{HCl}
\]

FIG. 2.1 Scheme for the synthesis of $[2'-^{14}\text{C}]$phenformin
(a) Benzyl[^14]C cyanide

Benzyl chloride (1.27g, 1 x 10^{-2} mole) in ethanol (2ml) was added slowly to an aqueous solution (1.0-1.5ml) of sodium[^14]C cyanide (0.49g, 1 x 10^{-2} mole, 500μCi) and the mixture refluxed for 4h. After filtering and washing the precipitate of NaCl with ether, the organic layer was washed successively with 55% (w/v) sulphuric acid (1ml), saturated sodium bicarbonate solution (1ml) and saturated sodium chloride solution (1ml). The organic phase was separated, dried (anhydrous sodium sulphate) and evaporated under reduced pressure to yield a pale yellow oil, benzyl[^14]C cyanide (0.97g, 83.0%).

(b) [2-[^14]C]Phenethylurethane hydrochloride

Lithium aluminium hydride (0.4g, 1.05 x 10^{-2} mole) in sodium-dried ether (20ml) was stirred under nitrogen while anhydrous aluminium chloride (1.40g, 1.05 x 10^{-2} mole) in sodium-dried ether was added over a period of 10 min. Benzyl[^14]C cyanide (0.97g, 0.83 x 10^{-2} mole) in sodium-dried ether (20ml) was added slowly over a period of 20 min and stirred for 2h. Excess hydride was destroyed by successive addition of water (0.8ml), 2N-sodium hydroxide (0.6ml) and water (2.0ml) and the pH finally adjusted to 11.0 with 30% (w/v) sodium hydroxide. The solid was filtered off and washed with water and ether and the filtrate extracted with ether. The combined ether extracts were dried over sodium sulphate and evaporated under reduced pressure to give an oil. This was dissolved in sodium-dried ether (2ml), cooled to 4°C and bubbled with dry hydrogen chloride gas for 5-10 min. The white solid was filtered off and dried in vacuo (0.80g, 61.3%).
(a) $[2'-^{14}C]$Phenethylbiguanide (phenformin)

$[2'-^{14}C]$Phenethylamine hydrochloride (0.80g, $5.1 \times 10^{-3}$ mole) was stirred in xylene (2ml) with dicyandiamide (0.43g, $5.1 \times 10^{-3}$ mole) and heated under reflux for 4h with the temperature of the oil bath being maintained above $140^\circ$C to ensure the best yield. The xylene was removed under reduced pressure and the solid crystallised once from propan-2-ol-water (95:5, by vol.) and once from butan-1-ol. The solid was filtered off and dried at $60^\circ$C in vacuo (0.480g, 39.1%), m.p. 169-174$^\circ$C and radiochemical purity 92.2%.

Radiochemical purity was determined by t.l.c. on silica-gel GF$_{254}$ in system B using 50-100$\mu$g loadings of radioactive phenformin co-chromatographed with authentic non-radioactive phenformin. After removal of the zones corresponding to the biguanide the remaining area in each run was divided into 1cm bands and taken for scintillation counting. The radiochemical purity was expressed as the percentage of the total radioactivity attributable to phenformin.

Further recrystallisation from propan-2-ol-water (95:5, by vol.) gave 0.155g of phenformin hydrochloride; radiochemical purity $>98\%$, m.p. 177-179$^\circ$C. Similar treatment of the mother liquor yielded an additional 0.075g of the compound; radiochemical purity $>98\%$, m.p. 176-179$^\circ$C.

The specific radioactivity of the $[2'-^{14}C]$phenformin hydrochloride was 223.2$\mu$Ci/g (53.9mCi/mole) and the total yield 51.3$\mu$Ci (0.230g).
Synthesis of 4-hydroxyphenformin hydrochloride

4-Hydroxyphenformin hydrochloride was prepared from tyramine hydrochloride and dicyandiamide analogously to the preparation of phenformin hydrochloride from phenethylamine and essentially as described by Beckmann (1967) and Murphy and Wick (1968).

The product of the reaction was crystallised from methanol, but contained considerable amounts of starting materials. Recrystallisation, twice from butan-1-ol and twice from propan-2-ol-water (95:5, by vol.) gave a sample of 4-hydroxyphenformin which produced a single zone when visualised under u.v. light (254nm) after t.l.c. on silica gel GF254 in systems B and D and gave a positive reaction with the nitroprusside-ferricyanide reagent for guanidines and biguanides.

The m.p. (179-181°C) agreed well with the value (180-183°C) reported by Murphy and Wick (1968).

Further samples of 4-hydroxyphenformin hydrochloride (and dihydrochloride) were kindly supplied by Sterling Winthrop Research Laboratories, Fawdon, Newcastle-upon-Tyne.

Determination of radioactivity

Several different scintillation fluids were used to accommodate the variety of samples counted. These are listed below.

1. Toluene, containing 2,5-diphenyloxazole (4g/l) and 1,4-di(2-methylstyryl)benzene (0.08g/l).

2. Toluene-Synperonic NXP (2:1, by vol.) (Wood et al., 1975) 2,5-diphenyloxazole (5.5g/l).

3. Cab-O-Sil scintillant
   Cab-O-Sil thixotropic gel-powder 4-5% (w/v) in scintillant system 1.

4. Instagel, commercially prepared scintillant.
Aqueous and organic samples (0.5ml; or adjusted to 0.5ml with water) were generally counted in scintillant system 1 (10ml) after addition of 2-ethoxyethanol (5ml) or system 2 (4ml). Highly quenching samples (0.5-4.0ml) such as digests of blood, tissues and faecal homogenates, or solutions of picrate salts from reverse isotope dilution analysis were counted in scintillant system 4 (4-10ml). Samples of silica-gel from t.l.c. plates or paper strips from descending paper-chromatography were suspended in scintillant system 3 for determination of radioactivity. Samples were stored in the dark and counted at 4°C in a Packard Tricarb 3320 or LKB Wallac 1210 scintillation counter.

Counting efficiencies for most samples were determined using an automatic, external standard, channels ratio calibration. Quench curves were prepared with n-[1-14C]hexadecane as the standard and carbon tetrachloride as the quenching agent. Different curves were prepared for different scintillant systems and counting conditions. The accuracy of the channels ratio correction procedure was checked periodically by internal standardisation of randomly selected samples. Separate quench curves were prepared for the coloured solutions of picrate salts, using non-radioactive phenformin picrate as the quenching agent.

Counting efficiencies of other highly quenching samples such as digests of blood, tissues and faecal homogenates were determined by internal standardisation.
Chromatography

Thin-layer chromatography was carried out using commercially prepared plates of silica gel GF₂₅₄ (0.25mm; Schleicher and Schull; Anderman and Co. Ltd., London) and alumina F₂₅₄ (type E, 0.25mm; E. Merck, Darmstadt, West Germany), preparative-layer chromatography on silica gel 6₀F₂₅₄ (2mm; E. Merck, Darmstadt, West Germany). The following solvent systems were used:

A. butan-1-ol-acetic acid-water (4:1:1, by vol.)
B. butan-1-ol-ammonia (0.88sp. gr.)-water (10:1:1, by vol.)
C. butan-1-ol-chloroform-methanol-25% (W/v)ammonia (45:15:15:15, by vol.)
D. propan-1-ol-ammonia (0.88sp. gr.) (7:3, by vol.)
E. chloroform-methanol (10:3, by vol.)
F. chloroform-methanol-25% (W/v)ammonia (10:2:1, by vol.)
G. ethanol-25% (W/v)ammonia (4:1, by vol.)

For paper chromatography the following solvent system was used:
H. propan-2-ol-ammonia (0.88sp. gr.)-water (80:1:20, by vol.).

All solvents used in the preparation of samples for mass spectrometry were of AnalaR quality and t.l.c. plates were pre-run in the solvent system to be used.

Authentic phenformin and 4-hydroxyphenformin were co-chromatographed with all samples and located by visualisation under u.v. light (254nm). Two spray reagents were used to detect guanidine and biguanide groupings:

1. Sodium nitroprusside-potassium ferricyanide-sodium hydroxide, each 10% (W/v) in water (1:1:1, by vol.) diluted with 9 volumes
50\% (\textit{v}/\textit{v}) aqueous ethanol (Murphy and Wick, 1968);

2. $\alpha$-naphthol, 1\% (\textit{w}/\textit{v}) in sodium hydroxide (8\%, \textit{w}/\textit{v} in water) - 1\% (\textit{w}/\textit{v}) diacetyl in water (1:1 by vol.) (Smith, 1969).

Glucuronic acid conjugates were detected by spraying plates with 1\% (\textit{w}/\textit{v}) naphthoresorcinol in acetone - 10\% (\textit{v}/\textit{v}) phosphoric acid (4:1, by vol.) followed by heating at 140\textdegree C for 15 min (Bridges et al., 1965).

Descending paper chromatography of guinea-pig urine extracts was carried out using Whatman 3MM paper in solvent system H. Authentic phenformin and 4-hydroxyphenformin were co-chromatographed with portions of the extracts and located using spray reagent 2.

Whole urine and urine extracts were chromatographed on silica gel in solvent systems B, C and D, and on alumina in solvent system G. Faecal extracts were chromatographed on silica gel in solvent systems E and F.

\textbf{Autoradiography}

Autoradiographs of t.l.c. plates were prepared by exposing to medical X-ray film (Kodak 'Blue Brand', BB54, 18 x 24\text{cm}) for a suitable period of time (typically 4-6 weeks) in a lightproof cassette. The films were developed in Kodak Universal developer for 3-4 min, rinsed in dil. acetic acid and fixed in Kodafix solution for 3-4 min before washing in running water for 30 min.

Contact prints of the autoradiographs were made on Agfa Rapidoprint paper (grade TP3 semi-matt finish) using an Agfa LD37 Rapidoprinter.
Where required, permanently fixed copies were made using Kodafix solution as described before.

**Animals**

Adult male Wistar albino rats and Dunkin-Hartley albino guinea-pigs were used in all experiments. For surgical procedures rats were anaesthetised by intraperitoneal injection of 1ml/kg sodium pentobarbitone solution (60mg/ml) and guinea-pigs received urethane (28% W/v aqueous solution, 8-10ml/kg, i.p.) as described by Schanker et al. (1958). All animals subjected to surgery were sacrificed before recovery from anaesthesia.

**Determination of phenformin in blood**

Guinea-pigs (350-500g) and rats (300-400g) were anaesthetised and the neck area shaved. A 3cm mid-line incision was made commencing in the region of the larynx and terminating before the ventral thorax wall. The common carotid artery was located and cannulated using PP50 (guinea-pig) or PP25 (rat) polypropylene tubing. The cannula was kept patent by frequent administration of small volumes of saline (0.9%, W/v) containing lithium heparin (1000U/ml) and was clamped between collections.

Samples (approx. 0.5ml) were collected at intervals into 1ml plastic auto-analyser cups which had been rinsed in heparinised saline and dried before use.

[2'-14C]Phenformin was administered intravenously through the cannula at a dose level of 10-12mg/kg or intraperitoneally (15-25mg/kg).
Aliquots of blood (0.1ml in triplicate) were digested at 50°C in glass scintillation vials with 62% (w/v) perchloric acid (0.2ml) and decolourised with 100 vol. (30%, w/v) hydrogen peroxide (0.2ml) before counting in scintillant 4.

**Excretion balance studies**

Rats (250-350g) and guinea-pigs (300-350g) were individually housed in all-glass metabolism cages (Metabowls, Jencon Scientific Ltd., Hemel Hempstead, Herts.).

Guinea-pigs were dosed with aqueous solutions of [2'-14C]phenformin hydrochloride at a level of 25mg/kg orally (1.5-1.8μCi) or 12.5mg/kg intraperitoneally (1.0-1.2μCi). Rats were dosed with aqueous solutions of [2'-14C]phenformin hydrochloride (7mg/kg, orally) or diluted with non-radioactive phenformin hydrochloride at doses equivalent to 100mg/kg (1.6-2.8μCi) either orally or intraperitoneally.

The urine and faeces were collected separately each 24h for 4 days and the expired air drawn at a rate of approximately 200ml/min through a series of carbon-dioxide traps containing ethanolamine and 2-ethoxyethanol (1:3, by vol.). After each collection of urine and faeces the cages were washed through with distilled water to remove any radioactivity adhering to the glass surfaces. Each washing was collected and assayed for radioactivity by liquid scintillation counting and the value obtained (<0.3% of the dose) was added to that of the appropriate urine sample. Aliquots (0.1-0.2ml) of each urine sample were assayed for radioactivity as described previously, usually after dilution in water.
Faecal samples were homogenised in water to give 5-10% (w/v) homogenates and triplicate aliquots (0.5 ml) were transferred into glass scintillation vials and digested with hyamine hydroxide (3 ml) at 50-60\degree\textnormal{C} for 6h. On cooling the samples were decolourised overnight at room temperature with 100 vol. (30% w/v) hydrogen peroxide (0.25 ml), neutralised with concentrated hydrochloric acid (0.25 ml) and the radioactivity determined by liquid scintillation counting in scintillant 4 (10 ml).

The solvent from each carbon dioxide trap was diluted with 2-ethoxyethanol and aliquots (4-5 ml) dispensed into vials and counted in scintillant 1 (10 ml).

**Biliary excretion studies**

The biliary excretion of radioactivity in rats (300-400g) and guinea-pigs (500-800g) was determined after a single intraduodenal administration of [2-\textsuperscript{14}\textsuperscript{C}]phenformin hydrochloride (20 mg/kg) in 0.25 ml saline (2.0-4.0 \mu\textnormal{Ci per animal}).

(a) **Collection of bile in the rat**

Rats were anaesthetised with sodium pentobarbitone, the abdominal region shaved and the limbs restrained. The common bile duct was cannulated essentially as described by Abou-el-Makarem et al. (1967a) except that the animals were anaesthetised throughout the experiment.

After a mid-line incision through the skin and peritoneum the duodenal region of the small intestine was located. The bile duct
was cleared of mesenteric fat and ligated at its entry to the duodenum. Two loose ligatures were placed in position around the duct and a 2 mm hole made in the side wall of the abdominal cavity using a trochar. A suitable length (20 cm) of PP10 or PP25 cannula tubing was fed through the hole in the abdominal wall and filled with saline. After making a small incision in the bile duct with iridectomy scissors the cannula was inserted and tied in.

When bile flow was evident a 20 cm length of PP25 tubing was tied into a small hole made in the duodenum, below the point of entry of the bile duct. The other end of the intraduodenal cannula was passed through the ventral mid-line incision which was then sutured using surgical thread. A third length of cannula (PP25) was inserted through the sutures into the peritoneal cavity for subsequent administration of saline and anaesthetic.

The outer skin was closed using suture clips and the exposed surface skin covered with cotton wool moistened with saline.

(b) Collection of bile in the guinea-pig

Guinea-pigs were anaesthetised and prepared in a similar fashion to rats. The gall bladder was located and tied off at its junction with the common bile duct. In cases where the gall bladder was highly distended the bile was withdrawn using a hypodermic needle and syringe. This improved access to the bile duct which was cannulated as described previously using PP25 cannula tubing.
Bile from rats and guinea-pigs was collected into preweighed 2 ml neutral glass tubes and the weight determined. Duplicate aliquots (50 μl) from each collection period were assayed for radioactivity in scintillant 2.

(c) Enterohepatic recirculation in the rat

The significance of enterohepatic recirculation of biliary metabolites in the rat was determined by inserting the bile duct cannula from one rat (donor) into a tight-fitting plastic sleeve which had been tied into the duodenum of a second bile duct-cannulated animal (recipient). The donor animal was dosed with [2'-14C]phenformin hydrochloride intraduodenally and the radioactive bile allowed to flow into the duodenum of the recipient rat whose bile was collected and assayed for radioactivity. Bile flow from the donor rat was checked periodically by removing the cannula from the plastic sleeve.

In a second experiment freshly collected radioactive bile (2 ml) was injected directly into the duodenum of a cannulated rat whose bile was collected and assayed for radioactivity.
Extraction of radioactivity from urine

(a) Solvent extraction

Duplicate aliquots (0.5-2.0ml) of the 24h urine from rats and guinea-pigs were adjusted to a range of pH values (1-11) and extracted two or three times with two volumes of ethyl acetate, diethyl ether or chloroform-methanol (85:15, by vol.). After incubating for 18h at 37° with β-glucuronidase (Ketodase, 5000 Units/ml in 0.2M-sodium acetate buffer, pH 5.0) the extraction procedure was repeated. The radioactivity in aliquots of the organic extracts from each sample was determined by liquid scintillation counting. The remaining portions of the extracts were evaporated under reduced pressure and the residues dissolved in methanol (50-100μl) for examination by t.l.c.

(b) Ion-exchange chromatography

The radioactivity in rat and guinea-pig urine collected 24h after administration of [2'-14C]phenformin was isolated using cation-exchange chromatography. Whole rat urine (10-25ml) and urine after extraction with chloroform-methanol (85:15, by vol.) was applied to columns of 200 mesh Amberlite CG-50 (a weakly acidic carboxylic acid resin) and Amberlite CG-120 (a strongly acidic sulphonated polystyrene resin) and eluted as described by Murphy and Wick (1968).

Guinea-pig urine (15-30ml) was passed through a column (35 x 1.5cm) of Zerolit 225 (a strongly acidic sulphonated polystyrene resin) in the H⁺ form. The column was eluted with water (50ml) and fractions collected until the eluate was pH 5.0. Weakly basic metabolites were eluted with 2N-aqueous ammonia (100ml) and strongly basic metabolites
with 50ml 4N-sodium hydroxide-ethanol (1:1, by vol.), 5-10ml fractions being collected. The fractions containing most of the radioactivity were pooled for each eluent, the sodium hydroxide solution neutralised and both samples concentrated for examination by t.l.c.

**Extraction of radioactivity from faeces**

**(a) Solvent extraction**

Homogenates (5-10%, w/v) of faeces containing radioactivity from guinea-pigs after oral dosing with [2'-14C]phenformin were centrifuged and the aqueous supernatant collected. The residues were extracted successively with 2 volumes of methanol and ethanol-25% (W/v) ammonia (9:1, by vol.). This procedure gave quantitative recovery of the radioactivity but further purification was necessary before examination by t.l.c. This was achieved by adsorption chromatography.

The extraction of radioactivity from rat faecal homogenates for reverse isotope dilution determination of phenformin is described later.

**(b) Adsorption chromatography**

A glass column (40 x 2.5cm) was packed with a slurry of silica gel (Kieselgel 60, 70-230mesh, E. Merck, Darmstadt, West Germany) in chloroform-methanol (10:1, by vol.). The faecal extract was evaporated to dryness, taken up in 1ml chloroform-methanol (1:4, by vol.) and applied to the top of the column. The column was eluted successively with chloroform-methanol mixtures of the following composition by volume; 10:1 (100ml), 5:1 (60ml) and 1:1 (70ml). The final eluent was methanol (80ml).

The eluate was collected as fractions of approx. 5ml and aliquots
taken for scintillation counting. The fractions containing radioactivity were pooled and concentrated before examination by t.l.c.

**Extraction of radioactivity from bile**

*(a) Solvent extraction*

Samples of bile (1.0-2.0ml) from rats after intraduodenal administration of \([2'-^{14}C]\)phenformin (20mg/kg) were pooled, diluted with water (2ml) and adjusted to pH values of 1, 7 or 11. Each sample was extracted with 3 x 5ml volumes of chloroform-methanol (85:15, by vol.) and the radioactivity in each extract determined by scintillation counting.

The residues after solvent extraction were subjected to ion-exchange chromatography.

*(b) Ion-exchange chromatography*

Residues of bile after solvent extraction were applied to 10 x 1.3cm columns of Amberlite CG-50 and Amberlite CG-120 (H⁺ form) as described for urinary extraction by Murphy and Wick (1968).

**Quantitative determination of metabolites**

*(a) After thin-layer chromatography*

Metabolites in rat and guinea-pig urine after oral and intraperitoneal administration of \([2'-^{14}C]\)phenformin were separated by t.l.c. of whole urine on silica gel (solvent systems B, C and D) and alumina (solvent system G) and located by autoradiography. The radioactive zones were removed and the silica suspended in Cab-O-Sil scintillant. The remaining areas of each run were divided into 1cm zones and treated in the same way. Radioactive metabolites were determined as a percentage
of the total activity recovered from the plates which corresponded to the total activity in the urine sample. Ion-exchange eluates of rat and guinea-pig urine after oral dosing and rat bile after intraduodenal dosing were examined in the same way.

Eluates from the adsorption chromatography of guinea-pig faecal extracts after oral administration of phenformin were examined by t.l.c. on silica gel in solvent systems E and F. The distribution of radioactivity was determined as described above.

(b) By reverse isotope dilution

Phenformin and 4-hydroxyphenformin were estimated as the picrate salts by reverse isotope dilution.

Whole urine samples (5-10ml) were diluted with an equal volume of water and aliquots taken to determine the total radioactivity (0.25-0.4μCi). Phenformin hydrochloride or 4-hydroxyphenformin dihydrochloride (0.4-0.5g) was added in aqueous solution (5-10ml) and a saturated solution of picric acid was slowly added with mixing, until no further yellow precipitate formed. The mixture was allowed to stand, with occasional mixing, for 30 min and tested with a further small amount of picric acid solution to ensure completion of the reaction. After filtration and drying, triplicate amounts (10-15mg) were dissolved in dimethylformamide (1ml), diluted with water (4ml) and aliquots (0.25ml) counted in scintillant 4 as previously described.

The picrate salts were recrystallised to constant specific radioactivity from water and dilute alcohol and a sample of the final product was submitted for elemental analysis.
For the determination of phenformin in rat faeces the biguanide (approx. 0.3g) was added to a suitable volume of faecal homogenate and freeze-dried. The powder was extracted three times with methanol (100ml), the solvent evaporated and the residue redissolved in water (20ml). The solution was adjusted to pH 12 and extracted four times with 100ml portions of chloroform-methanol (85:15, by vol.). The residue after evaporation of the solvents was dissolved in water (20ml) and converted to the picrate salt.

In the presence of a large excess of picric acid solution phenformin and 4-hydroxyphenformin formed dipicrate salts, the structures of which were assigned on the basis of elemental analyses for C, H and N. Examples of the analytical data are given below.

\[
\text{phenformin dipicrate, m.p. (from water) 188-192}^\circ
\]
\[
\text{C}_{10}\text{H}_{15}\text{N}_5 \cdot 2 \text{C}_6\text{H}_3\text{N}_3\text{O}_7 \text{ (mol.wt. 663.24)}
\]
\[
\text{calc. C, 39.80; H, 3.19; N, 23.23;}
\]
\[
\text{obs. C, 39.83; H, 3.15; N, 23.24;}
\]

\[
\text{4-hydroxyphenformin dipicrate, m.p. (from water) 220-224}^\circ
\]
\[
\text{C}_{10}\text{H}_{15}\text{N}_5\text{O} \cdot 2 \text{C}_6\text{H}_3\text{N}_3\text{O}_7 \text{ (mol.wt. 679.24)}
\]
\[
\text{calc. C, 38.87; H, 3.12; N, 22.68;}
\]
\[
\text{obs. C, 38.76; H, 3.15; N, 22.56;}
\]

**Isolation of metabolites for mass spectrometry**

Metabolites in the eluates from the ion-exchange chromatography of rat and guinea-pig urine and adsorption chromatography of guinea-pig faecal extracts were separated by repeated preparative-layer or paper chromatography and located by autoradiography or strip-counting respectively. Final separation was achieved using t.l.c. and the compounds were eluted from the silica with methanol into washed melting point tubes. The methanol was evaporated under nitrogen and the tubes
sealed for use as sample probes for direct insertion mass spectrometry.

In vitro metabolism studies

(a) Metabolism by isolated caecal microflora

Rats and guinea-pigs were killed by cervical dislocation and portions of the caecal contents removed rapidly and suspended in approx. 10 volumes of 0.1M-phosphate buffer, pH 7.4 by shaking. The coarse debris was removed by centrifugation at very low speed for 15-20 sec and the supernatant separated and used to inoculate aliquots of incubation medium which was prepared as follows. Glucose, yeast extract and proteose peptone (each 0.5%, w/v) were dissolved in the phosphate buffer and the solution boiled for 30 min and allowed to cool. Portions of the medium (10ml) and [2'-\textsuperscript{14}C]phenformin hydrochloride (2-3mg) in buffer (0.5ml) were transferred to Thunberg tubes. Each tube was inoculated with 0.5ml of caecal supernatant (test samples) or buffer (control samples). The tubes were evacuated and filled with nitrogen and incubated for 3 days at 37°.

After incubation portions (1ml) of medium from each tube were diluted with water (1ml), the pH adjusted to pH 2, 7 or 13 and the samples extracted with ethyl acetate (3 x 5ml) or chloroform-methanol (85:15, by vol., 3 x 5ml). Extracts containing appreciable quantities of radioactivity were concentrated and examined by t.l.c. on silica-gel in solvent systems B, C and E.

The amount of unchanged phenformin in control and test samples was determined as the picrate salt by reverse isotope dilution.
(b) Metabolism by isolated hepatocytes

Suspensions of isolated rat and guinea-pig hepatocytes were prepared according to Fry et al. (1976). The cells were suspended in L15 Leibovitz medium with glutamine (Gibco, Scotland) containing tryptose phosphate broth (10%, w/v) and foetal calf serum (10%, w/v) and the total yield of cells determined by counting in an improved Neubauer counting chamber. The viability of the cells was determined on the basis of the ability of the cells to exclude trypan blue dye. The viable cell concentration was adjusted to approximately $2 \times 10^6$ cells/ml.

The choice of substrate concentration ($1 \times 10^{-3} \text{M}$) was based upon preliminary studies on the cytotoxicity of phenformin in rat and guinea-pig liver cells. These experiments using $5 \times 10^{-5}$- $2 \times 10^{-3} \text{M}$ phenformin showed that concentrations of the biguanide up to $1 \times 10^{-3} \text{M}$ did not cause a significant decrease in viable cells after 90 min compared to control (phenformin-free) incubations. Viability of cells was in the order of 80-85% from rat liver and 70-80% from guinea-pig liver.

$[2'-^{14}\text{C}]$Phenformin hydrochloride (1.2mg, 0.3μCi, in 0.2ml Dulbecco's Ca$^{++}$- and Mg$^{++}$-free phosphate buffered saline) was incubated with the cell suspensions (5ml) in a conical flask in a shaking water bath at 37$^\circ$. Aliquots (0.1ml) of the incubates were taken at timed intervals and added to cold acetone (0.02ml) to stop the reaction, centrifuged and portions of the supernatants (0.02-0.03ml) examined by t.l.c. on silica and alumina in solvent systems C and E respectively. Radioactive zones were located by autoradiography and the radioactivity in each zone determined by scintillation counting in Cab-O-Sil.
2.2 RESULTS

Phenformin levels in blood

Determination of the radioactivity in whole blood after a single intravenous dose of \([2^-\text{H}]\)phenformin hydrochloride (10 mg/kg, 0.8-1.0 \(\mu\text{Ci}\)) to the rat and guinea-pig showed that phenformin was very rapidly removed from the circulation, falling to levels below the limit of detection of the assay (approx. 0.8 \(\mu\text{g - equivalent/ml blood}\)) within 10 min. Measurement of the plasma concentrations of phenformin in a single guinea-pig after a 10 mg/kg intravenous dose of the non-radioactive drug using the g.l.c. assay developed by Sterling Winthrop Research Laboratories (Fawdon) showed that after an initially rapid decline in the first 10 min, phenformin levels remained fairly constant over the next 30 min (see TABLE 2.1 and FIG. 2.1). The fall in blood and plasma levels of the drug showed two phases, the second of which was linear on semilogarithmic coordinates; see FIG. 2.2(b). The initial concentration \((C_0)\), elimination rate constant \((K_e)\), half-life \((t_\frac{1}{2})\) and apparent volume of distribution \((V_d)\) for the linear portion of the graph are shown in FIG. 2.2(b).

After intraperitoneal administration of 15 mg/kg phenformin hydrochloride (1.2-1.5 \(\mu\text{Ci}\)) to rats and guinea-pigs the blood concentration of radioactivity did not rise above the limit of detection (0.8 \(\mu\text{g-equivalents/ml}\)) during the course of the experiment (90 min).

Excretion of radioactivity

After oral administration of \([2^-\text{H}]\)phenformin hydrochloride to rats (100 mg/kg) and guinea-pigs (25 mg/kg) only 50-60% of the radioactivity was excreted in the first 24h. Large amounts of radioactivity were found in the 48h urine and faeces in both species and small quantities in the excreta up to 96h in the rat and 144h in the guinea-pig (see TABLE 2.2 and FIGS. 2.3 and 2.5).
TABLE 2.1  Blood concentrations of radioactivity or plasma phenformin concentrations after intravenous administration

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Radioactive assay (µg-equivalents/ml blood)</th>
<th>G.I.C. assay (µg phenformin/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>4.93</td>
<td>7.63 n.d.</td>
</tr>
<tr>
<td>1.5</td>
<td>4.04</td>
<td>4.80 3.00</td>
</tr>
<tr>
<td>2.0</td>
<td>3.49</td>
<td>4.00 n.d.</td>
</tr>
<tr>
<td>2.5</td>
<td>2.88</td>
<td>2.36 n.d.</td>
</tr>
<tr>
<td>3.5</td>
<td>2.29</td>
<td>2.08 n.d.</td>
</tr>
<tr>
<td>5.0</td>
<td>1.78</td>
<td>1.70 1.63</td>
</tr>
<tr>
<td>7.5</td>
<td>1.05</td>
<td>1.10 n.d.</td>
</tr>
<tr>
<td>10.0</td>
<td>0.85</td>
<td>&lt;0.80 0.81</td>
</tr>
<tr>
<td>15.0</td>
<td>&lt;0.80</td>
<td>&lt;0.80 0.79</td>
</tr>
<tr>
<td>30.0</td>
<td>&lt;0.80</td>
<td>&lt;0.80 0.73</td>
</tr>
<tr>
<td>45.0</td>
<td>&lt;0.80</td>
<td>&lt;0.80 0.69</td>
</tr>
</tbody>
</table>

(1) two individual experiments
(2) only one experiment
(3) n.d. = not determined

Phenformin hydrochloride (10 mg/kg) was administered in 0.25 ml saline.
FIG. 2.2 Blood concentrations of radioactivity in the rat and guinea-pig and plasma phenformin levels in the guinea-pig after an intravenous dose (10 mg/kg)

(a) Blood/plasma profiles in rat and guinea-pig

Radioactive assay in rat (○) and guinea-pig (□) expressed as μg-equivalents/ml blood, g.l.c. assay in guinea-pig (●) as μg/ml plasma

(b) ln concentration-time relationship in guinea-pig

\[ C_0 = 0.84 \, \mu g/ml; \, K_e = 0.6\% \, per \, min. \]
\[ t_\frac{1}{2} = 120 \, min.; \, V_d = 11.9 \, l/kg \]
In the rat urinary excretion accounted for 55-57% and faecal excretion the remaining 40-42% of the radioactivity after doses of 7 and 100 mg/kg, but the rate of elimination differed. After a dose of 7 mg/kg almost 90% of the dose was excreted in the first 24h while after 100 mg/kg only about 63% of the radioactivity was eliminated in this period, the difference being due to the more rapid faecal excretion at the lower dose (see FIG. 2.4). At the higher dose the 48h faeces contained as much radioactivity as the 24h faeces (15-16%) while at the lower dose almost all of the faecal excretion occurred in the first 24h; see TABLE 2.2.

In the guinea-pig after oral administration of phenformin hydrochloride (25 mg/kg) 37% of the dose was excreted in the urine and 20% in the faeces in the first 24h. Excretion in the urine and faeces in the second day was 6% and 14% respectively, but at 96h only some 80-90% of the dose was recovered which was significantly different from the recoveries achieved in the rat at 7 mg/kg and 100 mg/kg (p < 0.01 in both cases). The liver taken from one guinea-pig 96h after a single oral dose of phenformin contained a small amount of radioactivity (approx. 0.3% of the dose).

After intraperitoneal administration of the drug to rat and guinea-pig 81-87% of the dose was recovered in 24h, most of which appeared in the urine (see TABLE 2.2 and FIGS. 2.3 and 2.5). After 48h approximately 95-97% of the administered radioactivity had been eliminated in the urine and faeces. Faecal excretion in the first 24h was significantly greater (p < 0.001) in the guinea-pig (13-18%) than in the rat (7-10%) but elimination in the 24-48h period was significantly greater (p < 0.001) in the rat (9.5-13.5%) than in the guinea-pig (4-7%). The presence of
### Table 2.2
Excretion of radioactivity after a single administration of [2'-14C] phenformin hydrochloride

<table>
<thead>
<tr>
<th>species(1)</th>
<th>route of administration</th>
<th>dose (mg/kg)</th>
<th>mean percentage of dose excreted(2)</th>
<th>total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 - 24h</td>
<td>24 - 48h</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>urine</td>
<td>faeces</td>
</tr>
<tr>
<td>rat (4)</td>
<td>oral</td>
<td>7</td>
<td>51.5</td>
<td>36.7</td>
</tr>
<tr>
<td>rat (7)</td>
<td>oral</td>
<td>100</td>
<td>48.3</td>
<td>14.3</td>
</tr>
<tr>
<td>guinea-pig (6)</td>
<td>oral</td>
<td>25</td>
<td>37.3</td>
<td>20.1</td>
</tr>
<tr>
<td>rat (7)</td>
<td>intraperitoneal</td>
<td>100</td>
<td>72.3</td>
<td>8.7</td>
</tr>
<tr>
<td>guinea-pig (5)</td>
<td>intraperitoneal</td>
<td>12.5</td>
<td>72.6</td>
<td>14.9</td>
</tr>
</tbody>
</table>

(1) number of individual animal experiments are given in parentheses
(2) range of individual values are given in parentheses
(3) 96-144h excretion measured in a single guinea-pig was urine, 0.8%; faeces, 3.8%.

14CO2 production in rat and guinea-pig after oral administration at 24h and 48h was less than 0.1%, the detection limit.
Cumulative excretion of radioactivity in urine and faeces following a single dose (100 mg/kg) of [2-14C]phenformin orally or intraperitoneally to rats.

Phenformin hydrochloride (100 mg/kg, 1.6-2.8μCi) administered in water (0.5 ml approx.). Results expressed as means of 5-7 experiments ± S.E.M. represented by vertical bars. ●, urine - oral dose; ■, faeces - oral dose; ◊, urine - intraperitoneal dose; □, faeces - intraperitoneal dose.
Cumulative excretion of radioactivity in urine and faeces following a single oral dose of 7 mg/kg or 100 mg/kg [2'-14C]phenformin to rats.

Phenformin hydrochloride, 7 mg/kg (0.5-0.6µCi) or 100 mg/kg (1.6-2.8µCi) administered in water (0.5 ml approx.). Results expressed as means of 4-7 experiments + S.E.M. represented by vertical bars.

- , urine - 100 mg/kg; ▽, urine - 7 mg/kg;
- , faeces - 100 mg/kg; ▽, faeces - 7 mg/kg.
FIG. 2.5 Cumulative excretion of radioactivity in urine and faeces following a single oral (25 mg/kg) or intraperitoneal (12.5 mg/kg) dose of [2'-^CJphenformin to guinea-pigs.

Phenformin hydrochloride administered orally (25 mg/kg, 1.5-1.8 Ci) or intraperitoneally (12.5 mg/kg, 1.0-1.2μCi) in water (0.3-0.5 ml). Results expressed as means of 4-6 experiments ± S.E.M. represented by vertical bars.

●, urine - oral dose; ■, faeces - oral dose;
○, urine - intraperitoneal dose; □, faeces - intraperitoneal dose.
FIG. 2.6  Cumulative excretion of radioactivity in the bile following a single intraduodenal administration (20 mg/kg) of [2'-14C]phenformin to rats and guinea-pigs.

Phenformin hydrochloride (20 mg/kg, 2.0-3.0μCi) administered intraduodenally in water (0.5 ml). Results expressed as means of 4 experiments ± S.E.M. represented by vertical bars.

●, rat (mean bile flow rate 1.2 ml/h);
○, guinea-pig (mean bile flow rate 2.1 ml/h).
a substantial proportion of the dose in the faeces of both species after parenteral administration suggests that biliary excretion of radioactivity into the lumen of the gut or secretion from the plasma into the stomach or gut may occur.

The cumulative excretion of radioactivity in the bile after intraduodenal administration of \( [2'\text{-}^{14}\text{C}] \text{phenformin} \) (20 mg/kg) in the rat and guinea-pig is shown in FIG. 2.6. The rat excreted some 27% of the dose in 6h while the guinea-pig eliminated only 6% in the same period. The gradients of the respective rate curves imply that biliary excretion may play an important role in the elimination of radioactivity in the rat but that in the guinea-pig this route is of minor significance.

Experiments to determine the significance of enterohepatic recirculation of biliary radioactivity in the rat showed that when bile from a \( [2'\text{-}^{14}\text{C}] \text{phenformin} \) dosed rat was directed continuously into the duodenum of a second rat, < 0.1% of the original dose was re-excreted in the bile in 7h. If a portion of radioactive bile was administered as a single intraduodenal injection to the rat no radioactivity ( < 1.5%) was detected in the bile in 8h.

**Metabolism of \([2'\text{-}^{14}\text{C}]\text{phenformin in vivo}\)**

(a) Urinary metabolites

Metabolites in rat and guinea-pig urine were identified by t.l.c. of whole urine in at least 3 different solvent systems and where possible by reverse isotope dilution and mass spectrometry. The compounds were estimated by scintillation counting of the radioactive areas after t.l.c. and where possible by reverse isotope dilution.
Whole urine from rat and guinea-pig after oral and intraperitoneal administration of [2'-14C]phenformin contained a total of 5 different radioactive metabolites; see FIGS. 2.7 - 2.12. Metabolites 1 and 2 gave positive reactions with spray reagents 1 and 2 for biguanide and guanidine functions, and co-chromatographed with authentic phenformin and 4-hydroxyphenformin respectively. Metabolite 3 gave a blue colour with the naphthoresorcinol spray reagent for glucuronides and hydrolysed on incubation with β-glucuronidase to metabolite 2. The hydrolysis was inhibited by saccharic acid 1,4-lactone (25mM), see FIGS. 2.11 and 2.12, suggesting that metabolite 3 is the glucuronic acid conjugate of 4-hydroxyphenformin. Confirmation of the presence of phenformin and 4-hydroxyphenformin was obtained by reverse isotope dilution via formation of the dipicrate salts.

Mass spectrometric analysis of metabolites 1 and 2 isolated from urine by ion-exchange chromatography, solvent extraction and t.l.c. produced electron impact spectra which were comparable with those obtained from samples of authentic phenformin and 4-hydroxyphenformin respectively. Examples of positive ion fragmentation patterns obtained from samples of phenformin and 4-hydroxyphenformin isolated from urine are compared with spectra from samples of the authentic materials in FIGS. 2.13 - 2.16. A more detailed discussion of these spectra is presented in the APPENDIX.

Metabolites 4 and 5 were found in guinea-pig urine and have not been identified. Metabolite 5 gave a blue colour with the naphthoresorcinol spray reagent for glucuronide conjugates and hydrolysed on incubation with β-glucuronidase to metabolite 4. The hydrolysis was inhibited by saccharic acid 1,4-lactone (25 mM) (see FIG. 2.9) indicating that metabolite
5 is probably the glucuronic acid conjugate of metabolite 4. Metabolite 4 did not co-chromatograph with authentic 4-hydroxyphenformin on silica in solvent systems B, C, D and G or on paper in solvent system H but showed a slightly higher $R_F$ value in each case; see FIGS. 2.19-2.21. Attempts to determine the structure of this compound by mass spectrometry have proved unsuccessful but the spectra have demonstrated the absence of the positive ions characteristic of the phenolic function of 4-hydroxyphenformin and the absence of a triazine structure such as formed by phenformin and 4-hydroxyphenformin; see APPENDIX.

Solvent extraction of rat and guinea-pig urine was most effective at pH 11-12 using chloroform or chloroform-methanol (85:15, by vol.) under which conditions some 30-45% of the radioactivity was removed. This extract was shown by t.l.c. to contain almost exclusively phenformin. The more polar metabolites 2-5 could not be extracted from aqueous solution with organic solvents and were isolated from urine by ion-exchange chromatography. Ion-exchange chromatography of rat urine did not result in quantitative recovery of radioactivity unless phenformin was previously extracted from the urine. Ion-exchange chromatography of guinea-pig urine as described earlier separated metabolite 5 from phenformin (see FIG. 2.17) but the appearance of radioactive compounds not seen in whole urine suggests that elution with sodium hydroxide may have caused some breakdown of the metabolites; see FIG. 2.17A. These procedures proved useful for the isolation of metabolites for mass spectrometry.

Details of the urinary metabolic patterns of rat and guinea-pig are summarised in TABLE 2.3 and examples of the distribution of radioactivity after t.l.c. of whole urine are shown in FIGS. 2.19 - 2.21.
Rat urine contained unchanged phenformin as well as 4-hydroxyphenformin and its glucuronic acid conjugate but the relative contribution of these 3 compounds varied with the dose level and route of administration. Metabolites 4 and 5 were not detected in rat urine. After oral administration of 100 mg/kg almost one-quarter of the 24h urinary radioactivity (12.5% of the dose) was excreted as unchanged phenformin and the remaining radioactivity as 4-hydroxyphenformin and its glucuronide in a 1:3 ratio, see FIG. 2.19(a).

After the lower dose of 7 mg/kg orally metabolism of phenformin to the phenol was more extensive, the parent compound accounting for an average of only 5% of the 24h urinary radioactivity (2.5% of the dose); see FIG. 2.20. Of the 4 rats studied two excreted no phenformin in the urine while the others excreted only 6-13% of the unchanged drug; see FIGS. 2.11 and 2.12. The remaining radioactivity was attributable to 4-hydroxyphenformin and its glucuronide in a 1:8 ratio.

After intraperitoneal administration to rats of phenformin (100 mg/kg) almost one-half of the 24h urinary radioactivity (32.5% of the dose) was excreted as the parent compound, the remainder being eliminated as 4-hydroxyphenformin and its glucuronic acid conjugate in a 1:2 ratio; see FIG.2.19(b).

Following a 25 mg/kg oral dose to guinea-pigs an average of 50% of the 24h urinary radioactivity (18.5% of the dose) was eliminated as phenformin. The remaining radioactivity was excreted as metabolite 4 and its glucuronide (metabolite 5) in approximately a 1:3 ratio; see FIG. 2.21(a). No significant amount of 4-hydroxyphenformin was detected before or after β-glucuronidase hydrolysis; see FIG. 2.9.

After intraperitoneal administration (12.5 mg/kg) the guinea-pig
<table>
<thead>
<tr>
<th>species (no. of animals)</th>
<th>dose</th>
<th>mean % of 24h urinary radioactivity(^1) (range in parentheses) as:-</th>
<th>4-hydroxyphenformin-glucuronide(^2)</th>
<th>unidentified metabolite</th>
<th>unidentified metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dose</td>
<td>phenformin (metabolite 1)</td>
<td>4-hydroxyphenformin (metabolite 2)</td>
<td>glucose (metabolite 3)</td>
<td>glucose (metabolite 4)</td>
</tr>
<tr>
<td>rat (6)</td>
<td>100 mg/kg (oral)</td>
<td>24 (14 - 30)</td>
<td>18 (11 - 27)</td>
<td>53 (40 - 60)</td>
<td>n.d.</td>
</tr>
<tr>
<td>rat (4)</td>
<td>7 mg/kg (oral)</td>
<td>5 (0 - 13)</td>
<td>10 (8 - 12)</td>
<td>78 (73 - 80)</td>
<td>n.d.</td>
</tr>
<tr>
<td>guinea-pig (5)</td>
<td>25 mg/kg (oral)</td>
<td>50 (42 - 58)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>13 (11 - 16)</td>
</tr>
<tr>
<td>rat (6)</td>
<td>100 mg/kg (intraperitoneal)</td>
<td>45 (40 - 52)</td>
<td>16.4 (12 - 20)</td>
<td>33 (28 - 41)</td>
<td>n.d.</td>
</tr>
<tr>
<td>guinea-pig (3)</td>
<td>12.5 mg/kg (intraperitoneal)</td>
<td>55 (51 - 58)</td>
<td>4 (3 - 5)</td>
<td>16 (13 - 20)</td>
<td>7 (6 - 8)</td>
</tr>
</tbody>
</table>

1 - phenformin and 4-hydroxyphenformin determined by t.l.c. counting and reverse isotope dilution; metabolite 4 and 5 determined by t.l.c. counting only.
2 - % glucuronide determined as the increase in aglycone concentration after β-glucuronidase hydrolysis.

n.d. = not detected.
FIG. 2.7 - oral dose

Urines incubated with:
A, β-glucuronidase and saccharolactone
B, β-glucuronidase

FIG. 2.8 - intraperitoneal dose

Urines incubated with:
A, β-glucuronidase and saccharolactone
B, β-glucuronidase

T.l.c.-autoradiography of whole rat 24h urine after administration of [2'-14C]phenformin (100 mg/kg).
FIG. 2.9 - oral dose (25 mg/kg)

Urine incubated with:
A, buffer
B, β-glucuronidase and saccharolactone
C, β-glucuronidase

FIG. 2.10 - intraperitoneal dose (12.5 mg/kg)

Urine incubated with:
A, β-glucuronidase and saccharolactone
B, β-glucuronidase

T.l.c. - autoradiography of whole guinea-pig 24h urine after administration of [2'-14C]phenformin.
FIG. 2.11
Urines incubated with:
A, buffer
B, β-glucuronidase and saccharolactone
C, β-glucuronidase

FIG. 2.12
Urines incubated with:
A, buffer
B, β-glucuronidase and saccharolactone
C, β-glucuronidase

T.l.c.-autoradiography of whole rat 24h urine after oral administration of [2'-14C]phenformin (7 mg/kg).
FIG. 2.17  T.1.c.-autoradiography of metabolites from 24h urine and faeces of guinea-pigs after oral dosing with [2'-14C]phenformin (25 mg/kg).

For explanation of symbols, see overleaf.

FIG. 2.18  T.1.c.-autoradiography of rat bile.
Explanation of symbols in FIGS. 2.17 and 2.18

FIG. 2.17
A, eluted from ion-exchange resin with 4N-sodium hydroxide-ethanol (1:1, by vol.)
B, eluted from ion-exchange resin with 2N-aqueous ammonia
C, eluate B after incubation with β-glucuronidase
D, faecal metabolite isolated by solvent extraction and adsorption chromatography

FIG. 2.18
A, metabolites isolated by ion-exchange chromatography of aqueous residue after solvent extraction.
B, as for A, but including a representative portion of organic solvent extract.
FIG. 2.19 Distribution of radioactivity after t.l.c. of whole 24h urine of rats following a single oral dose or intraperitoneal administration of [2'-14C]phenformin (100 mg/kg)

(a) oral administration

(b) intraperitoneal administration

Results expressed as means of 4 experiments; t.l.c. on silica gel in solvent system D.

1, phenformin; 2, 4-hydroxyphenformin; 3, glucuronic acid conjugate of 2.
FIG. 2.20 Distribution of radioactivity after t.l.c. of whole 24h urine of rats following a single oral administration of [2'-\(^{14}\)C]phenformin (7 mg/kg).

Results expressed as means of 4 experiments; t.l.c. on silica gel in solvent system C.

1, phenformin; 2, 4-hydroxyphenformin; 3, glucuronic acid conjugate of 2.
FIG. 2.21  Distribution of radioactivity after t.l.c. of whole 24h urine of guinea-pigs following a single oral or intraperitoneal administration of \([2'\text{-}^{14}\text{C}]\)phenformin.

(a) oral administration (25 mg/kg)

(b) intraperitoneal administration (12.5 mg/kg)

Results expressed as means of 2-3 experiments; t.l.c. on silica gel in solvent system D.
1, phenformin; 2, 4-hydroxyphenformin; 3, glucuronic acid conjugate of 2; 4, unidentified metabolite; 5, glucuronic acid conjugate of 4.
eliminated some 55% of the urinary radioactivity (40% of the dose) as the parent compound. The urine also contained 4-hydroxyphenformin (4%), 4-hydroxyphenformin glucuronide (16%) and some 7% of a further compound, probably metabolite 4, and its glucuronide (15%); see FIGS. 2.10 and 2.21(b).

(b) Faecal metabolites

Solvent extraction gave approximately quantitative recovery of radioactivity from the 24h faeces of guinea-pigs after an oral dose (25 mg/kg). Ion-exchange chromatography using Zerolit-225 cation resin as described for the extraction of urinary radioactivity was unsatisfactory for separation of the radioactivity in faecal extracts as > 60% of the radioactivity was retained by the resin, even after elution with sodium hydroxide or hydrochloric acid. Column chromatography of the faecal extract on silica-gel eluted with chloroform-methanol mixtures gave > 96% recovery of the radioactivity and subsequent t.l.c. of the radioactive fractions showed the presence of a single radioactive compound (metabolite 6) which did not co-chromatograph with phenformin or 4-hydroxyphenformin, see FIG. 2.17D. This metabolite accounted for a total of approx. 75% of the 24h faecal radioactivity in the guinea-pig and showed similar t.l.c. characteristics to metabolite 4 on silica in solvent systems B, E and F. Attempts to determine the structure of metabolite 6 by mass spectrometry proved unsuccessful but the spectra obtained demonstrated the absence of the positive ions characteristic of the phenolic function of 4-hydroxyphenformin and the absence of the triazine structure typical of the biguanide moiety and shown by phenformin and 4-hydroxyphenformin under these conditions, see APPENDIX.

Phenformin, determined by reverse isotope dilution, accounted for
52 and 71% of the radioactivity in the 24h faeces of two rats dosed at 100 mg/kg orally.

(a) Biliary metabolites

Extraction of the 6h bile of 3 rats at pH 11 with chloroform-methanol (85:15, by vol.) removed 59-72% of the radioactivity which was shown by t.l.c. to be almost exclusively phenformin. The metabolites remaining in the aqueous phase were isolated by ion-exchange chromatography and the combined eluates examined by t.l.c. in solvent systems C and D. A typical t.l.c.-autoradiograph of the biliary ion-exchange eluates alone (A) and co-chromatographed with an appropriate portion of the organic extract in order to represent the distribution of radioactivity in whole bile (B) is shown in FIG. 2.18.

The combined organic and ion-exchange extracts accounted for 96-98% of the biliary radioactivity and contained largely unchanged phenformin (75%), 4-hydroxyphenformin (13%) and 4-hydroxyphenformin glucuronide (8%). Small quantities (4-5%) of other radioactive compounds were also detected.

In vitro metabolism of [2'-14C]phenformin

(a) Metabolism by isolated caecal microflora

Phenformin was not appreciably metabolised by isolated caecal-microflora from rat or guinea-pig. Examination by t.l.c. of organic extracts of the incubation medium after 3 days showed a single radioactive component which co-chromatographed with authentic phenformin and accounted for > 98% of the radioactivity. Reverse isotope dilution analysis for phenformin accounted for > 95% of the radioactivity.

(b) Metabolism by isolated hepatocytes

Phenformin was partially metabolised in vitro by hepatocytes from rat
and guinea-pig; see FIGS. 2.22 and 2.23. After incubation of $1 \times 10^{-3}$M $[2'-^{14}C]$phenformin hydrochloride (0.3μCi) for 90 min some 15% metabolism occurred in the guinea-pig cells and 20% in rat cells; see FIG. 2.24 (a).

In rat hepatocytes metabolism of phenformin produced 5 radioactive-metabolites in measurable quantities ($>0.5\%$ of total radioactivity). Metabolite 2 corresponded chromatographically with 4-hydroxyphenformin and metabolite 3 to its glucuronic acid conjugate. The structures of metabolites 7, 8 and 9 have not been determined. After t.l.c. on silica gel in solvent system C the three unidentified compounds had higher $R_F$ values than phenformin but on alumina in solvent system G they showed $R_F$ values lower than the parent compound. This suggests that the metabolites may be less basic in nature than phenformin. Such a change may result from alteration in or loss of part of the biguanide moiety.

A qualitatively similar metabolic pattern was produced by guinea-pig hepatocytes except that 4-hydroxyphenformin glucuronide was not detected ($<0.6\%$) at any of the time intervals studied; see FIG. 2.24(b).

Quantitatively there were a number of differences in the metabolic patterns produced by the liver cells of the two species; see FIG. 2.24. Initially metabolism was very rapid with metabolites 7-9 being detectable almost immediately on addition of the substrate, accounting for 8-10% of the radioactivity at 0min (i.e. the time taken to add the substrate, mix, remove an aliquot of the incubation and stop the metabolism with acetone). T.l.c. of $[2'-^{14}C]$phenformin in phosphate buffered saline produced a single radioactive zone indicating that the appearance of other compounds at 0min was not due to the presence of other radioactive components in the substrate.
In both species the concentrations of metabolite 7 decreased rapidly; see FIG. 2.24(c) and (d). This decrease may be due to further metabolism or to a rapid removal of the metabolite from the medium through binding to the hepatocytes. The fact that the concentrations of metabolites 8 and 9 changed in such a way that the combined contributions of the three unidentified metabolites to the total radioactivity remained fairly constant (8-10% in the rat, 11-13% in the guinea-pig) may be an indication that metabolite 7 was gradually converted to metabolites 8 and 9; see FIG. 2.24 (c) and (d).

The guinea-pig hepatocytes produced very little 4-hydroxyphenformin (1% at 90 min) and no significant amount of its glucuronide while in the rat these compounds accounted for 10% of the radioactivity by 90 min; see FIG. 2.24(b). The increase in the concentration of these two metabolites corresponded fairly well with the decrease in phenformin levels in both species; see FIG. 2.24 (a) and (b).

The results suggest, therefore, that two discrete metabolic sequences may have occurred. Initially a very rapid formation of metabolite 7 was observed, followed by the more gradual appearance of metabolites 8 and 9 which may have been formed from metabolite 7. 4-Hydroxyphenformin was produced more slowly and to a significant extent only in rat hepatocytes, and was probably formed from [2'-14C]phenformin rather than from metabolites 7, 8 and 9.

These latter three metabolites did not correspond chromatographically with any of the radioactive metabolites in urine and faeces of rat and guinea-pig after oral or intraperitoneal administration of [2'-14C]-phenformin.
T.l.c.-autoradiography of hepatocyte incubation medium various times after addition of [2'-14C]phenformin (10^{-3}M).
FIG. 2.24 Metabolism of [2'-14C]phenformin by rat and guinea-pig hepatocytes.

Results expressed as average of two determinations. Phenformin concentration 1 x 10^3 M. Open symbols represent values from guinea-pig, closed symbols represent values from rat.

○, ●, phenformin; □, ■, 4-hydroxyphenformin; ▲, 4-hydroxyphenformin glucuronide; ○, ●, metabolite 7; ▼, ▼, metabolite 8; ○, ●, metabolite 9.
2.3 DISCUSSION

In both rat and guinea-pig the radioactivity was rapidly cleared from the blood during the first 10 min after intravenous administration of [2\(^1\text{C}\)]phenformin hydrochloride (10 mg/kg); see FIG. 2.2(a). Phenformin concentrations measured in guinea-pig plasma were very similar to those calculated from the radioactivity in whole blood (see TABLE 2.1) indicating that very little metabolism or plasma protein binding occurred in the first 10 min after systemic administration. A semilogarithmic plot of the combined results for the guinea-pig showed that phenformin removal from blood occurred in two distinct phases; see FIG. 2.2(b). The second phase followed first-order kinetics.

An initial rapid fall in plasma levels of the drug is typical of intravascularly administered foreign compounds (Goldstein et al., 1968) but the rate of decrease appears to be particularly rapid with phenformin. After intravenous administration of [\(^{1}\text{C}\)]buformin (1.2 mg/kg) in man the serum concentrations of radioactivity showed an initial disappearance with a half-life of some 75 min (Beckmann, 1968). In comparison the half-life of the first phase in FIG. 2.2(b) is less than 2 min. Extrapolation of the linear portion of the curve in FIG. 2.2(b) to the ordinate gave an initial serum concentration for this portion (C\(_0\)) of 0.84 \(\mu\)g/ml and a half-life of 120 min. The rate of decrease was slow with an elimination rate constant (K\(_e\)) of approximately 0.6\% per min. The apparent volume of distribution (V\(_d\)) was 11.9 l/kg indicating that with phenformin the parameter does not represent a real anatomical volume and that the drug was extensively localised outside the plasma. After intravenous administration of [\(^{1}\text{C}\)]buformin (1.2 mg/kg) in man the serum concentrations of radioactivity in the linear portion of the curve decreased at about half
the rate shown for phenformin in FIG. 2.2(b), with a half-life of 230 min 
\(K_e = 0.3\% \text{ per min}\) and an initial concentration of 0.42 \(\mu\text{g/ml}\) (Beckmann, 1968). These figures give a value for \(V_d\) for buformin of 2.9 1/kg in man indicating that this biguanide is also concentrated extravascularly.

The first portion of the curve in FIG. 2.2(a) and (b) probably represents distribution of phenformin in the tissues. This is supported by the high value of \(V_d\) obtained for the linear section of the graph. Hall et al. (1968) have reported that large proportions of an intraperitoneal dose of \([^{14}\text{C}-\text{biguanide}]\text{phenformin}\) to rats (100 mg/kg) and guinea-pigs (15 mg/kg) are associated with the liver (20-30\%) and intestines (15-20\%) for 3-4h after administration, and smaller amounts (2-7\%) are detected in the stomach. This rapid tissue localisation of parenterally administered \([^{14}\text{C}]\text{phenformin}\) may explain why blood levels of radioactivity after intraperitoneal injection of \([2^-{14}\text{C}]\text{phenformin}\) (15-25 mg/kg) did not reach the limit of detection (equivalent to 0.8 \(\mu\text{g phenformin/ml}\) blood). The high levels in the liver indicate that the drug is rapidly absorbed into the portal system and retained by hepatic tissue, presumably for gradual metabolism and release into bile and blood. According to Hall et al. (1968) most of the radioactivity associated with rat liver and about 60\% of that associated with guinea-pig liver is accounted for by metabolic products.

The presence of radioactivity in the stomach and intestines 30-60 min after parenteral administration (Wick et al., 1960; Hall et al., 1968) suggests that the biguanide is secreted into the stomach and perhaps the gut. This process usually displays first-order kinetics (Goldstein et al., 1968) and is favoured by the basic nature of the drug. Phenformin is a di-acidic base with a strongly basic primary dissociation constant \((pK_b = 11.8)\) and a much weaker secondary dissociation constant \((pK_a = 2.7)\)
(Ray, 1961). The drug exists in the plasma and gut (effective pH 5.3; Schanker, 1971) almost exclusively in the protonated form as an internal, 6-membered, hydrogen-bonded ring (Shapiro, 1959); see FIG. 2.25. Although this structure is fairly polar it is readily absorbed through the various tissue membranes as the drug is effective after both oral and intravenous administration. In the stomach (pH 1.4; Cohn, 1971) phenformin exists largely as the di-protonated species which is probably less readily reabsorbed into the plasma. Assuming that membrane barriers are relatively impermeable to the di-protonated phenformin molecule the stomach juices may concentrate large amounts of the drug from the plasma, although the values in FIG. 2.25 represent a static equilibrium and should only be regarded as indications of the values existing under dynamic equilibrium conditions. Wick et al. (1960) have shown that the radioactivity in rat stomachs 30-60 min after intraperitoneal administration of \([2^14\text{C}]\)phenformin is chromatographically identifiable as the parent compound and is associated with the gastric juices rather than bound to the tissue. The gastric secretion of a number of parenterally administered basic foreign compounds such as aniline, aminopyrine and mecamylamine in the rat (Shore et al., 1957) and nicotine in rat and mouse (Andersson et al., 1965) has been reported.

The rapid clearance of radioactivity from the circulation may also be due in part to renal excretion. The renal clearance of phenformin in man is 567 ml/min (Sterling Winthrop Research Laboratories, personal communication, 1977) which is considerably greater than the average glomerular filtration rate of 120 ml/min (Harper, 1965) and indicates that the drug may be actively secreted in the kidney, probably in the proximal tubule (Cafruny, 1971). Butormin is actively secreted by the kidneys in normal (Beckmann, 1968) and diabetic (Lintz et al., 1974) human volunteers. Active tubular secretion of ionised foreign compounds is extremely rapid.
FIG. 2.25 The pH dependent distribution of phenformin between plasma, gut and stomach.

<table>
<thead>
<tr>
<th>intestinal epithelium</th>
<th>plasma</th>
<th>gastric juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.3</td>
<td>pH 7.4</td>
<td>pH 1.4</td>
</tr>
<tr>
<td>P</td>
<td>(3 x 10^{-7})</td>
<td>(4.0 x 10^{-11})</td>
</tr>
<tr>
<td>( pK_b ) 11.8</td>
<td>(4.0 x 10^{-5}) (P)</td>
<td>( pK_b ) 11.8</td>
</tr>
<tr>
<td>( pK_a ) 2.7</td>
<td>(PH^+)</td>
<td>(PH^+)</td>
</tr>
<tr>
<td>( \text{PH}^+ ) 1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>( \text{PH}_2^+ ) 2.5 x 10^{-3}</td>
<td>2.0 x 10^{-5} (PH^2+)</td>
<td>20.0</td>
</tr>
<tr>
<td>Total = 1.0</td>
<td>Total = 1.0</td>
<td>Total = 21.0</td>
</tr>
</tbody>
</table>

Structures of protonated species as described by Shapiro et al. (1959a) \( R = C_{6\,5}\text{H}_2\text{CH}_2\text{CH}_2^- \). Relative concentrations based on the assumption that the compartments are separated by a barrier that is not readily permeable to the di-protonated form of the molecule.
Chasseaud, 1970) and this may contribute to the precipitous fall in blood concentrations of phenformin.

The slower rate of decline ($K_e = 0.6\%$/min) in guinea-pig plasma levels of unchanged phenformin in the linear phase of FIG. 2.2(b) may correspond to the rate of metabolism of the drug and excretion of the metabolic products after distribution equilibrium has been established.

In the rat after oral administration of [2'-¹⁴C]phenformin the pattern of excretion and metabolism were different after low and high doses. After a 7 mg/kg dose about 88% of the dose was eliminated within 24h, compared with 63% after 100 mg/kg. Excretion in the 24h-48h period was more significant after the high dose than after the low dose (see TABLE 2.2) and was predominantly in the faeces. These findings differ considerably from those obtained by Wick et al. (1960), Beckmann (1967, 1968) and Murphy and Wick (1968); see TABLE 1.4. Faecal excretion of radioactivity was approximately equal (14-16% of the dose) in the first two 24h periods after a 100 mg/kg dose while after 7 mg/kg doses most of the faecal radioactivity appeared in the first 24h. The presence of radioactivity in the faeces may not have been due entirely to unabsorbed [2'-¹⁴C]phenformin as only 50-70% of the faecal radioactivity was accounted for as unchanged drug. Rat caecal microflora did not metabolise [2'-¹⁴C]phenformin under conditions which result in the metabolism of a number of foreign compounds (Scheline, 1966) but it is still possible that metabolism of the drug by the intestinal wall could account for the presence of metabolites in the faeces. However, if the biguanide was not readily absorbed from the gut the proportion of radioactivity excreted in the 24h faeces would be greater after a 100 mg/kg dose than after a 7 mg/kg dose. The equal proportion of the dose in the 24h and 48h faeces after administration of [2'-¹⁴C]phenformin (100 mg/kg) may be an indication that the radioactivity
was being eliminated in the bile and that the process was saturated and was therefore rate limiting.

Relatively little is known about the biliary excretion of bases but studies with some quaternary ammonium compounds have shown that these substances are excreted in the bile in large amounts. Such molecules usually contain one quaternary nitrogen (Smith, 1966; Hughes et al., 1973). The quaternary ammonium structure may be important in this respect as the biliary excretion of the tertiary analogue of benzomethamine is negligible whilst quaternary benzomethamine itself is extensively eliminated in the bile by man, rat and rabbit (Levine and Clark, 1955). Certain organic cations appear to be excreted into the bile by active processes which are different from those for anions. Procaineamide ethobromide was rapidly secreted into the bile of rats with bile to plasma concentration ratios of about 80, 1h after dosing (Schanker and Solomon, 1963). The transfer mechanism could be saturated by large doses of the drug and depressed by administration of other cations which were eliminated in the bile, but was unaffected after administration of anions. After \([2'-^{14}C]\)phenformin administration intraduodenally (20 mg/kg) more than 27% of the dose in rats was excreted in the bile in 6h while in the guinea-pig only about 6% was excreted; see FIG. 2.6. In the rat the rate of biliary excretion was essentially constant and the mean concentration of radioactivity for the 6h period was equivalent to approximately 250 \(\mu g\) phenformin/ml bile. This is very much greater than the expected blood concentration since intravenous administration of \([2'-^{14}C]\)phenformin (10 mg/kg) gave a blood level of radioactivity after the first 10 min equivalent to less than 1 \(\mu g\) phenformin/ml; see FIG. 2.2(a). It seems likely, therefore, that the rat actively eliminates phenformin and probably its metabolites in the bile. Pooled
0-6h rat bile contained mainly unchanged phenformin (75%) as well as its metabolites 4-hydroxyphenformin (13%) and 4-hydroxyphenformin glucuronide (8%) all of which contain a quaternary nitrogen moiety at physiological pH. The glucuronide conjugate probably exists as the zwitterion (1) as the pK values of the acid portion of most glucuronides are in the range 3-4 and are therefore ionised at physiological pH (Smith and Williams, 1966). The other two biguanides probably exist as the hydrogen bonded structures (2) described earlier. These three compounds may be actively secreted by the mechanism described by Schanker and Solomon (1963) for hepatic transport of cations in the rat. All three compounds have molecular weights greater than the threshold value (200 + 50) for appreciable biliary excretion of monoquaternary organic cations in rat and guinea-pig (Hughes et al., 1973). The pattern of biliary

\[
\text{4-hydroxyphenformin glucuronide} \\
\text{mol. wt. 397}
\]

\[
\text{(4-hydroxy)phenformin} \\
\text{mol. wt. 206 (222)}
\]
elimination of cations appears to be in contrast to the excretion of organic anions which shows a species dependent biliary threshold for molecular weights, being about 325 ± 50 in rat and 400 ± 50 in guinea-pig (Abou-el-Makarem et al., 1967b; Millburn et al., 1967; Hirom et al., 1972). The existence of an active transport mechanism for biliary elimination of phenformin and its metabolites could explain the linear rate of biliary excretion of radioactivity in the rat over the 6h period studied.

Although a significant portion of the biliary radioactivity in rats was undoubtedly reabsorbed in the gut (otherwise 100% of a 20 mg/kg dose would have been excreted in the faeces) no enterohepatic circulation was demonstrated. In view of the large proportion of the dose excreted in the 48h faeces after oral and intraperitoneal administration of [2'-1H]phenformin (100 mg/kg), it is more likely that the failure to demonstrate recycling was due to the limitations of the techniques employed. Absorption of basic biliary radioactive components may have been impaired in animals anaesthetised with pentobarbitone. Phenobarbitone has been shown to stimulate bile acid synthesis and bile-salt secretion (Redinger and Small, 1973) and if such an effect was produced by sodium pentobarbitone the absorption of basic drugs might be hindered. Lack of food in the gut, such as would result from the inhibition of gastric-emptying by anaesthesia also increases the flow of bile salts into the intestines. Even though the membrane boundary between the blood and bile is extremely porous (Parke, 1968) it is doubtful that the intestinal epithelium would be permeable to the polar metabolites of phenformin and only the parent compound is likely to be reabsorbed from the gut.

In addition to enterohepatic circulation the secretion of phenformin from the plasma into the stomach could contribute to the delayed excretion
of the drug. As the biguanide passes from the stomach to the intestines it may be reabsorbed into the plasma and re-excreted either into the stomach or in the bile.

The reason for the profound species difference in the biliary excretion of phenformin is not known. Hughes et al. (1973) found no significant difference in the excretion of a range of monoquaternary ammonium cations in the bile of rat and guinea-pig; all were extensively excreted provided the molecular weights exceeded 200 ± 50. The rate of biliary excretion of radioactivity in the guinea-pig was not constant. Initially the rate of excretion was high, with some 4% of the dose being excreted in the first hour. During this time the mean concentration of radioactivity was equivalent to some 150 μg phenformin/ml bile. After the first hour the rate of elimination fell such that only about 2% of the dose was excreted in the next 5h; see FIG. 2.6. During this period the mean concentration of radioactivity was equivalent to less than 20 μg phenformin/ml bile. A possible explanation for this variable rate of excretion may lie with the particular sensitivity of the guinea-pig to the pharmacological actions of phenformin (see CHAPTER 3). It may be that at this dose of the drug mitochondrial respiration in guinea-pig liver was depressed sufficiently to lower the availability of ATP (see SECTION 1.4) and impair the active biliary excretion of phenformin and its metabolites.

In view of the low degree of biliary elimination of radioactivity in the guinea-pig it is difficult to explain how a large proportion of the dose was excreted in the faeces after 24h (20% of the dose) and 48h (14% of the dose); see TABLE 2.2. About 75% of the 24h faecal radioactivity (15% of the dose) was due to an unidentified compound (metabolite 6) which was probably more polar than phenformin (see FIG. 2.17 D) and as [2'-14C] phenformin was not metabolised on incubation with guinea-pig caecal micro-
flora under conditions which metabolise a variety of foreign compounds (Scheline, 1966) it would seem that, unless the intestinal epithelium is capable of extensively metabolising the drug, unabsorbed $[2'{}^{-14}C]$-phenformin does not contribute significantly to the presence of radioactivity in guinea-pig faeces. This suggests that most of the radioactivity was derived from biliary excretion and/or gastric secretion. As the metabolic pattern in guinea-pig bile was not determined it is not possible to say whether the faecal metabolite originated in the bile or stomach juices, but in view of the well-established stability of the biguanides in acid solution (Shapiro, 1959; Wick et al., 1960; Cohen and Costerousse, 1961; Beckmann, 1966, 1967) the metabolite is more likely to be of hepatic origin. The discrepancy between the rate of biliary excretion of radioactivity and its appearance in the faeces may only be apparent. It has already been suggested that active biliary secretion of phenformin and/or its metabolites in the guinea-pig may have been impaired by the drug. The depletion of ATP levels by phenformin is related to its hypoglycaemic and hyperlactataemic actions (see SECTION 1.4), both of which are temporary effects (see CHAPTER 3). If the pharmacological effects diminished at some time after the 6h period of bile collection, the active elimination of phenformin and/or its metabolites may have resumed and resulted in higher faecal levels of radioactivity.

A difference in the urinary ratio of phenformin to metabolic products occurred in the rat after different oral doses of $[2'{}^{-14}C]$-phenformin; see FIGS. 2.19(a) and 2.20 and TABLE 2.3. After oral administration of 7 mg/kg an average of only 5% of the urinary radioactivity (2.5% of the dose) was excreted in the urine as unchanged phenformin, the remainder being accounted for as 4-hydroxyphenformin and its glucuronic acid conjugate. In some rats receiving this low dose no
parent compound was found in the urine; see FIG. 2.12. After a high
dose (100 mg/kg) almost a quarter of the radioactivity in the urine
(12% of the dose) was unchanged phenformin. These findings are in
contrast to earlier reports which have found that after 50 mg/kg
$[^{3}H]$phenformin (Beckmann, 1967, 1968) or 100 mg/kg $[^{2'}-^{14}C]$phenformin
(Murphy and Wick, 1968) only 4-hydroxyphenformin and its glucuronide
were present in rat urine.

The liver is probably the major site of metabolism of phenformin
in the rat (Hall et al., 1968) and Wick et al. (1970) have shown that
rat liver cytochrome P-450 is involved in the aromatic hydroxylation
of the drug. The intestinal wall may also contribute to the metabolism
of the biguanide, particularly in view of the high concentrations which
arise after oral and intraperitoneal dosing.

Drugs given orally or intraperitoneally (assuming no lymphatic
absorption) gain access to the peripheral venous circulation almost
exclusively by way of the hepatic portal system (Gibaldi and Perrier,1975).
Extensive metabolism and elimination of a drug may occur in the intestinal
wall and liver during the first passage of the compound into the circulation;
this is generally referred to as the 'first-pass effect'. First-pass
extraction and metabolism of foreign compounds can occur in the liver,
as with lidocaine (Boyes et al., 1970; Gugler et al., 1975) or in the
intestinal wall, as with isoproteronol (Dolley et al., 1971). Both
tissues have been implicated in the metabolism of acetylsalicylic acid
(Harris and Riegelman, 1969). Grundin et al. (1974) have suggested that
hepatic cytochrome P-450 may be, at least partly, responsible for the
marked first-pass extraction and metabolism of alprenolol in rats.

The presence of unmetabolised phenformin in rat urine after a large
oral dose of $[2'-^{14}C]$phenformin (100 mg/kg) may reflect the limited capacity of the first-pass extraction process in gut and/or liver. The extraction of propranolol was shown to be saturable (Evans and Shand, 1973). This explanation is supported by the results obtained after intraperitoneal administration of $[2'-^{14}C]$phenformin (100 mg/kg); see TABLES 2.2 and 2.3. Although a greater percentage of the administered radioactivity was eliminated in the 24h urine after the parenteral dose than after a similar oral dose, the increase was accounted for as unchanged drug rather than as metabolic products; see FIG. 2.26. This could be the result of a more rapid absorption of the drug into the portal blood supply following intraperitoneal injection and saturation of the hepatic extraction capacity for the drug with the result that a higher proportion of the dose is eliminated unchanged by the kidneys.

Significant first-pass metabolism reduces the fraction of the dose ultimately excreted in the urine as unchanged drug (Gibaldi and Perrier, 1975) and reduces the availability of the compound in the peripheral tissues. In cases where metabolism results in loss of biological activity first-pass elimination may significantly reduce the pharmacological effectiveness of a drug. Saturation of the extraction process may lead to dose-dependent disposition kinetics of drugs (Grundin et al., 1974). The implications of this effect in relation to the hyperlactataemic and hypoglycaemic actions of phenformin and 4-hydroxyphenformin are discussed in CHAPTER 3.

Active biliary excretion may be an important route of elimination of phenformin in the rat but if this process is saturated after administration of large enteral or parenteral doses of the biguanides, renal excretion of the parent compound could be increased. Urinary and
biliary pathways of excretion may be complementary to each other in the elimination of a number of anionic foreign organic compounds and when one of these routes is blocked the other may be increased (Hirom et al., 1976). This interrelationship between the two excretory mechanisms may also occur for cations such as phenformin and in circumstances where one of the routes is saturated rather than blocked. The relatively slower rate (on a % dose basis) of metabolism and elimination in the rat of high doses of phenformin may have important implications in the pharmacological behaviour of the drug (see also CHAPTER 3).

In the guinea-pig excretion of a 25 mg/kg oral dose of [2'-14C]-
phenformin was incomplete even after 6 days; see TABLE 2.2. The 96h cumulative excretion in this species (86% of the dose) was significantly lower (p < 0.01) than in rats after 7 or 100 mg/kg doses. In one guinea-pig studied for 144h after dosing the total recovery of radioactivity was only 92%. The presence of significant amounts of the dose in the faeces at 144h suggests that extensive recycling of radioactivity occurred in the guinea-pig, despite the apparently low degree of biliary excretion. This possibility has been discussed earlier.

Guinea-pig urine 24h after oral administration of [2'-14C]phenformin (25 mg/kg) contained about 37% of the dose, half of which was identified as unchanged phenformin. 4-Hydroxyphenformin and its glucuronic acid conjugate were not detected. The metabolite 4 which, along with its glucuronide (metabolite 5) accounted for the remaining radioactivity in the 24h urine was not identified. Mass spectrometric analysis indicated that the metabolite was not a phenolic compound. The hydrolysis of metabolite 5 by β-glucuronidase was inhibited by saccharic acid 1,4-lactone and the conjugate was stable to acid and alkali, indicating that it was an O-glucosiduronic acid rather than an N-glucosiduronic acid conjugate (Fishman, 1961). The aglycone (metabolite 4), therefore, may have been hydroxylated on the aliphatic side chain, either α- or β- to N1 of the biguanide moiety. The absence of evidence for the s-triazine grouping in the mass spectrum suggests that the biguanide portion of the molecule may be incomplete or structurally altered. Beckmann (1967) has reported that the guinea-pig excreted only traces of metabolic products, which were identified as 4-hydroxyphenformin and its glucuronide conjugate, and that the urine contained almost exclusively unchanged phenformin after oral administration of [3H]phenformin (50 mg/kg). This finding was based on the result from a single animal and identification relied solely on comparison
of \( R_F \) values. In addition the dose used was greater than the oral \( LD_{50} \) for phenformin in the guinea-pig (38 mg/kg; Ungar et al., 1957; see TABLE 1.2). Small amounts of 4-hydroxyphenformin, free and conjugated with glucuronic acid, were present in guinea-pig urine after intraperitoneal administration of \([2'-^{14}C]\)phenformin (12.5 mg/kg); see TABLE 2.3.

The hepatocytes from rat and guinea-pig also produced different metabolic patterns after incubation with \([2'-^{14}C]\)phenformin (10\(^{-3}\)M). Both species rapidly formed three unidentified compounds (metabolites 7, 8 and 9) which were not present in urine after in vivo administration. Some 7-9% of the substrate was converted to metabolite 7 within the short space of time required to stop the reaction. This time varies with liver cells from different species according to their fragility, but is probably in the order of 2 minutes (Jones, 1978). Rat hepatocytes produced 4-hydroxyphenformin, free and conjugated with glucuronic acid (10% in 90 min) while guinea-pig hepatocytes produced only a trace amount of the phenol (1% in 90 min) and no detectable conjugate; see FIGS. 2.23 and 2.24.

Differences in the metabolic fate of phenformin in the rat and guinea-pig are not totally unexpected. Although aromatic hydroxylation occurs in most species, there are "considerable species variations in the extent to which it occurs in a given compound and also in the extent to which it occurs with different compounds in the same species" (Williams, 1971). This is illustrated by the metabolism in different species of amphetamine, a molecule with some structural similarities to phenformin. Aromatic hydroxylation is the major route of metabolism in rats but a minor one in guinea-pigs, rabbits and in man. Deamination and subsequent oxidation giving benzoic acid and its conjugates is the major route in
guinea-pigs and a significant route in rabbits and in man, but not in rats. Rabbit urine also contains acetophenone as the sulphate conjugate of the enol form of the molecule and some 1-phenylpropan-2-ol (Dring et al., 1966, 1970); see FIG. 2.27. Similarly, after administration of methamphetamine (N-methylamphetamine) the rat excretes mainly products of aromatic hydroxylation while the guinea-pig excretes large amounts of benzoic acid and its conjugates, but no products of aromatic hydroxylation (Caldwell et al., 1972a).

FIG. 2.27 Metabolism of amphetamine (Dring et al., 1966, 1970)

Phenethylamine derivatives are hydroxylated on the methylene group adjacent to the aromatic ring as an important stage in the anabolism of
biologically active amines, such as the transformation of dopamine to noradrenaline. Some exogenous substrates also undergo β-hydroxylation to a limited extent. Dopamine-β-hydroxylase is believed to catalyse hydroxylation of both endogenous and exogenous substrates (Testa and Jenner, 1976). An example of β-hydroxylation has also been reported with the anticonvulsant drug N-2-[5-(m-chlorophenyl)1,2,4-oxadiazol-3-yl]-ethyl-N-methylacetamide (3) which contains an N-substituted ethylamino side chain adjacent to a heterocyclic ring. In man the secondary alcohol (4) is one of the major metabolites (Allen et al., 1971). As the molecular structure of (3) differs greatly from that of the endogenous phenethylamines it is doubtful whether this drug acts as a substrate for dopamine-β-hydroxylase (Testa and Jenner, 1976).

\[
\begin{align*}
\text{CH}_2\text{-CH}_2\text{N} & \quad \text{CH}_3 \\
\text{Cl} & \quad \text{COCH}_3 \\
\end{align*}
\]

(3)

\[
\begin{align*}
\text{CH}_2\text{-CH}_2\text{-NH} & \quad \text{OH} \\
\text{Cl} & \quad \text{COCH}_3 \\
\end{align*}
\]

(4)

Amphetamine in man and rat (Dring et al., 1970; Caldwell et al., 1972b) and methamphetamine in man, rat and guinea-pig (Caldwell et al., 1972a) are partly metabolised by β-hydroxylation. In man the products in each case are norephedrine (5) and 4-hydroxynorephedrine (6) which total only some 2-4% of the dose. In rats the major product from methamphetamine (16% of a 45 mg/kg oral dose) is 4-hydroxynorephedrine while the guinea-pig excretes only norephedrine. In the guinea-pig the β-hydroxylation reaction appears to be dose dependent with norephedrine accounting for 1% of a 10 mg/kg dose and 19% of a 45 mg/kg dose of
Deamination of a number of phenethylamine derivatives occurs readily in various species and proceeds via cleavage of the C–N bond. Thus mescaline (3,4,5-trimethoxyphenethylamine) is metabolised in rat and man to give both benzoic acid and phenylethanol derivatives (Schreiber, 1970). In a comparative study of the aromatic oxidation and deamination of a series of amphetamine-related compounds in several species, it was shown that the rat demonstrated marked aromatic oxidation while the guinea-pig displayed efficient deamination (Williams et al., 1973).

In the main the guanidine and biguanide groupings appear relatively inert to most metabolic processes. No metabolic changes in the biguanide moiety of phenformin, buformin or metformin have been reported previously, although on heating with alkali phenformin is rapidly degraded to give phenethylguanidine, phenethylurea and phenethylamine (Shapiro, 1959). The guanidine grouping of Guanethidine (7) is unchanged in man while N-oxidation of the alicyclic nitrogen occurs to a significant extent (McMartin and Simpson, 1971) and the diuretic amiloride hydrochloride (8) and carcino-chemotherapeutic agent methylglyoxal-bis-guanylhydrazone (9) are not biotransformed to a significant extent in several species.
(Chasseaud, 1970). In contrast the guanidino-group of Guanoxan (10)

\[
\begin{align*}
\text{NH} & \quad \text{N-CH}_2\text{CH}_2\text{NH-C-NH}_2 \\
\end{align*}
\]

undergoes transamidination in dogs in the presence of ornithine and glycine to give arginine and guanidino-acetic acid (Cañas-Rodriguez, 1966).
CHAPTER 3

STUDIES ON PHENFORMIN-ASSOCIATED LACTIC ACIDOSIS
3.1 MATERIALS AND METHODS

Chemicals

Lithium lactate, lactate dehydrogenase, NAD⁺ and thiamine pyrophosphate were obtained from Sigma (London) Chemical Co. Ltd. Guaiacum, peroxidase and glucose oxidase (Fermcozyme 653AM) reagents were purchased from Hughes and Hughes (Enzymes) Ltd., Romford, Essex. Dichloroacetic acid (reagent grade) was supplied by Fisons, Loughborough, Leics. and was converted to the sodium salt by neutralizing the aqueous solution with sodium hydroxide and freeze-drying at 30°C. The solid was pulverised and stored in a dark glass bottle until used. Phenformin hydrochloride, 4-hydroxyphenformin dihydrochloride and chlorpropamide were kindly donated by Sterling Winthrop Research Laboratories, Fawdon, Newcastle-upon-Tyne. Sodium pentobarbitone anaesthetic solution was obtained from Abbot Laboratories, Queenborough, Kent (Nembutal) or May and Baker Ltd., Dagenham, Essex (Sagattal). Urethane anaesthetic solution was prepared as described in CHAPTER 2 from the reagent grade material obtained from British Drug Houses Ltd., Poole, Dorset. All other chemicals were purchased from common laboratory suppliers and, unless otherwise specified, were of reagent grade.

Materials

Cannula tubing was supplied by Portex Ltd., Hythe, Kent. Neutral glass collection tubes (2ml capacity) were obtained from Glass Wholesale Supplies Ltd., London and 1ml capacity auto-analyser cups prefilled with fluoride-oxalate with concave base (F07/C) from Stayne Laboratories Ltd., Marlow, Bucks. Butterfly infusion catheters (short-25 pediatric infusion set, 0.5mm ext. diameter, length 9.6mm; 25-G thin wall siliconed
needle) were supplied by Abbot Laboratories, Queenborough, Kent.

Collection of blood

Male Wistar albino rats (200g-300g) and Dunkin-Hartley albino guinea-pigs (280g-400g) were anaesthetised as described in CHAPTER 2.

Rat blood samples were collected at 0.5h intervals from the tail vein which was exposed by removing the tip of the tail using a scalpel. The end of the tail was cleaned to prevent surface contamination of the blood and the first sample discarded as tissue damage and venous stasis may cause an elevation of blood lactate levels. Clotting of the tail vein between collection periods was encouraged by wrapping the exposed tip of the tail in cotton wool.

Guinea-pig blood samples were collected by cardiac puncture at 1.0h intervals, using heparinised syringes and needles.

All blood samples were transferred to fluoride-oxalate treated autoanalyser cups containing heparin, mixed and appropriate aliquots dispensed for assay of lactic acid and glucose.

Determination of lactate

Lactate concentrations were determined by the method of Höhorst (see Henry et al., 1974) modified for small blood volumes.

Perchloric acid (7%, W/v, 0.2ml) was transferred to a neutral glass 2ml test tube and blood or standard (0.2ml) added and mixed thoroughly. After standing for 5-10 min the tube was centrifuged and 0.1ml supernatant dispensed into a similar 2ml tube containing 1ml buffer (0.4M-hydrazine sulphate and 0.5M-glycine adjusted to pH 9.0 with 5N-NaOH), 0.1ml NAD+ solution (20mg/ml in water) and 0.02 ml lactate dehydrogenase (400U/ml).
Reagent blanks containing 0.1ml 3.5% (W/v) perchloric acid and lactic acid standards (10 and 20mg/100ml 2.5 x 10^{-3}M sulphuric acid) were prepared. Blood samples which were expected to have a value greater than 35 mg/100ml were diluted with water before assay.

The tubes were incubated for 1h at 25° and the absorbance measured at 340nm in 1ml quartz cuvettes (1cm light path).

**Determination of glucose**

Blood glucose concentrations were determined by the glucose oxidase method of Marks *et al.* (1968) using 0.1 volumes of heparinised blood. This method permits determination of glucose over the range 0-200mg/100ml blood.

**Analysis of data**

Significant differences between the blood lactate or glucose concentrations in the different groups were determined using a modified form of Student's t-test for small samples (Bailey, 1959). Where variances in the populations were shown by a variance-ratio test to be statistically unequal, a second modified form of t-test was used (Bailey, 1959).

3.2 **RESULTS**

(a) **Studies in the non-fasted rat**

*Preliminary studies with phenformin*

Anaesthetised rats were given phenformin hydrochloride intraperitoneally and the lactate and glucose levels in the blood measured at 0.5h intervals up to 4 or 5h. The maximum effect upon blood lactate and glucose concentrations occurred at approx. 2.5h after administration (see FIG. 3.1).
FIG. 3.1 Blood lactate and glucose concentrations in the rat following a single intraperitoneal administration of phenformin.

Points represent the means of 3-9 experiments ± S.E.M. represented by vertical bars. Phenformin hydrochloride administered in saline (0.5 ml)

\[ \n\begin{align*}
\n\uparrow & \quad 75 \text{ mg/kg}; \\
\square & \quad 120 \text{ mg/kg}; \\
\bigcirc & \quad \text{saline alone.}
\end{align*}
\n\]
After a single dose of phenformin (75mg/kg) lactate levels were increased from a resting level of 12mg/100ml (1.3mM) to 17mg/100ml (1.9mM) at 2.5h and glucose levels were decreased from some 100mg/100ml (5.6mM) to 70mg/100ml (3.9mM). After a single dose of 120mg phenformin/kg blood lactate concentrations reached 24mg/100ml (2.7mM) at 2.5h while glucose levels fell to 65mg/100ml (3.6mM). Thus while the hyperlactataemic responses were significantly different at the two dose levels (p<0.001 from 1.0h) the hypoglycaemic effects were indistinguishable (p>0.1 at all time intervals).

Dose-response studies with phenformin

The dependence of the hyperlactataemic and hypoglycaemic responses on the dose of phenformin in the rat was determined in groups of 3 animals given 50, 75, 90, 105, 120, 135 or 150mg/kg intraperitoneally. The experiment was performed over a period of 3 days and the doses were randomised for each day. Saline injected control animals were examined at the beginning and end of each day to allow for any changes in the resting levels of lactate and glucose.

Blood samples were taken immediately before and 2.5h after dosing and the lactate and glucose concentrations determined. A larger sample of blood (2.0-3.0ml) was removed from each animal by cardiac puncture and the plasma sample analysed for phenformin by g.l.c. at Sterling Winthrop Research Laboratories, Fawdon.

The mean percentage change in lactate and glucose concentrations for each dose and the corresponding mean plasma phenformin level are shown in FIG. 3.2. The values obtained were subjected to computer analysis in order to calculate the nature of the best curve-fit.
FIG. 3.2 The effect of increasing dose of phenformin on blood lactate and glucose and plasma phenformin levels in the rat at 2.5h.

Points represent the means of 3 experiments ± S.E.M. represented by vertical bars; blood lactate and glucose concentrations expressed as mean % change from individual resting levels. Phenformin hydrochloride administered intraperitoneally in saline (0.5 ml).
The blood concentration of lactic acid in the rat did not increase dramatically with doses of 50-105mg/kg. Lactate levels after a 50mg/kg dose of phenformin were not significantly different from those in the control animals, but after doses of 75, 90 and 105mg/kg small but significant increases of up to 40% were measured (p<0.05-p<0.001 compared to controls). At doses of 120-150mg/kg much greater rises in lactate concentrations occurred, ranging from 100% at 120mg/kg to 225% at 150mg/kg.

The hypoglycaemic response followed a similar pattern although it should be noted that a dose of 50mg/kg produced a 20% decrease in blood glucose levels without producing hyperlactataemia. Glucose concentrations after doses of 75-120mg/kg showed a fairly uniform reduction to 65-70%, while 135 and 150mg/kg produced a sharp fall to only some 40% of resting levels.

These findings suggest that the hyperlactataemic and hypoglycaemic responses in the rat to increasing doses of phenformin may exhibit a biphasic nature with a critical dose level in the region of 120-135mg/kg.

The effects of dichloroacetate

The effects of dichloroacetate on the hyperlactataemic and hypoglycaemic actions of phenformin in the rat were studied. Rats were anaesthetised throughout the experiment and the right femoral vein was catheterised using a butterfly infusion catheter. Sodium dichloroacetate (50mg/ml isotonicsaline, pH7.4) was administered intravenously as boluses at 10 min intervals (equivalent to 300mg/kg/h) commencing 1h before administration of phenformin (120mg/kg) or saline (control animals). Blood lactate and glucose concentrations were determined immediately
before commencing the infusion of dichloroacetate, immediately before administration of the challenging dose of phenformin or saline and at 0.5h intervals for the following 4h.

Dichloroacetate infusion produced a significant (p<0.001) 40% reduction in the blood lactate concentrations to 5.6mg/100ml (0.6mM) after 1h; see FIG. 3.3. Subsequent administration of phenformin (120mg/kg) resulted in an increase in lactate levels of almost 30% in 2h, but the concentration did not exceed the normal resting levels. In the absence of dichloroacetate the same dose of phenformin produced a 100% increase over normal resting concentrations.

Dichloroacetate had a slight but significant effect on resting blood glucose concentrations in the non-fasted rat; see FIG. 3.3. In the dichloroacetate treated, saline injected animals throughout the 5h period of the experiment the mean blood glucose (90mg/100ml, 5.0mM) was significantly lower (p<0.001) than that of rats studied for 4h after administration of saline alone (103mg/100ml, 5.7mM).

Administration of phenformin (120mg/kg) to dichloroacetate infused rats resulted in a severe hyopglycaemia reaching a maximum effect at 2h at which time blood glucose levels were reduced to 30% of normal levels (27mg/100ml, 1.5mM). In phenformin treated animals not receiving dichloroacetate blood glucose levels were reduced to 65-70% of resting levels at 2h; see FIG. 3.3. The blood glucose concentrations in rats receiving phenformin and dichloroacetate were significantly lower (p<0.01-p<0.001) than those in rats given phenformin alone from 0.5h after dosing.
FIG. 3.3 Blood lactate and glucose concentrations in the rat following a single intraperitoneal administration of phenformin alone or in conjunction with an intravenous infusion of dichloroacetate

Points represent the means of 3-6 experiments ± S.E.M. represented by vertical bars. Phenformin hydrochloride (120 mg/kg) administered in saline (0.5 ml); sodium dichloroacetate (300 mg/kg/h) in saline, pH 7.4 infused intravenously (1.2-1.4 ml/h) starting 1h before administration of phenformin or saline. □, phenformin alone; ■, phenformin and dichloroacetate; ○, saline alone; •, saline and dichloroacetate.
The effects of thiamine and thiamine pyrophosphate

The effects of thiamine hydrochloride and thiamine pyrophosphate chloride on phenformin-associated hyperlactataemia and of thiamine hydrochloride on phenformin-associated hypoglycaemia were investigated. The right femoral veins of anaesthetised rats were cannulated with polypropylene cannula tubing (PP10 cannula, Portex Ltd.) or butterfly infusion catheters. Equimolar doses of thiamine hydrochloride (1.8-2.0mg/ml saline, pH 7.4) or thiamine pyrophosphate chloride (2.0-3.0mg/ml saline, pH 7.4) were administered intravenously as regular boluses at 10min intervals (equivalent to 7mg/kg/h and 10mg/kg/h respectively) commencing 1h before administration of phenformin.

Thiamine hydrochloride had no significant effect on phenformin-induced changes in blood lactate or glucose concentrations; see FIG. 3.4.

Administration of thiamine pyrophosphate in conjunction with phenformin produced small but statistically significant changes in the blood lactate profile compared to that in animals receiving only phenformin; see FIG. 3.4. Peak lactate concentrations were slightly higher in animals given both compounds (p<0.001) and declined at a faster rate so that the 4h concentrations in animals receiving dual therapy (14mg/100ml, 1.53mM) were significantly lower (p<0.001) than those of rats receiving phenformin alone (18mg/100ml, 2.0mM).

Adjuvant effects of phenformin and chlorpropamide

The effects on the blood concentrations of lactic acid and glucose of chlorpropamide (150mg/kg) administration alone and in conjunction with phenformin (75mg/kg) were investigated.
Blood lactate and glucose concentrations in the rat following a single intraperitoneal administration of phenformin alone and in conjunction with an intravenous infusion of thiamine or thiamine pyrophosphate.

Points represent the means of 3-8 experiments ± S.E.M. represented by vertical bars. Phenformin hydrochloride (120 mg/kg) administered in saline (0.5 ml); thiamine hydrochloride (7 mg/kg/h) and thiamine pyrophosphate chloride (10 mg/kg/h) in saline, pH 7.4, infused intravenously (1.0-1.2 ml/h) starting 1h before administration of phenformin. □, phenformin alone; o, saline alone; ▼, thiamine and phenformin; ▽, thiamine pyrophosphate and phenformin.
Chlorpropamide was administered intraperitoneally as a suspension in corn-oil (0.4-0.5ml) to two groups of rats and was immediately followed by an injection of phenformin (75mg/kg) in saline or saline alone. Two other groups of rats received either phenformin alone or saline only (controls). Blood lactate and glucose concentrations were determined at 0.5h intervals for 4h.

Blood lactate concentrations in rats given chlorpropamide alone were significantly lower (p<0.05-P<0.001) than those of control animals within 0.5h of administration, decreasing from a resting level of 11mg/100ml (1.2mM) to 7.5mg/100ml (0.83mM) at 4h; see FIG. 3.5. The blood lactate profiles in rats receiving chlorpropamide and phenformin together and rats given phenformin alone were very similar except the hyperlactataemic response appeared to be prolonged in animals on dual therapy (see FIG. 3.5) giving rise to significant differences between the two groups at 3h-4h (p<0.05).

Chlorpropamide, alone and in conjunction with phenformin, produced a marked hypoglycaemia with blood glucose levels falling by some 60-70% to 30-40mg/100ml (1.7-2.2mM) in 2.5-3h, but the responses of the two groups were indistinguishable. Phenformin alone caused a reduction of 30-35% in blood glucose concentrations in the same period; see FIG. 3.5.

The effects of 4-hydroxyphenformin

After a single intraperitoneal administration of 4-hydroxyphenformin dihydrochloride in saline (150mg/kg or 183mg/kg, the molar equivalent of 120mg/kg and 150mg/kg phenformin hydrochloride respectively) blood concentrations of lactic acid were unchanged; see FIG. 3.6. Molar equivalent doses of phenformin produced 100-225% increases in blood
FIG. 3.5  Blood lactate and glucose concentrations in the rat following intraperitoneal administration of phenformin alone or in conjunction with chlorpropamide.

Blood lactate (mg/100 ml)

Blood glucose (mg/100 ml)

Points represent the means of 3-6 experiments ± S.E.M. represented by vertical bars. Phenformin hydrochloride (75 mg/kg) administered in saline (0.5 ml), chlorpropamide (150 mg/kg) in corn-oil (0.5 ml). □, phenformin alone; ■, phenformin and chlorpropamide; v, chlorpropamide alone; o, saline alone.
FIG. 3.6 Blood lactate and glucose concentrations in the rat following a single intraperitoneal administration of 4-hydroxyphenformin.

![Graph of blood lactate and glucose concentrations.](image)

Blood lactate (mg/100 ml)

Blood glucose (mg/100 ml)

Time after administration (h)

Points represent the means of 4-8 experiments ± S.E.M. represented by vertical bars. 4-Hydroxyphenformin dihydrochloride administered in saline (0.5 ml). ■, 150 mg/kg; □, 183 mg/kg; ○, saline.
lactic acid in 2.5h; see FIGS. 3.1 and 3.2.

After a higher dose of 4-hydroxyphenformin (305mg/kg equivalent to 250mg/kg phenformin) to 5 rats, all of the animals died within 1.0-1.5h but the cause of death was unknown.

No significant changes in blood glucose concentrations were observed in rats given a single dose of 183mg/kg 4-hydroxyphenformin. The equivalent dose of phenformin (150mg/kg) produced a 55-60% reduction in blood glucose levels in 2.5h; see FIG. 3.2.

(b) Studies in the non-fasted guinea-pig

The effects of phenformin

Anaesthetised guinea-pigs were given phenformin hydrochloride (12.5mg/kg) intraperitoneally and the lactate and glucose levels in the blood measured at 1h intervals for 5h.

Blood lactate concentrations increased very rapidly after phenformin administration. After 1h lactate concentrations (25mg/100ml, 2.7mM) were double the resting levels (12mg/100ml, 1.3mM) and by 5h had reached 132mg/100ml (14.6mM); see FIG. 3.7.

Blood glucose concentrations increased by 200% from a resting level of some 128mg/100ml (7.1mM) to 392mg/100ml (21.8mM) in 2h. The hyperglycaemia decreased rapidly and the animals showed an 80% hypoglycaemia at 5h with blood glucose levels of 26mg/100ml (1.4mM); see FIG. 3.7.

The effects of 4-hydroxyphenformin

Administration of 4-hydroxyphenformin dihydrochloride (15.2mg/kg, the molar equivalent of 12.5mg/kg phenformin hydrochloride) caused a
FIG. 3.7  Blood lactate and glucose concentrations in the non-fasted guinea-pig following a single intraperitoneal administration of phenformin.

![Graph of blood lactate and glucose concentrations over time after administration of phenformin.]

Points represent the means of 3-4 experiments (treated group) or 6 experiments (control group) ± S.E.M. represented by vertical bars. Phenformin hydrochloride (12.5 mg/kg) administered in saline (0.5 ml) □, phenformin; ○, saline.
FIG. 3.8  Blood lactate and glucose concentrations in the non-fasted guinea-pig following a single intra-peritoneal administration of 4-hydroxyphenformin.

Blood lactate (mg/100 ml)

Blood glucose (mg/100 ml)

Time after administration (h)

Points represent the means of 6 experiments ± S.E.M. represented by vertical bars. 4-Hydroxyphenformin dihydrochloride (15.2 mg/kg) administered in saline (0.5 ml). □, 4-hydroxyphenformin; ○, saline.
140% increase in blood lactic acid levels in 4h (30mg/100ml, 3.3mM) compared to control animals (12mg/100ml, 1.3 mM); see FIG. 3.8.

No significant effects on blood glucose concentrations were seen over the 4h period studied; see FIG. 3.8.

(c) Studies in the fasted guinea-pig

The effects of phenformin

Guinea-pigs were deprived of food for 24h before use but were allowed free access to water. Phenformin hydrochloride (12.5mg/kg) was administered intraperitoneally and the blood concentrations of lactic acid and glucose determined at 1h intervals for 4h.

Blood lactate levels increased rapidly after administration of phenformin from a resting level of 9mg/100ml (1.0mM) to 58mg/100ml (6.5mM) at 4h; see FIG. 3.9.

The increase in lactic acid levels in the 24h-fasted guinea-pig was less marked than in the non-fasted guinea-pig; see FIGS. 3.7 and 3.9. Lactate levels were significantly different (p<0.01-p<0.001) between the two groups from 1h.

Phenformin administration in the fasted guinea-pig was accompanied by a fall in blood glucose concentration from 140mg/100ml (7.8mM) to 56mg/100ml (3.1mM) at 4h; see FIG. 3.9. This response with no hyperglycaemic phase was very different from that observed in the non-fasted guinea-pig; see FIG. 3.7. Blood glucose concentrations at 4h in the fasted guinea-pig (56mg/100ml, 3.1mM) were marginally lower (p<0.05) than in the non-fasted animal (92mg/100ml, 5.1mM).
Blood lactate and glucose concentrations in the 24h fasted guinea-pig following a single intraperitoneal administration of phenformin alone or in conjunction with an intraperitoneal infusion of dichloroacetate.

Points represent the means of 3 experiments ± S.E.M. represented by vertical bars. Phenformin hydrochloride (12.5 mg/kg) administered in saline (0.5 ml); sodium dichloroacetate (300 mg/kg/h) in saline, pH 7.4 infused intraperitoneally (0.5-0.6 ml/h) starting 1h before administration of phenformin or saline. ◯, phenformin alone; ■, phenformin and dichloroacetate; ○, saline alone; ●, saline and dichloroacetate.
Glucose concentrations in the fasted control animals rose significantly during the 4h period of the experiments from some 137mg/100ml (7.6mM) to 170mg/100ml (9.4mM); see FIG. 3.9. No significant changes in blood glucose levels in non-fasted guinea-pigs were observed; see FIG. 3.8.

**The effects of dichloroacetate and phenformin**

The effects of an intraperitoneal infusion of sodium dichloroacetate on blood lactate and glucose concentrations in the 24h-fasted guinea-pig after a single intraperitoneal administration of phenformin (12.5mg/kg) were determined.

Sodium dichloroacetate was administered in saline at pH 7.4 (180-200mg/ml) at a rate of 300mg/kg/h. The solution was infused through an in-dwelling intraperitoneal catheter (PP50 cannula tubing, Portex Ltd.) which was inserted through a 0.5cm mid-line incision in the abdominal wall and fixed with sutures.

Phenformin hydrochloride (12.5mg/kg) was administered in 0.5ml saline as a single intraperitoneal injection after 1h of infusion with dichloroacetate.

In the fasted control guinea-pig dichloroacetate administration had no effect on lactate levels compared to untreated control animals; see FIG. 3.9. Glucose levels, however, were considerably reduced in animals receiving dichloroacetate (pre-infusion levels 130mg/100ml, 7.3mM, compared with 98mg/100ml, 5.5mM, after 5h infusion) while in untreated starved control animals the blood glucose concentrations increased from a resting level of 137mg/100ml (7.6mM) to a 4h level of 170mg/100ml (9.4mM). Comparison of the means at each time interval showed
significant differences from 2h (p<0.05-p<0.002); see FIGS. 3.9 and 3.10.

Dichloroacetate infusion partially prevented the hyperlactataemia produced by phenformin (see FIG. 3.9) giving a 4h lactate concentration of 38mg/100ml (4.2mM) compared to 58mg/100ml (6.5mM) in animals receiving phenformin alone. Differences between the two phenformin-treated groups were significant at 2, 3 and 4h (p<0.05-p<0.001).

The hypoglycaemic action of phenformin was considerably enhanced after infusion with dichloroacetate. Blood glucose concentrations fell to 23mg/100ml (1.3mM) at 4h compared with 56mg/100ml (3.1mM) in animals receiving only phenformin (≤ 0.05 at 3h and 4h); see FIG. 3.9.

The effects of 4-hydroxyphenformin

Administration of 4-hydroxyphenformin (15.2mg/kg, the molar equivalent of 12.5mg/kg phenformin) produced a significant increase in lactate concentrations to 20mg/100ml (2.2mM) at 4h compared to controls (11mg/100ml, 1.2mM; p<0.005); see FIG. 3.10. The increase in lactic acid levels in fasted guinea-pigs was significantly lower than in non-fasted guinea-pigs (p<0.05 at 3h and 4h); see also FIG. 3.8.

Blood glucose levels in the fasted guinea-pig after administration of 4-hydroxyphenformin were not statistically different from those of control animals; see FIG. 3.10.

The effects of dichloroacetate and 4-hydroxyphenformin

The effects of an intraperitoneal infusion of sodium dichloroacetate (300mg/kg/h) on blood lactate and glucose concentrations after intraperitoneal administration of 4-hydroxyphenformin (15.2mg/kg) were studied. Sodium dichloroacetate was infused as previously described and after 1h infusion the biguanide was administered in saline (0.5ml approx.).
FIG. 3.10 Blood lactate and glucose concentrations in the 24h fasted guinea-pig following a single intraperitoneal administration of 4-hydroxyphenformin alone or in conjunction with an intraperitoneal infusion of dichloroacetate.

Blood lactate (mg/100 ml)

Blood glucose (mg/100 ml)

Points represent the means of 3 experiments ± S.E.M. represented by vertical bars. 4-Hydroxyphenformin dihydrochloride (15.2 mg/kg) administered in saline (0.5 ml); sodium dichloroacetate (300 mg/kg/h) in saline, pH 7.4, infused intraperitoneally (0.5-0.6 ml/h) starting 1h before administration of 4-hydroxyphenformin or saline. □, 4-hydroxyphenformin alone; ■, 4-hydroxyphenformin and dichloroacetate; ○, saline alone; ●, saline and dichloroacetate.
Dichloroacetate completely abolished the hyperlactataemic response associated with 4-hydroxyphenformin; see FIG. 3.10. Blood lactate levels in guinea-pigs receiving both drugs were not statistically different from levels in untreated controls or dichloroacetate treated controls.

Administration of dichloroacetate in conjunction with 4-hydroxyphenformin failed to elicit a hypoglycaemic response. Blood glucose levels appeared to decrease at a slightly slower rate than in animals receiving dichloroacetate alone (see FIG. 3.10) but the differences are of doubtful significance, with 0.05<p<0.10 at 4h.
3.3 DISCUSSION

Because of the difficulties involved in developing a suitable diabetic animal model with which to examine phenformin-associated lactic acidosis the present studies were performed using non-diabetic normal and fasted animals. The effects of the two commonly used diabetogenic agents, alloxan and streptozotocin, result from their cytotoxic actions on the pancreatic $\beta$-cells, causing rapid and essentially irreversible necrosis (Lukens, 1948; Hellman and Diderholm, 1955; Scherer, 1955; Miller and Wurster, 1956; Rakieten et al., 1963; Brosky and Logothetopoulos, 1969; Dulin and Wyse, 1969). Treatment with these compounds produces insulin deficiency and in severe cases ketoacidosis. As the biguanides are not generally recommended for the treatment of insulin-deficient diabetic patients, because of the inability of the drugs to suppress lipolysis leading to ketosis, the experimentally-induced diabetic animal is not wholly representative of the clinical situation. In addition there have been several reports of extrapancreatic damage with both of these diabetogenic agents which cast doubts on their value in studying biguanide-associated lactic acidosis. In view of the involvement of renal and hepatic dysfunction in the aetiology of phenformin-associated lactic acidosis (see SECTION 1.5) the finding that alloxan and streptozotocin may cause a number of renal and hepatic lesions in animals and man (Lukens, 1948; Burwell and Paley, 1955; Scherer, 1955; Webb, 1966; Livingston and Carter, 1969; Vargas et al., 1970; Hohenegger and Rudas, 1971) means that careful characterisation of experimentally-induced diabetes in animals is essential in pharmacological studies with the biguanides.

In the normal, fed rat the hyperlactataemic and hypoglycaemic effects of phenformin were dose related; see FIGS. 3.1 and 3.2. The maximum effect on lactate and glucose concentrations occurred at around 2.5h and would
probably have returned to normal after about 6h. The dose-effect relationships in Fig. 3.2 suggest the existence of a critical dose of phenformin in the normal rat of 120-135 mg/kg at which point lactate concentrations rise sharply to 200-320% of the normal level and glucose concentrations fall to only 40% of resting levels. The plasma phenformin level after doses of 120-135 mg/kg was 2-3 μg/ml (1.0-1.5 x 10^{-5} M) which is considerably lower than the concentrations of drug used in most in vitro experiments demonstrating glycogen depletion and respiratory inhibition (10^{-3}-10^{-4} M; Steiner and Williams, 1958), inhibition of enzymes in the citric acid cycle (2 x 10^{-3} M; Wick et al., 1958; Kruger et al., 1960) and inhibition of oxidative phosphorylation and hepatic gluconeogenesis (4 x 10^{-4} M; Altschuld and Kruger, 1968). There is, however, some dispute as to the concentration of phenformin required to produce some of these inhibitory effects. Thus while Söling (1969) has reported that no evidence of reduced oxidative phosphorylation is seen in rat livers perfused in vitro with biguanides at 2.5 x 10^{-5} M, Woods and Alberti (1973) showed that 10^{-5} M phenformin causes a 45% inhibition of gluconeogenesis in rat liver.

It remains possible, therefore, that the rapid onset of hyperlactataemia and hypoglycaemia as seen in Fig. 3.2 indicates the point at which inhibition of oxidative phosphorylation and gluconeogenesis become evident. The possible mechanisms by which phenformin exhibits its hypoglycaemic action are discussed in detail in Section 1.4. It has been reported that at low doses (e.g. in therapeutic use) phenformin causes an increased rate of anaerobic and oxidative metabolism of glucose and gluconeogenesis may be slightly enhanced, possibly as a compensatory mechanism for the increased production of lactate (Searle and Cavalieri, 1968; Losert et al., 1971). The increase in lactate oxidation may be insufficient to counter the
enhanced production via anaerobic metabolism (Searle and Siperstein, 1975) causing blood levels to rise. In the non-diabetic subject only slight changes in blood lactate and glucose occur because increased gluconeogenesis acts as a regulatory mechanism. Diabetic subjects are probably unable to make such a metabolic adjustment as gluconeogenesis is already increased or even maximal in diabetes (Forbath and Hetenyi, 1966; Hermann, 1973; Ray, 1976).

With higher doses of phenformin the inhibition of oxidative phosphorylation, such as that seen in in vitro experiments, becomes a distinct possibility. Such an effect would prevent the recycling of lactate and other gluconeogenic substrates to glucose and produce hypoglycaemia and hyperlactataemia in normal and diabetic subjects. A decrease in lactate uptake by the liver may cause a drop in hepatic intracellular pH (Lloyd et al., 1973) which in turn could further inhibit gluconeogenesis due to the pH-dependent activation of pyruvate carboxylase by acetyl-CoA (see SECTION 1.5). Such a series of events could produce a self-perpetuating cycle which might explain the rapid-onset of lactic acidosis once the process has started. The data in FIG.3.2 support the current view that lactic acidosis only occurs in the presence of abnormally high plasma levels of phenformin (Cohen and Woods, 1976; Alberti and Nattrass, 1977). This situation may occur in cases of frank overdose as represented in these animal studies with high doses of biguanide, or in cases of hepatic or renal insufficiency where metabolism and excretion of phenformin are impaired. Karam et al. (1974) have reported a plasma concentration of $1 \times 10^{-5}$ M phenformin in a patient with lactic acidosis resulting from suicidal self-poisoning and Heuclin et al. (1975) have demonstrated that phenformin accumulation and reduced gluconeogenesis from lactate occurs
in rats with experimentally-induced renal failure.

Experiments in rats treated with microsomal hydroxylase inhibitors have suggested that metabolism of phenformin reduces its pharmacological activity (Holloway and Alberti, 1976; Alberti and Holloway, 1977). The major metabolite of phenformin in the rat is 4-hydroxyphenformin which, together with its glucuronic acid conjugate, accounts for more than half the radioactivity eliminated in the 24h urine (35-40% of the dose) after a 100 mg/kg intraperitoneal dose of [2'-\(^{14}\)C]phenformin (see CHAPTER 2). 4-Hydroxyphenformin had no significant effect on blood lactate or glucose concentrations in the normal rat (see FIG. 3.6) when administered at doses of 150 or 183 mg/kg (the molar equivalents of 120 and 150 mg/kg phenformin respectively). The molecule was not without biological activity as a higher dose (305 mg/kg, equivalent on a molar basis to 250 mg/kg phenformin) was lethal to all of 5 rats studied. Beckmann (1967) reported that oral administration of 4-hydroxyphenformin (200-400 mg/kg) does not produce hypoglycaemia in the mouse and Heuclin et al. (1975) found no change in blood lactate levels in rats with surgically-induced renal insufficiency after administration of 4-hydroxyphenformin (unspecified dose) for 8 days. In contrast Wick et al. (1970) have reported that the 4-hydroxy metabolite possesses half the hypoglycaemic activity of phenformin in the rat, but no details of this study were given.

It therefore appears that the rat can effectively inactivate phenformin by aromatic oxidation and subsequent conjugation of the phenol to a varying extent (see CHAPTER 2). After high doses of the biguanide to rats the ability of the liver to extract and/or metabolise phenformin may be saturated and a higher proportion of the dose may be excreted unchanged in the urine (see TABLE 2.3). The existence of a first-pass elimination process for phenformin in the rat has been discussed in more detail in
SECTION 2.3. The saturation of such a system may lead to dose-dependent disposition kinetics (Grundin et al., 1974) and could explain the relatively constant increase in plasma phenformin concentrations with each dose increment above 50 mg/kg; see FIG. 3.2. Thus a 50 mg/kg intraperitoneal dose may result in portal blood concentrations which approximate to the maximum that can be effectively extracted by the liver and higher doses lead to the appearance of the drug in the peripheral circulation and the onset of the conditions leading to lactic acidosis.

Sodium dichloroacetate infusion in the normal rat reduced blood lactate levels by almost 50% in 1h. Although subsequent injection of phenformin caused a slight elevation, lactate concentrations did not exceed those in untreated control rats (see FIG. 3.3) indicating that dichloroacetate can effectively prevent the occurrence of phenformin-associated lactic acidosis. Similar findings have been reported in the fasted rat treated with SKF525A to prevent the hepatic inactivation of phenformin (Holloway and Alberti, 1976) and in streptozotocin-induced diabetic rats treated for 28 days with phenformin and dichloroacetate (Man and Alberti, 1976). Dichloroacetate can also reverse established phenformin-associated lactic acidosis in the starved rat (Alberti and Holloway, 1977).

The slight but significant ($p < 0.001$) reduction in blood glucose levels in dichloroacetate-infused rats is in contrast to a number of earlier reports that the compound only lowers blood glucose concentrations in diabetic and fasted non-diabetic animals (Lorini and Ciman, 1961; Stacpoole and Felts, 1970; Eichner, 1974). In those studies where no reduction in blood glucose levels was found, dichloroacetate was
administered orally or intraperitoneally, while in the present studies the compound was given intravenously. As the hypoglycaemic effect of dichloroacetate is due at least partly to a reduction in the peripheral release of gluconeogenic precursors (Blackshear et al., 1974, 1975; Stacpoole et al., 1976) the fall in blood glucose levels in rats after systemic administration of dichloroacetate may be the result of an enhanced peripheral effect of the compound due to the higher blood concentrations and increased tissue distribution which occur after intravenous injection.

Administration of phenformin to dichloroacetate-infused rats caused a 70% fall in blood glucose levels in 2h to 27 mg/100 ml (1.5 mM) compared with 65 mg/100 ml (3.6 mM) after phenformin alone. Holloway and Alberti (1976) have reported that simultaneous infusion of phenformin and dichloroacetate in SKF525A-treated, starved rats produces a slightly greater hypoglycaemic effect than phenformin alone.

The mechanism of action of dichloroacetate has been discussed in SECTION 1.5. The compound is believed to activate hepatic and extrahepatic pyruvate dehydrogenase and to decrease the release of gluconeogenic precursors from peripheral tissues (Stacpoole et al., 1970, 1976; Blackshear et al., 1974, 1975; Whitehouse et al., 1974). Dichloroacetate probably prevents hyperlactataemia by decreasing the flow of gluconeogenic substrates to the liver rather than by directly counteracting the hepatic action of high doses of phenformin on gluconeogenesis as Stacpoole (1977) has shown that dichloroacetate significantly reduces the rate of glucose formation from a range of substrates in isolated parenchymal cells obtained from the livers of fasted rats. The fall in lactate levels in control infused animals may be due to the enhanced activity of pyruvate
dehydrogenase. This is reflected by the observation that after

treatment with phenformin and dichloroacetate the ketone body concentration

in rat liver is increased (Man and Alberti, 1976; Alberti and Holloway,
1977) probably due to an increased production of acetyl-CoA and an

inhibition of citric acid cycle activity caused by dichloroacetate and

phenformin respectively. The enhanced glycolytic activity and reduced

gluconeogenesis which results from the inhibition of oxidative phosphor-

ylation and the activation of pyruvate dehydrogenase may also account for

the increased hypoglycaemic effect observed after combined therapy.

The hyperlactataemic and hypoglycaemic responses to phenformin in

the guinea-pig differed greatly from those in the rat; cf. FIGS 3.1 and

3.7. Blood lactate levels in the normal, fed guinea-pig appeared to peak

after 5h, increasing from a resting concentration of 12 mg/100 ml (1.3 mM)

to 132 mg/100 ml (14.6 mM). Blood glucose levels showed a massive hyper-

glycaemia lasting almost 4h, followed by a severe hypoglycaemia. The

period of hyperglycaemia after biguanide administration corresponds to the

mobilisation of the liver glycogen reserves of the animal to combat the

hypoglycaemic action of the drug (Sterne, 1969). Glycogen depletion in

the non-fasting animal precedes hypoglycaemia, survives it and appears in

some animals without changes in blood glucose (Sterne, 1969). In the

normal guinea-pig the blood glucose concentration at 5h (26 mg/100 ml;

1.4 mM) was only 20% of the resting level (128 mg/100 ml; 7.1 mM).

In the 24h-fasted guinea-pig blood lactate levels rose to less than

50% of the lactate concentration in the non-fasted guinea-pig. Blood

glucose concentrations showed no hyperglycaemic phase but fell steadily

from 140 mg/100 ml (7.8 mM) to 56 mg/100 ml (3.1 mM) at 4h; see FIG. 3.9.

In the normal guinea-pig the hyperglycaemia which results from phenformin-

induced glycogenolysis probably triggers the secretion of insulin. Such

a response could explain the extremely rapid fall in blood glucose levels
due to the stimulation by insulin of glycogen synthesis and the glycolytic and oxidative pathways of carbohydrate metabolism and the inhibition of gluconeogenesis from lactate and glucogenic amino acids (West et al., 1966; Ashmore and Weber, 1968; Felig, 1972; Weber, 1972). Insulin alone may cause slight rises in blood lactate concentrations by causing lactate to accumulate at a greater rate than it can be disposed of by oxidation (Alberti and Nattrass, 1977). The combined actions of insulin and toxic levels of phenformin (which also causes the accumulation of lactate by inhibiting its elimination by oxidation and gluconeogenesis) could explain the exaggerated response in the normal guinea-pig. In the fasted animal the absence of hepatic glycogen deposits obviates the occurrence of a hyperglycaemic, insulinogenic response.

Dichloroacetate infusion alone did not reduce the normal blood lactate concentrations in the fasted guinea-pig but did significantly lower blood glucose levels; see FIG. 3.9. The fasted guinea-pig therefore behaves differently to the fasted rat in which dichloroacetate lowers blood lactate concentrations by 50% (Blackshear et al., 1974). It should also be noted that blood lactate concentrations in untreated control fasted guinea-pigs were not lower than those in control normal guinea-pigs, while lactate levels in cardiac blood of fasted rats (6.8 ± 1.7 mg/100 ml) are lower than those in normal rats (13.6 ± 1.4 mg/100 ml) (Ramachander et al., 1968).

Dichloroacetate increased the hypoglycaemia and decreased the hyperlactataemia associated with phenformin administration in the fasted guinea-pig; see FIG. 3.9. Blood lactate levels were not completely prevented from rising above normal but it should be noted that dichloroacetate was administered intraperitoneally in this species and intravenously in the rat. The difference in effect in the two species may therefore reflect only the influence of route of administration on the pharmacological
effectiveness of dichloroacetate. The dose administered (300 mg/kg/h) was that reported as being effective in preventing the development of lactic acidosis in fasted, SKF525A-treated rats infused simultaneously with phenformin (50 mg/kg/h) and dichloroacetate intravenously (Holloway and Alberti, 1976). In view of the much greater increases in blood lactate which occur in phenformin treated guinea-pigs compared with rats, larger doses of dichloroacetate may be required to prevent hyperlactataemia in the guinea-pig.

Blood lactate levels in both the normal and fasted guinea-pig were elevated after administration of 4-hydroxyphenformin (15.2 mg/kg, molar equivalent to 12.5 mg phenformin/kg); see FIGS. 3.8 and 3.10. No hypoglycaemic response was observed. The hyperlactataemia accompanying normoglycaemia is unlikely to have arisen from inhibition of oxidative phosphorylation or inhibition of gluconeogenesis. A lowering of the ATP/ADP ratio which would reduce pyruvate oxidation would also reduce gluconeogenesis and increase the rate of glycolysis. These changes would lead to a fall in blood glucose concentrations in fasted animals. Impaired gluconeogenesis in the presence of a normal level of glucose metabolism would also produce hypoglycaemia in the fasted animal. The increases in blood lactate after 4-hydroxy-phenformin administration could have resulted from an enhanced peripheral glycolysis without a corresponding increase in oxidative metabolism, similar to the observations of Searle and Siperstein (1975) with phenformin. Glucose concentrations may be maintained by gluconeogenesis from lactate and other glucogenic substrates and in normal animal by the breakdown of hepatic glycogen deposits. The absence of glycogen reserves in the fasted guinea-pig is probably reflected by an increased rate of gluconeogenesis. This could explain the slower rate of increase in blood lactate concentrations in fasted guinea-pigs compared with non-fasted animals.
4-Hydroxyphenformin-associated hyperlactataemia in the fasted guinea-pig was prevented by dichloroacetate. This could be due to the enhanced activity of pyruvate dehydrogenase which follows dichloroacetate administration (Whitehouse and Randle, 1973; Whitehouse et al., 1974).

The absence of an effect of 4-hydroxyphenformin on blood lactate and glucose levels in the normal rat may be attributed to a more efficient hepatic extraction and elimination of the compound, or may reflect a species difference in susceptibility to the effects of 4-hydroxyphenformin on carbohydrate metabolism.

The blood lactate and glucose concentrations in fasted guinea-pigs treated with phenformin and 4-hydroxyphenformin alone and in conjunction with dichloroacetate are summarised in FIGS. 3.11 and 3.12.

The effect of thiamine pyrophosphate on the hyperlactataemic response to phenformin in the rat was studied in the light of a report by Valette et al. (1975) that intravenous infusion of the compound (5mg/kg/h) in the dog counteracted the increase in blood lactate produced by intraduodenal administration of phenformin (30mg/kg). In the rat thiamine pyrophosphate (10mg/kg/h) did not reduce the increase in blood lactate associated with phenformin administration (120mg/kg); see FIG. 3.4. Slight changes were seen in the blood lactate profile in that the peak level occurred earlier and declined more rapidly. No explanation has been found for the slightly higher lactate levels but the more rapid clearance may be due to enhanced activity of pyruvate dehydrogenase. Thiamine pyrophosphate is a coenzyme for the pyruvate dehydrogenase enzyme complex and thiamine deficiency causes elevations in blood lactate levels due to pyruvate accumulation (Harper, 1965;
Blood lactate concentration in the 24h-fasted guinea-pig receiving phenformin, 4-hydroxyphenformin or saline intraperitoneally, alone (a) or in conjunction with dichloroacetate (b).

For further experimental details see FIGS. 3.9 and 3.10.
FIG. 3.12  Blood glucose concentrations in the 24h-fasted guinea-pig receiving phenformin, 4-hydroxyphenformin or saline intraperitoneally, alone (a) or in conjunction with dichloroacetate (b).

( △ , ▲ ) - phenformin
( □ , ■ ) - 4-hydroxyphenformin
( ○ , ● ) - saline

For further experimental details see FIGS. 3.9 and 3.10.
Harken, 1976). The coenzyme is formed in the cell by transfer of pyrophosphate from ATP to thiamine which is absorbed from the diet. It would be surprising to find that thiamine pyrophosphate is readily distributed in the tissues after intravenous administration.

Thiamine itself had no effect on the blood lactate or glucose profiles following phenformin administration; see FIG. 3.4.

The effects of chlorpropamide and phenformin alone and in conjunction with each other on blood lactate and glucose concentrations in the rat (see FIG. 3.5) reflect the independent actions of the drugs on carbohydrate metabolism. The sulphonylureas such as chlorpropamide produce hypoglycaemia by stimulating the release of insulin from the pancreatic β-cells (Pfeiffer et al., 1969).

The fall in blood lactate concentrations seen after administration of chlorpropamide to rats (see FIG. 3.5) probably results from an increased oxidation of pyruvate, as gluconeogenesis is inhibited by insulin. Insulin alone, or with glucose in patients with hypoglycaemia, has been recommended for the treatment of biguanide-associated lactic acidosis (Johnson and Waterhouse, 1968; Dembo et al., 1975; Fulop and Hoberman, 1976). The rationale for this is that by activating pyruvate dehydrogenase the hormone will enhance lactate clearance. However by stimulating glycolysis and inhibiting gluconeogenesis insulin may cause lactate to accumulate more rapidly than it can be oxidised (Alberti and Nattrass, 1977). This may explain the prolonged hyperlactataemia produced in rats after treatment with both phenformin and chlorpropamide. The impaired oxidative phosphorylation caused by phenformin may partially counteract the enhanced oxidation of pyruvate associated with insulin
secretion with the result that combined therapy causes accumulation of lactate. Bramanti et al. (1971a) have reported that in blood samples taken from guinea-pigs 4h after administration of phenformin (0-40 mg/kg orally) and chlorpropamide (50 or 100 mg/kg orally) the blood lactate levels were about double those occurring after phenformin alone.

The increased blood lactate concentrations in the guinea-pig on combined therapy were accompanied by an enhanced hypoglycaemic effect (Bramanti et al., 1971a, b). This was not seen in the rat (see FIG.3.5) where the hypoglycaemic effect of chlorpropamide alone was as great as that measured after combined therapy with phenformin. Such contrasts between the rat and guinea-pig are not surprising in view of the marked species differences in pharmacological susceptibility to phenformin (Üngar et al., 1957) and the differences in the metabolism and excretion of both phenformin (see CHAPTER 2) and chlorpropamide (Pfeiffer et al., 1969).
CHAPTER 4

FINAL DISCUSSION
The aim of these studies has been to investigate the role of the metabolism of phenformin in the species differences in the hypoglycaemic and hyperlactataemic responses to the drug and in the aetiology of phenformin-associated lactic acidosis. The metabolism and excretion of [2'-¹⁴C]phenformin in the rat and guinea-pig have been studied and the effects of phenformin and 4-hydroxyphenformin on blood lactate and glucose concentrations have been determined.

The view widely expressed in the literature is that the differences in hypoglycaemic response to phenformin in the rat and guinea-pig arise from differences in the degree of metabolism of the drug in the two species. This belief stems from the reports of Beckman (1967, 1968) that rat urine after a 50 mg/kg oral dose of [³H]phenformin contained only 4-hydroxyphenformin (and its glucuronide) while guinea-pig urine contained almost exclusively parent compound with only traces of the metabolites. In contrast, the present studies have shown that although the drug was less extensively metabolised by the guinea-pig, guinea-pig urine contained approximately equal amounts of unchanged phenformin and metabolites after oral dosing and that 4-hydroxyphenformin was not excreted; see TABLE 2.3 and FIG. 2.9. Guinea-pig faeces also contained large amounts of a novel metabolite, but as neither the faecal nor the urinary metabolite have been identified it is not possible to state whether these are biologically active metabolites or inactive. Beckmann (1966) has reported that an inverse correlation exists for buformin between the hypoglycaemic activity in different species and the degree of metabolism. The present findings, together with the report that phenformin (50 mg/kg) produced hypoglycaemia in the mouse which excreted predominantly 4-hydroxyphenformin glucuronide, but not in the rabbit which excreted large amounts of the parent compound
along with the same metabolites (Beckmann, 1967) indicate that with phenformin such a simple relationship does not hold.

In contrast to the report of Beckmann (1967) the guinea-pig showed a slower rate of excretion of radioactivity than the rat after low oral doses of \([2\textsuperscript{14}C]\)phenformin; see TABLE 2.2. The differences in the urinary excretion of unchanged parent compound (see TABLE 2.3) suggest a slower rate of metabolism in the guinea-pig and this is supported by the differences in the rate of metabolism in isolated hepatocytes; see FIG. 2.24a. These metabolic and dispositional differences may contribute significantly to the increased pharmacological response of the guinea-pig to a given dose of the drug.

Differences in the rates of metabolism and excretion, however, are unlikely to account entirely for the species differences in the hypoglycaemic and hyperlactataemic responses. The effects of phenformin on blood glucose and lactate concentrations were more pronounced in the guinea-pig after administration of 12.5 mg/kg intraperitoneally than in the rat after doses of 100-120 mg/kg (see CHAPTER 3) although in both cases most of the dose was rapidly eliminated in the urine as an approximately equal mixture of the parent compound and metabolites. As some 22% of the guinea-pig urinary radioactivity (16% of the dose) was accounted for as the unidentified metabolite (metabolite 4) and its glucuronide it remains possible that the sensitivity of this species to the pharmacological actions of phenformin may be due to the formation of a biologically active metabolite.

The variations in pharmacological response to phenformin in different animals may be the result of differences in the mechanism of glucose regulation. Species differences exist in the regulation of hepatic gluconeogenesis, partly due to the intracellular distribution
of the enzyme phosphoenolpyruvate carboxykinase. This enzyme is present in the mitochondria and cytosol in varying proportions in different animals (Nordlie and Lardy, 1963). The two forms of the enzyme are separate proteins regulated in different manners (Ballard and Hanson, 1969). Guinea-pig and man fall into the same group with the enzyme equally distributed between the cytosol and mitochondria while the rat enzyme is predominantly cytosolic (Nordlie and Lardy, 1963; Hanson and Garber, 1972).

In comparison to rat liver the cytosolic redox state (calculated from the lactate dehydrogenase couple) is more reduced in the guinea-pig and the mitochondrial redox state (calculated from the 3-hydroxybutyrate dehydrogenase couple) is more oxidised (Willms et al., 1970; Garber and Hanson, 1971a,b; Söl ling and Kleineke, 1976). In species with mitochondrial phosphoenolpyruvate carboxykinase activity (e.g. guinea-pig and man) conditions which lead to oxidation of the mitochondrial NAD\(^+\)/NADH system, such as fasting (Garber and Hanson, 1971a,b) favour mitochondrial synthesis of phosphoenolpyruvate by increasing the mitochondrial concentration of oxaloacetate. Similarly, conditions which lead to reduction of the mitochondrial NAD\(^+\)/NADH system, such as fatty acid oxidation, impair mitochondrial synthesis of phosphoenolpyruvate by increasing the mitochondrial concentration of malate at the expense of oxaloacetate (Garber and Hanson, 1971a,b; Arinze et al., 1973). This may explain the fact that fatty acids have an inhibitory effect on gluconeogenesis from lactate in guinea-pig liver. In rat liver on the other hand, fatty acids stimulate gluconeogenesis mainly by causing an increase in the activity of pyruvate carboxylase due to an increased level of acetyl-CoA and by decreasing the activity of pyruvate dehydrogenase (Söl ling et al., 1970; Arinze
Alternatively, hepatic gluconeogenesis in the guinea-pig may be inhibited by fatty acid oxidation due to a decrease in the concentration of pyruvate resulting from an increased reduction of the cytosolic NAD\(^+\)/NADH system (Söling and Kleineke, 1976). The lactate/pyruvate ratio in guinea-pig liver is considerably higher than in rat liver and increases further in the presence of fatty acids (Willms et al., 1970). For a given concentration of lactate the concentration of pyruvate is always less with isolated, perfused guinea-pig livers than with rat livers under the same conditions (Willms et al., 1970).

The susceptibility of the gluconeogenic enzymes in guinea-pig liver to inhibition by changes in the cytosolic or mitochondrial redox states may explain the particular sensitivity of this animal to the pharmacological effects of phenformin. Haeckel and Haeckel (1972) have shown that in perfused livers from guinea-pigs pretreated with phenformin the ATP/ADP ratio was lowered but gluconeogenesis was unaffected. At the same time small increases in the 3-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios were measured, and the concentration of malate but not oxaloacetate was increased. Addition of phenformin (2 x 10\(^{-5}\)M) to the perfusate produced a 60% inhibition of gluconeogenesis without further depressing the ATP/ADP ratio but with further increases in the cytosolic and mitochondrial redox couples. These results suggest that in the guinea-pig a small decrease in the liver ATP/ADP ratio, which is insufficient to inhibit gluconeogenesis by directly limiting the supply of ATP, may still be sufficient to increase the reductive state of the cytosolic and mitochondrial NAD\(^+\)/NADH systems which in turn would impair glucose synthesis by limiting
the substrate available for either or both of the key enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxykinase.

Rat liver, on the other hand, is less susceptible to these reductive changes (as shown by the effects of fatty acid administration) and in this species phenformin probably has little effect on gluconeogenesis until high doses of the drug are administered which cause a substantial depletion of hepatic ATP levels. As human liver is similar to guinea-pig liver in the intracellular distribution of phosphoenolpyruvate carboxykinase it is possible that a similar series of changes may occur in man in response to intermediate doses of phenformin.

The present studies indicate that a number of factors are involved in the species differences in response to phenformin and that the widely held view that the rate of metabolism is the controlling factor in the pharmacological activity of the drug in different species (Beckmann, 1967; Cook et al., 1973a) is an oversimplification.

The differences in pharmacological activity in the rat and guinea-pig can be explained by the results in this thesis, which show a slower rate of metabolism and excretion in the guinea-pig, together with the established biochemical differences in the mechanisms of carbohydrate regulation in these species. Contrary to the report of Beckmann (1967) the guinea-pig was found partly to metabolise phenformin but excreted an oral dose more slowly than the rat. Biliary excretion was also shown to be less significant in the guinea-pig. Although intrinsically this may be an important route for the elimination of phenformin in the guinea-pig, it appears to be easily inhibited, at least by the dose level used in this study. At lower dose levels it may be that biliary excretion is more efficient as it may not be inhibited.
The possibility that the guinea-pig may produce a biologically active metabolite has not been resolved. The previously unreported metabolites have been detected in guinea-pig urine and faeces after oral dosing. The urinary metabolite is probably the product of aliphatic hydroxylation rather than aromatic hydroxylation and both metabolites have modified biguanide portions (see APPENDIX), but their complete characterisation and pharmacological properties remain to be determined.

The hyperlactataemic activity of 4-hydroxyphenformin in the guinea-pig is of interest. Man reportedly excretes equal quantities of 4-hydroxyphenformin and parent compound after administration of [3H]phenformin (Beckmann, 1967). The pharmacological activity of this metabolite in man requires investigation.

The existence of a critical dose level for the hypoglycaemic and hyperlactataemic actions of phenformin together with the dose-related degree of metabolism (see SECTION 2.3) of the drug provide further indications that the toxic actions of phenformin are related to accumulation of the active drug. These findings may explain the predisposition to lacticacidosis of patients with renal and hepatic dysfunction.

Sodium dichloroacetate was shown to be effective in controlling phenformin-associated hyperlactataemia and enhanced the hypoglycaemic action of the drug. A combination of sodium dichloroacetate and a reduced dose of phenformin may therefore be valuable in the treatment of some forms of diabetes. However, more extensive biochemical and toxicological studies are required to determine the therapeutic potential of the combined treatment. The risks of ketosis are increased (see SECTION 3.3) but this may cause less difficulty in ketoacidosis-resistant diabetes.
APPENDIX

INTERPRETATION OF MASS SPECTRA
Appendix

Confirmation of the structures of metabolites 1 and 2 in rat urine and metabolite 1 in guinea-pig urine was obtained by mass spectrometry as described in Chapter 2. Examples of the positive-ion distribution spectra for authentic and isolated samples of phenformin and 4-hydroxyphenformin are presented in FIGS. 2.13-2.16.

The biguanides are insufficiently volatile to produce spectra until subjected to high temperatures when they form triazine derivatives by internal condensation. Suggested structural assignments for the major positive ions in the electron-impact spectra of phenformin and 4-hydroxyphenformin are given in Fig. 1. The symbol $m^*/e$ is used to denote the occurrence of a metastable ion accompanying a particular transition. The position of $m^*$ accompanying the transition of ion $m_1$ to $m_2$ is given by equation (1) (Budzikiewicz et al., 1967). In FIG. 1 homolytic bond fission

$$m^* = \frac{m_2}{m_1}$$

involving one-electron movements are signified using the "fishhook" symbol (→) introduced by Budzikiewicz et al. (1964, 1967). For the sake of simplicity only one "fishhook" is written when homolysis of a bond is indicated so that the expressions in (2) are synonymous. The usual arrow (→) denotes a two-electron shift as in (3).

$$\text{C-C} \rightarrow \text{C}^+ + \text{C}^- \quad (2)$$

$$\text{C-C} \rightarrow \text{C}^+ + \text{C}^- \quad (3)$$

Wickramasinghe and Shaw (1972) have reported that after mass spectrometric analysis of the single g.l.c. peak produced by phenformin at 225-230°C,
Produced a t 220-230 (70eV) 

Phenformin (mol.wt. 205)

\[
\text{m/e 334 (I)}
\]

\[
\text{m/e 243 (II)}
\]

\[
\text{m/e 230 (III)}
\]

\[
\text{m/e 139 (V)}
\]
FIG. 1 (continued)

\[ \begin{align*}
\text{H}_2\text{N} \cdot \text{C} \cdot \text{NH}_2 \text{CN} & \xrightarrow{-\text{H}_2\text{NCN}} \text{CN}_2\text{CN} \\
\text{m/e 85 (XII)} & \quad \text{m/e 68 (XIII)}
\end{align*} \]

\[ \begin{align*}
\text{m/e 110 (VII)} & \quad \text{m/e 68 (XIII)}
\end{align*} \]

\[ \begin{align*}
\text{R = H (III) or PhCH}_2\text{CH}_2(\text{I}) & \xrightarrow{+ \text{C}_2\text{H}_2} \text{m/e 91 (XI)} \\
\text{m/e 65 (XIV)}
\end{align*} \]

\[ \begin{align*}
\text{m/e 246 (XVI)} & \xrightarrow{+ \text{C}_2\text{H}_2} \text{m/e 139 (V)} \\
\text{m/e 162 (XVII)} & \xrightarrow{+ \text{HCHO?}} \text{m/e 107 (XIX)} \\
\text{m/e 77 (XX)}
\end{align*} \]
the parent ion occurred at \( m/e \) 230 and prominent peaks were found at \( m/e \) values of 139, 110, 91 and 68. The mass spectrum of 4-hydroxyphenformin does not appear to have been reported previously.

After direct insertion mass spectrometry at 220-230°, the parent ion for phenformin occurred at \( m/e \) 334 corresponding to the disubstituted triamino-s-triazine ion (I). Cleavage at the C-C bond adjacent to a nitrogen atom (a-cleavage) whose non-bonding electrons provide resonance stabilisation (Silverstein and Bassler, 1967) resulted in the base peak at \( m/e \) 243 (II); see FIG. 1.b. Under the same conditions the parent ion for 4-hydroxyphenformin occurred at \( m/e \) 246 and corresponded to the mono-substituted triamino-s-triazine (XVI) while the base peak was due to the fragment at \( m/e \) 43 (XV); see FIG. 1.i. When 4-hydroxyphenformin was heated at 220-230° for several minutes the ions (XXI) and (XXII) corresponding to (I) and (II) from phenformin could also be detected and the spectrum resembled that of phenformin more closely.

\begin{align*}
\text{m/e 366 (XXI)} & \quad \text{m/e 259 (XXII)} \\
\text{CH}_2\text{CH}_2\text{NH} & \quad \text{CH}_2\text{CH}_2\text{NH} \\
\text{NH}_2 & \quad \text{NH} = \text{CH}_2 \\
\text{m*} & \quad \text{obs. 183.3} \\
\text{c} & \quad \text{calc. 183.28}
\end{align*}
The ions (III), (V), (VI) and (IX) appear to be produced by McLafferty rearrangements which involve the transfer of γ-hydrogen radicals in six-membered transition states (Budzikiewicz et al., 1967). Styrene (IX) may retain the charge if the nitrogen containing fragment is eliminated as a neutral molecule (FIG. 1.h) or may itself be expelled as a neutral fragment (FIG. 1.c, d and g).

The ions at m/e 139 (V) and m/e 126 (VI) or 127 (XVIII) appear to be characteristic of the fragmentation of the triazine structures formed by phenformin and 4-hydroxyphenformin. The ion (V) may be formed by α-cleavage (FIG. 1.e and m) or by McLafferty rearrangement (FIG. 1.d) and produces a number of smaller fragments by successive elimination of small neutral molecules (FIG. 1.f, i and j).

The phenolic portion of 4-hydroxyphenformin gives rise to the hydroxy-tropylium ion (XIX) at m/e 107 which fragments further to give the C₆H₅⁺ ion (m/e 77). Whether this occurs by the elimination of formaldehyde as depicted in FIG. 1.n, or by successive elimination of CO and two hydrogens as shown below (Budzikiewicz et al., 1967) is a matter for conjecture. The appearance of a metastable ion is generally regarded as evidence that the neutral atoms are ejected in a one-step process as a single entity. However, a number of examples of decomposition reactions which occur with appropriate metastable peaks, but are likely to correspond to two-step
processes have been reported (Budzikiewicz et al., 1967).

The mass spectra of guinea-pig metabolites 4 and 6 contained no evidence of the positive ions characteristic of phenolic or triazine groupings. As metabolite 4 is also excreted as an O-glucosiduronic acid conjugate (metabolite 5) the absence of the hydroxytropylium ion (XIX) in the mass spectrum suggests that hydroxylation has occurred on the aliphatic side chain of the molecule. The absence of the ions (V), (VI), (XII) and (XIII) which characterise the triazine structures formed by phenformin and 4-hydroxyphenformin may be an indication that the biguanide portions of metabolites 4 and 6 have been modified. The spectra of both metabolites contained ions at m/e values of 77 (XX) and 65 (XIV) which were related by the presence of metastable ions. This transition indicates the presence of the aromatic phenyl moiety (Budzikiewicz et al., 1967; Silverstein and Bassler, 1967).

Treatment of the urinary aglycone with acetic anhydride in pyridine did not result in the appearance in the mass spectrum of positive ions characteristic of the triazine structure which would be expected to form in the presence of an intact biguanide grouping; see FIG. 1.3.
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