THE INTER-RELATIONSHIP OF LACTATE METABOLISM AND CELL pH IN THE ISOLATED PERFUSED LIVER

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ABSTRACT

The relationship between cell pH and lactate metabolism has been studied in the isolated perfused mammalian liver under conditions of simulated metabolic acidosis and during the administration of phenethylbiguanide (phenformin).

In the first series of experiments isolated rat livers were perfused with media, the pH of which was varied by altering the bicarbonate concentration. Cell pH was measured using 5,5-dimethyloxazolidine-2,4-dione (DMO). Under the conditions of simulated metabolic acidosis, it was found that hepatic cells are partially protected against external acidosis. The lactate uptake of the livers was determined and it was found that above a cell pH of 7.0 lactate uptake did not vary with pH. However, below pH 7.0 lactate uptake was inhibited and in some cases lactate was actually produced by the liver.

In the second series of experiments isolated guinea pig livers were perfused with media containing the hypoglycaemic drug phenethylbiguanide (phenformin). This inhibited lactate uptake by the livers and a positive relationship between lactate uptake and cell pH was demonstrated. Glucose production was also inhibited although the expected stoichiometric relationship between lactate uptake and glucose production was not observed.

These results indicate that hepatic lactate uptake and cell pH are inter-related. Firstly, lowering cell pH has been shown to inhibit lactate uptake and under severely acidotic conditions lactate may be produced by the liver.

Secondly, the most likely explanation of the lowering of cell pH by phenformin is that this is a consequence of inhibition of lactate uptake and metabolism. This suggests that there is a positive feedback mechanism controlling hepatic lactate metabolism and hepatic cell pH. The feedback mechanism may be relevant to the pathogenesis of clinical lactic acidosis and may provide a rationale for the therapeutic effect of large amounts of alkali in this condition.

GLOSSARY OF ABBREVIATIONS

Acetyl CoA	acetyl coenzyme A
ADP	adenosine 5'-pyrophosphate
AMP	adenosine 5'-phosphate
Cyclic AMP	adenosine 3':5'-cyclic monophosphate
ATP	adenosine 5'-triphosphate
DMO	5,5-dimethyloxazolidine-2,4-dione
FDP	fructose 1,6-diphosphate
F6P	fructose 6-phosphate
GTP	guanosine 5'-triphosphate
L	lactic acid (lactate)
NAD^+	oxidized nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinucleotide
P	pyruvic acid (pyruvate)
PC	pyruvate carboxylase
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
pH_i	intracellular pH
pH_e	extracellular pH

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1. INTRODUCTION

1.1 RATIONALE OF THE STUDY

Lactic acid is the end product of the anaerobic metabolism of glucose. The main tissue sources are skeletal muscle, erythrocytes, brain and skin. Under resting conditions lactate is cleared from the circulation mainly by the liver and renal cortex. Both of these tissues have the ability to convert lactate to glucose. Within the liver the greatest proportion of lactate metabolised follows this pathway, only a minor proportion being oxidised to carbon dioxide and water; within the kidney the latter pathway is probably the major one. The synthesis of glucose from lactate and other substrates, i.e. gluconeogenesis, is of great importance in the glucose homeostasis of the organism, particularly during starvation.

Under normal circumstances the blood lactate level is low and reflects the balance between lactic acid production and utilization. Lactic acid is produced in large amounts during maximal exercise and major elevation of blood lactate may occur; the circulating lactate is removed by submaximally exercising muscle (Hermansen and Stensvold, 1972) and by liver (Rowell, Kraning, Evans, Kennedy, Blackman and Kusami, 1966) and probably kidney.

Lactic acid levels in blood may be raised as a result of disease processes. Any condition which produces tissue hypoxia may be accompanied by a rise in blood lactate with the

consequent development of a metabolic acidosis. In recent years a group of patients with a similar biochemical picture and yet with no clinical evidence of tissue hypoxia has been identified (Huckabee, 1961b). The mechanism of production of the high blood lactate levels in these patients is not understood. Two of the most striking features of this condition are its rapid development and high mortality rate.

The liver plays a central role in lactate metabolism and hepatic dysfunction has been implicated in the pathogenesis of lactic acidosis (Mulhausen, Eichenholz and Blumentals, 1967; Sriussadaporn and Cohn, 1968). The present work was designed to investigate further the role of the liver in the development of this condition using the isolated perfused rat and guinea pig livers as animal models. Special attention was focused on the relationship between hepatic cell pH and lactate uptake and metabolism. It has been shown previously that lactate uptake by the liver is accompanied by alkalinisation of the hepatic cell (Cohen, Iles, Barnett, Howell and Strunin, 1971); conversely, a fall in lactate uptake would render the hepatocyte more acid. Since a rate limiting enzyme in the gluconeogenic pathway from lactate is effectively inhibited at low pH, it was suggested that a fall in lactate uptake, caused by an inhibition of gluconeogenesis would result in further inhibition of the enzyme, due to the fall in pH. This would create a 'vicious circle' which eventually would result in cessation of lactate uptake. Two consequences follow from such an hypothesis; firstly, acidification of the liver cell should result in a reduction in lactate uptake and, secondly, liver cell pH should fall in the presence of an inhibitor of

gluconeogenesis, such as the antidiabetic drug phenformin.

It was the primary object of the work described in this thesis to test these two predictions and in so doing to cast light on possible mechanisms of clinical lactic acidosis. The first group of experiments were designed to investigate the effect of simulated metabolic acidosis on hepatic intracellular pH and lactate uptake in the isolated, perfused rat liver. In the second group of experiments, the isolated perfused guinea pig liver was used to investigate the effect of phenformin on lactate uptake, glucose production and hepatic intracellular pH.

1.2 LACTIC ACID METABOLISM

1.2.1 Historical background

Lactic acid was first detected in human and animal muscle tissue by Bertzalius in 1807 (Filachione, 1952). It had been originally isolated from sour milk by Scheele in 1780 (Schertel, 1965). A century later Wislicenus proved that lactic acid isolated from these two sources had the same chemical structure but had different chemical properties and suggested that lactic acid existed in two isomeric forms. Cori and Cori (1929) found that L(+) lactic acid was the metabolically active form in animals.

Claude Bernard (1877) was the first to suggest that lactic acid present in tissues after death was produced by the fermentation of sugar and glycogen. The term glycolysis was introduced by Lepine (1909) to describe the disappearance of carbohydrate during tissue metabolism. The glycolytic pathway was elucidated by the work of Fletcher and Hopkins (1906-7), Hill (1926) and Meyerhof (1930). Quantitative investigations of lactic acid production were made first by Warburg (1923) using tissue slice techniques. He demonstrated that the breakdown of one molecule of glucose resulted in the formation of two molecules of lactic acid. He also showed that lactate production is greater under anaerobic conditions than it is in the presence of oxygen.

The synthesis of glycogen from lactic acid in the liver was first demonstrated by Cori and Cori (1929) in fasted rats. On the basis of this observation they suggested that lactic acid

produced during muscular exercise was transported from muscle to liver where it was converted to glycogen. This liver glycogen could subsequently be broken down to glucose and provide an energy source for exercising muscle. Muscle and liver glycogen were thus shown to be linked via lactic acid.

1.2.2 Production of lactate

Lactic acid is the end product of glycolysis; the overall equation for this process is:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \longrightarrow 2\text{Lactic acid} + 2\text{ATP}$$

The main tissue sources of lactate are erythrocytes, brain, skeletal muscle and skin. Estimates of the contribution of these tissues to total lactate production in resting man have been made by various workers. Cahill and Owen (1968) estimated lactate production by incubated erythrocytes to be 0.30 mol/24 hr/70 Kg man. McIlwain and Bachelard (1971) determined lactate production of brain by measuring the arterio-venous difference and calculated the output to be 0.22 mol/24 hr/70 Kg man. Skeletal muscle output under resting conditions was estimated to be 0.20 mol/24 hr/70 Kg man (Andres, Cader and Zierler 1956), and the output of skin to be 0.37 mol/24 hr/70 Kg man (R.M.Fusaro and J.A. Johnson quoted by Kreisberg 1972). Other tissues, i.e. leucocytes, platelets and renal medulla, have been found to produce small amounts of lactate (Cahill and Owen 1968). These estimates, all of which must be regarded as being very approximate, suggest that the total lactate production in a resting 70 Kg man is in the order of 1.27 mol/24 h. Under non-resting conditions lactate production increases and this is

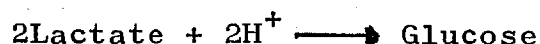
seen particularly during strenuous exercise when the blood lactate concentration can reach 22 mmol/l (Turrell and Robinson 1942).

The importance of glycolysis in mammalian tissue is twofold. Firstly, it provides a source of energy under anaerobic conditions; this is particularly important in exercising skeletal muscle and Margaria (1967) has shown that during maximal exercise, when the tissues are relatively anoxic, muscle is partly dependent on glycolysis as an energy source. Secondly, glycolysis is a source of precursors for other metabolic pathways principally the Krebs cycle. However, in the liver glycolysis is of little importance as a source of energy, but provides precursors for lipogenesis (Woods and Krebs 1971).

1.2.3 Utilization of lactate

Under aerobic conditions, lactate can either be oxidized to carbon dioxide and water or be resynthesised to glucose. Oxidation of lactate occurs in submaximally exercising muscle following maximal exercise and is probably important in clearing lactate from the circulation (Hermansen and Stensvold 1972).

Gluconeogenesis, i.e. the synthesis of carbohydrate from non-hexose sources of which the most important are lactate, glycerol and aminoacids, principally alanine and glutamine, occurs mainly in the liver and renal cortex. The overall equation for gluconeogenesis from lactate is



$$2\text{Lactate} + 2H^{+} \longrightarrow \text{Glucose}$$

Studies on lactate uptake by the isolated perfused,

starved rat liver have shown that the majority of lactate metabolized is converted to glucose (Hems, Ross, Berry and Krebs 1966; Exton and Park 1967). In the kidney at physiological pH, approximately 40% of the lactate metabolized is converted to glucose and the remainder is oxidized to carbon dioxide and water (Nishiitsutsuji-Uwo, Ross and Krebs 1967). Very few quantitative studies of lactate removal in man have been carried out. It can be calculated from the data of Rowell et al (1966) that the liver removes 0.72 mol/24 h /70 Kg man under resting conditions. This represents 57% of the estimated daily lactate production of 1.27 mol/70 Kg man.

Estimates of the rough order of magnitude of lactate uptake under non-resting conditions can be obtained by the extrapolation of the results of animal experiments to man. In the majority of these studies lactate uptake by the isolated perfused rat liver or kidney has been investigated under conditions in which lactate uptake would be expected to be increased (Woods and Krebs 1971; Söling, Willms, Kleineke and Gehlhoff 1970; Nishiitsutsuji-Uwo et al 1967). When these results are extrapolated to man they indicate that capacity of the lactate removal mechanisms enormously exceeds the resting lactate turnover. Yudkin and Cohen (1975) found that lactate uptake in the conscious fed rat was much greater than the sum of individual organ lactate removal. Extrapolation of their results to man gives a value for lactate uptake of 17.2 mol/24 h / 70 Kg man at a blood level of 10 mmol/l.

1.2.4 The control of lactate metabolism

The resting blood lactate level in man is maintained relatively

constant at 1 mmol/l and represents the balance between lactate production, i.e. glycolysis, and lactate utilization, i.e. gluconeogenesis and lactate oxidation. A knowledge of the pathways of glycolysis and gluconeogenesis and the rate-limiting steps in these pathways is essential to an understanding of the factors involved in the control of lactate metabolism.

1.2.4.1 The pathways of glycolysis and gluconeogenesis and their rate-limiting steps

The glycolytic and gluconeogenic pathways are shown in a simplified form in Fig. 1.

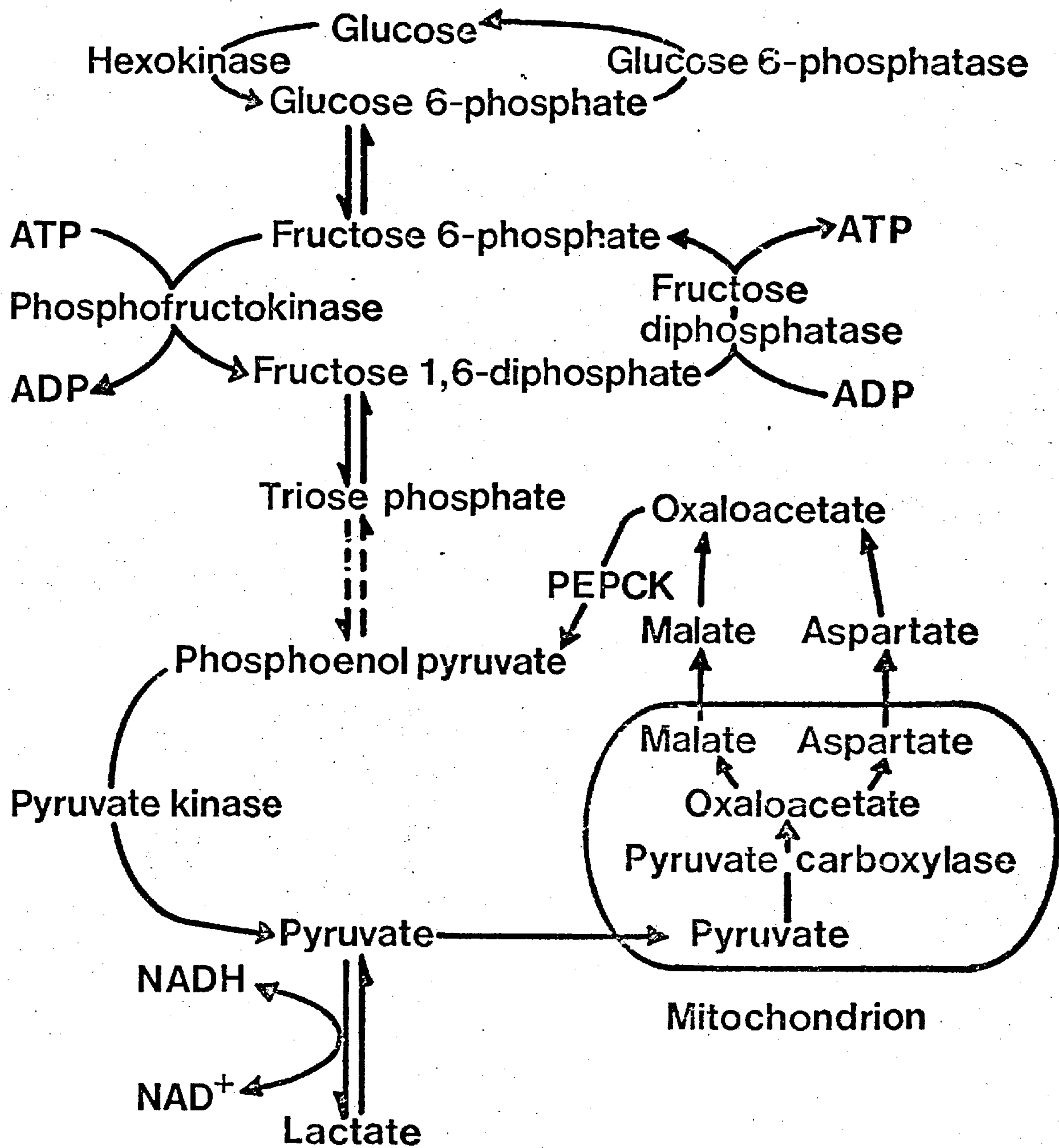
The majority of the reactions of the gluconeogenic pathway are the direct reversal of those of the glycolytic pathway. There are four non-equilibrium reactions which involve enzymes other than those of the glycolytic sequence. These reactions are:

(a) The conversion of pyruvate to oxaloacetate.
(b) The conversion of oxaloacetate to phosphoenolpyruvate.
(c) The conversion of fructose 1,6-diphosphate to fructose 6-phosphate.
(d) The hydrolysis of glucose 6-phosphate.

Evidence suggests that these are the rate-limiting steps in the glycolytic/gluconeogenic pathways (Newsholme and Gevers 1967).

1.2.4.1 (a) The conversion of pyruvate to phosphoenolpyruvate

During glycolysis, pyruvate kinase catalyses the



Pathways of glycolysis and gluconeogenesis in rat liver

Fig.1

conversion of phosphoenolpyruvate (PEP) to pyruvate. This reaction is strongly exergonic and therefore is not directly reversible. The direction of gluconeogenesis involves the enzymes pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK).

Pyruvate carboxylase catalyses the conversion of pyruvate to oxaloacetate and is one of the key regulatory enzymes in the gluconeogenic pathway (Scrutton and Utter 1968). It has an absolute requirement under physiological conditions for acetyl CoA and is inhibited by acetoacetate which destroys its interaction with acetyl CoA (Utter and Fung 1971). It is activated in the presence of hydroxybutyryl CoA. The rate of conversion of pyruvate to oxaloacetate is also influenced by substrate concentration, although the effect of activators and inhibitors is superimposed upon this (Utter and Fung 1971). Pyruvate carboxylase activity has important pH dependent characteristics which will be discussed later.

Evidence suggests that pyruvate carboxylase is confined to the mitochondria in rat, guinea pig and human liver (Böttger, Wieland, Brdiezka and Petts 1968; Scrutton and White 1974).

The next enzyme in the sequence, PEPCK, is present only in the cytosol in the rat liver. The mitochondrial membrane is impermeable to oxaloacetate (Haslam and Krebs 1968) and further reactions are therefore necessary to effect its transfer to the cytosol. Oxaloacetate is carried into the cytosol as malate when pyruvate is the substrate and as aspartate when the substrate is lactate (Shrago and Lardy 1966). Malate and aspartate are then reconverted to oxaloacetate. When malate is involved, reducing equivalents needed for the

further course of gluconeogenesis are also transferred. In the guinea pig and probably also in man, the sequence is slightly different as PEPCK is present in both the mitochondrion and cytosol (Nordlie and Lardy 1963) and there is evidence that both forms actively function during gluconeogenesis from lactate (Arinze, Garber and Hanson 1973).

Phosphoenolpyruvate carboxykinase, which is GTP dependent, converts oxaloacetate to phosphoenolpyruvate.

1.2.4.1 (b) The interconversion of fructose 6-phosphate and fructose 1,6-diphosphate

During glycolysis, formation of fructose 1,6-diphosphate (FDP) from fructose 6-phosphate (F6P) is catalysed by phosphofructokinase (PFK). The activity of this enzyme is inhibited by ATP and citrate and the inhibition is reduced by AMP, F6P, FDP and inorganic phosphate. The *in vitro* inhibitory effect of a given concentration of ATP increases with falling pH, within the physiological ranges of ATP concentration and cell pH (Ui 1966). There is much evidence that PFK is the rate limiting enzyme in the glycolytic pathway (Gevers and Dowdle 1963; Ui 1966; Scheuer and Berry 1967).

The enzyme involved in the gluconeogenic pathway is fructose diphosphatase which is inhibited by AMP. In the rat liver, its maximum activity is three times greater than PFK.

1.2.4.1 (c) Hydrolysis of glucose 6-phosphate

This reaction, which is catalysed by glucose 6-phosphatase, is important in the control of glucose

release by the liver. It is not relevant to the present discussion and will not be considered further.

1.2.4.2 The regulation of gluconeogenesis

The majority of the lactate taken up and metabolized by the liver is converted to glucose. Inhibition of this process of gluconeogenesis could lead to lactate accumulation and clinical lactic acidosis. As the work to be described in this thesis explores certain aspects of this hypothesis, factors which influence the rate of gluconeogenesis will be discussed in some detail.

1.2.4.2 (a) Factors acting on the rate limiting enzymes

The most important control site in the gluconeogenic pathway is between pyruvate and phosphoenol pyruvate (PEP) (Exton and Park 1969). In the liver, pyruvate carboxylase is a likely rate limiting enzyme (Scrutton and Utter 1968; Exton and Park 1969). The next enzyme in the gluconeogenic pathway, phosphoenolpyruvate carboxykinase (PEPCK), may also be rate limiting in the liver under certain conditions. In the kidney, evidence suggests that PEPCK may be the most important control site (Alleyne and Scullard 1969; Alleyne 1970).

Gluconeogenesis may be stimulated or inhibited by factors which act directly on these rate limiting enzymes. The pyruvate to PEP conversion step in the liver has been shown to be the most likely site of action of the hormones which stimulate gluconeogenesis, i.e. glucagon and adrenaline.

The effect of these hormones is probably mediated by adenosine 3':5'-cyclic monophosphate (cyclic AMP) which has been shown to exert its stimulatory action on the gluconeogenic pathway at the same site (Exton, Mallette, Jefferson, Wong, Friedman, Miller and Park 1970). The increased rates of gluconeogenesis which occurs during exercise and starvation is produced, at least in part, by the action of hormones on the gluconeogenic pathway. In the rat liver gluconeogenesis is stimulated by fatty acid oxidation (Ross, Hems, Freedland and Krebs 1967; Söling et al 1970). This may be explained by the activating effect of increased acetyl CoA levels, resulting from fatty acid breakdown on pyruvate carboxylase which, as discussed previously, has an absolute requirement for this cofactor. In the guinea pig liver gluconeogenesis is inhibited by fatty acid oxidation; the reasons for this will be discussed in the next section.

Inhibition of gluconeogenesis, which may be important in the pathogenesis of lactic acidosis, may result from direct inhibition of rate controlling enzymes. This will be discussed in greater detail later.

1.2.4.2 (b) <u>Hepatic redox status and the rate of gluconeogenesis</u>

The formation of lactate from pyruvate is expressed by the equation:

$$\text{Lactate} + \text{NAD}^+ \rightleftharpoons \text{Pyruvate} + \text{NADH} + \text{H}^+$$

At equilibrium:

$$\frac{[\text{Lactate}]}{[\text{Pyruvate}]} = K. \frac{[\text{NADH}].[\text{H}^+]}{[\text{NAD}^+]}$$

where K is the dissociation constant.

As lactate dehydrogenase is confined to the hepatic cytosol, determination of the lactate/pyruvate (L/P) ratio gives an indirect measurement of the cytoplasmic redox status (Hohorst, Kreutz and Bucher 1959).

The L/P ratio in the liver has been shown to be raised in conditions in which gluconeogenesis is increased, i.e. diabetes mellitus (Hohorst, Kreutz, Reim & Hübener 1961), and starvation (Wieland and Loffler 1963). This poses the question whether the change in the hepatic cytoplasmic redox status is the cause or the result of the increased rate of gluconeogenesis. Theoretically, an increase in the $\frac{NADH}{NAD^+}$ ratio could produce an increase in the rate of gluconeogenesis because of the requirement for NADH at the step involving the conversion of 1,3-diphosphoglycerate to 3-phosphoglyceraldehyde. If this were so, it could be postulated that the hepatic redox status was a controlling factor in the rate of gluconeogenesis.

However, evidence now suggests that the increase in the L/P ratio is the result of, rather than the cause of, increased gluconeogenesis. Ross, Hems and Krebs (1967), using the perfused rat liver, determined the rate of gluconeogenesis from lactate and then from pyruvate (each at a concentration of 10 mmol/l) and found that the rate of glucose formation was independent of the nature of the substrate and hence of the $\frac{NADH}{NAD^+}$ ratio. Further evidence is provided by the work of Hawkins, Houghton and Williamson (1973) who showed that the rate of gluconeogenesis from lactate in the rat liver is unaffected by the injection of crotonate which lowers

hepatic redox status.

The increase of the L/P ratio accompanying increased gluconeogenesis is now considered to be the effect of the decreased phosphorylation state of the adenine nucleotides on the $\frac{NADH}{NAD^+}$ ratio (Krebs 1971a). During gluconeogenesis 6 molecules of ATP are used, resulting in a decrease in the $\frac{ATP}{ADP}$ ratio in the liver. Theoretically, this can be shown to lead to an increase in the $\frac{NADH}{NAD^+}$ ratio, the phosphorylation and the cytoplasmic redox state of the cell being linked together by the reactions involving glyceraldehyde phosphate dehydrogenase, 3-phosphoglycerate kinase and lactate dehydrogenase.

Whilst there is no evidence that the redox state of the cytoplasmic NAD couple is important in the regulation of gluconeogenesis in the rat liver, it may have a controlling influence under some conditions in the guinea pig liver. Söling et al (1970), using the isolated perfused guinea pig liver, showed that gluconeogenesis from lactate was inhibited when hexanoate or oleate was added to the medium. They concluded from their series of experiments that the concentration of pyruvate was the most important controlling factor of gluconeogenesis from lactate or pyruvate in the guinea pig liver. An increase in the supply of reducing equivalents, as occurs in fatty acid oxidation, inhibits gluconeogenesis by shifting the lactate-pyruvate equilibrium and reducing the concentration of pyruvate.

Gluconeogenesis in the guinea pig liver may also be influenced by the $\frac{NADH}{NAD^+}$ ratio at the 1,3-diphosphoglycerate

to 3-phosphoglyceraldehyde conversion step. Söling et al (1970) demonstrated, again using the isolated perfused guinea pig liver, that gluconeogenesis from pyruvate was stimulated by the addition of ethanol or hexanoate to the perfusate. This suggests that gluconeogenesis from pyruvate is submaximal because of an inadequate supply of reducing equivalents and may therefore be stimulated by an increase in the $\frac{NADH}{NAD^+}$ ratio. However, the physiological significance must be interpreted with reservation in view of the fact that a high concentration of pyruvate (20 mmol/l) was used.

1.2.4.2 (c) The role of futile cycles in the regulation of gluconeogenesis

Under physiological conditions both enzymes involved in the interconversion of fructose 6-phosphate and fructose 1,6-diphosphate are active and there is continuous recycling of substrate through the pathway. Similarly there is substrate recycling through the pyruvate carboxylase/ phosphoenolpyruvate carboxykinase/pyruvate kinase pathway. These futile cycles involve some wastage of energy, but they do provide a possible control site of gluconeogenesis and also a site at which inhibitors of gluconeogenesis could operate.

Changes in futile cycles may be important in the production of an increased rate of gluconeogenesis during starvation. Friedman, Goodman, Saunders, Kostos and Weinhouse (1971) using the perfused rat liver preparation, showed that in fed rats 25% of ^{14}C labelled pyruvate introduced into the medium was converted to glucose and 75% was recycled. In

contrast to this, in fasted animals twice as much pyruvate was converted to glucose as was recycled. They concluded that pyruvate kinase was inhibited in the fasted rat and that this contributed to the observed increased rate of gluconeogenesis. Söling et al (1971) suggested that the observed differences in the regulation of gluconeogenesis in the rat and guinea pig may be due, at least in part, to the higher pyruvate kinase activity in the rat liver which results in a higher rate of futile cycling along the pyruvate/phosphoenolpyruvate pathway.

1.3 LACTIC ACIDOSIS

In the normal resting human subject the blood concentration of lactate is about 1 mmol/l. Under certain physiological conditions, e.g. exercise, this level may rise but then fall rapidly as the lactate is metabolized by the tissues. The blood lactate level may also be raised above normal and be associated with a metabolic acidosis as a result of pathological processes or the administration of drugs. This abnormal accumulation of lactic acid is termed lactic acidosis.

1.3.1 Historical background

The recognition of lactic acidosis as a clinical entity followed the development of a technique for the estimation of lactic acid in blood by Clausen in 1922. In the following year Barr, Himwich and Green (1923) showed that the metabolic acidosis which accompanied severe exercise was due to the accumulation of lactic acid in the blood and used the term lactic acid acidosis to describe the condition. The first cases of lactate accumulation associated with a disease process were described by Clausen (1925). He reported cases of children who had become dehydrated as a result of diarrhoea and who were found to have metabolic acidosis due to lactate accumulation. Studies on animals later demonstrated that lactic acidosis could be caused by drugs. Minot, Dodd and Saunders (1934) demonstrated that guanidine (a hypoglycaemic compound) produced lactate accumulation and a metabolic

acidosis in animals.

The current interest in lactic acidosis follows the studies of Huckabee (1961 a; 1961 b) on a group of patients who were found to have hyperlactaemia. As a result of these studies he produced a classification of lactic acidosis which will be discussed later.

1.3.2 Definition

There is no widely accepted definition of lactic acidosis and the criteria used in making the diagnosis vary considerably. Tranquada (1964) confined the term to those cases in whom the blood lactate level exceeded 7 mmol/l and there was a significant, but not defined, lowering of arterial pH. Other authors have accepted a lower lactate level and have defined the degree of acidosis. Peretz, Scott, Duff, Dossetor, MacLean and McGregor (1965) used the criteria of an arterial lactate concentration exceeding 1.3 mmol/l and an arterial pH below 7.30. The corresponding levels used by Oliva (1970) were an arterial lactate concentration in excess of 2 mmol/l and an arterial pH below 7.37.

Huckabee (1961 a) used the term lactic acidosis in the context of a metabolic acidosis which was entirely due to lactate accumulation. It is now recognised that organic acids other than lactate may contribute to the metabolic acidosis. In view of this and the fact that he excluded cases of metabolic acidosis associated with diabetes mellitus and renal failure, Huckabee's definition is considered to be too narrow. The same criticism may be levelled against the definitions which express blood lactate concentration in

numerical terms. A simpler and more useful definition would be 'a raised blood lactate concentration associated with a metabolic acidosis'.

1.3.3 Classification

During his systematic study of blood lactate and pyruvate levels in patients, Huckabee (1961a) identified a group of 37 patients with hyperlactaemia and divided them into two main groups on the basis of their lactate/pyruvate (L/P) ratios.

Group I had raised lactate and pyruvate levels, the L/P ratio being maintained within normal limits. The administration of glucose or intravenous bicarbonate may produce this metabolic picture.

Group II had increased lactate levels but pyruvate was not raised to the same extent and the L/P ratio was therefore above normal. This group of 24 patients was further divided into two types.

Type IIA included those patients in whom there was evidence of circulatory failure and tissue hypoxia resulting in increased production of lactate.

Type IIB included nine patients in whom there was no evidence of circulatory failure. They did however have other diseases, i.e. pneumonia, subacute bacterial endocarditis and poliomyelitis with respiratory muscle involvement, but the cause of their hyperlactaemia was not immediately obvious. Huckabee (1961b) used the term idiopathic or spontaneous lactic acidosis to describe this group of patients. Although they had no clinical evidence of circulatory failure,

Huckabee considered that the raised L/P ratio indicated tissue underperfusion which was subclinical. This conclusion has been questioned by many workers and their reasons for doing so will be discussed in greater detail later. In this thesis it is Type IIB lactic acidosis which is of the greatest interest, but the findings in the experimental models used are also relevant to Type IIA lactic acidosis.

1.3.4 Type IIB lactic acidosis

Lactic acidosis which occurs in the absence of signs of tissue underperfusion and hypoxia (i.e. Type IIB) is always associated with other conditions or drugs and can be further classified on this basis.

1.3.4.1 Subclassification of Type IIB lactic acidosis

There are three main subgroups:

(a) Lactic acidosis associated with common medical conditions.

(b) Lactic acidosis associated with the administration of drugs or toxic substances.

(c) Hereditary forms of lactic acidosis.

1.3.4.1(a) Common medical conditions associated with Type IIB lactic acidosis

Cohen and Woods (1976) in their survey of the literature on lactic acidosis identified 65 cases which fulfilled the criteria they listed for the diagnosis of Type IIB (or Type B as they termed it) and which were associated either with common diseases or drug administration. The associated conditions

recorded most frequently were diabetes mellitus, hepatic disease, uraemia and infections.

Of these 65 patients, 35 had diabetes and of these 28 were receiving phenformin. The association between lactic acidosis and phenformin therapy will be discussed in a separate chapter. Several series suggest that hyperlactaemia contributes to the metabolic acidosis in a significant proportion (about 10%) of acutely ill diabetic patients with hyperglycaemic coma but no clinical evidence of shock (Watkins, Smith, Fitzgerald and Malins 1969; Arieff and Carroll 1972).

Patients with liver disease often have somewhat elevated blood lactate and pyruvate concentrations (Mulhausen, Eichenholz and Blumentals 1967; Alberti 1972; Record, Alberti, Williamson and Wright 1973). However, it is usually only in severe liver disease (most frequently hepatic necrosis) that really high blood lactate concentrations are seen and these are usually associated with an alkalosis rather than a metabolic acidosis (Record, Iles, Cohen and Williams 1975).

Uraemia is frequently associated with Type IIB lactic acidosis and the association is most marked in patients who have received phenformin. Acute infections associated with lactic acidosis have most commonly involved the urinary tract. Septicaemia and bacterial endocarditis have also been recorded in association with lactic acidosis.

1.3.4.1(b) Drugs associated with lactic acidosis

A survey of the literature has shown that patients who develop Type IIB lactic acidosis had been receiving a wide variety of drugs at the time of onset of the condition (Cohen and Woods 1976). Of these drugs only phenformin, ethanol, fructose, sorbitol, xylitol and possibly metformin are considered to be a direct cause of lactic acidosis.

The drug most commonly associated with the development of lactic acidosis is phenformin: as this association is of particular interest in the work described in this thesis it will be discussed in a separate chapter. In contrast to phenformin, it is uncertain whether the other biguanide used in clinical practice, metformin, is associated with lactic acidosis. Only three cases of an apparent association have been reported (Lebacq and Tirzmalis 1972; Hayat 1974) and in only two of these can metformin definitely be implicated as the cause of lactic acidosis. Investigation of the effect of metformin on lactate metabolism in animals has produced conflicting results. Woods and Alberti (1973) found that its effects on lactate metabolism in the isolated perfused rat liver were similar to those of phenformin. Meyer, Ipaktchi and Clauser (1967) showed that metformin and phenformin inhibited gluconeogenesis in the intact rat and guinea pig and in rat kidney slices, although there was evidence that the mechanism of their inhibition was not identical.

Ethanol raises the blood lactate concentration in normal and alcoholic subjects but the increase is usually

small (Lieber, Jones, Losowsky and Davidson 1962). One severe case of lactic acidosis associated with ethanol intoxication has been reported (Oliva 1970). The hyperlactaemia produced by ethanol is probably the result of impaired hepatic gluconeogenesis from lactate (Krebs, Freedland, Hems and Stubbs 1969).

The association between fructose administration and lactic acidosis, hyperlactaemia or metabolic acidosis has been reported in seventeen patients (Cohen and Woods 1976). The high rate of lactate production from fructose in these patients is thought to be due to the activation of pyruvate kinase which results from the accumulation of fructose 1-phosphate and the depletion of adenine nucleotides which occur in the liver during fructose metabolism (Eggleston and Woods 1970).

1.3.4.1 (c) Hereditary forms of lactic acidosis

These are very rare and usually present in childhood. The episodes of lactic acidosis are commonly recurrent and the associated conditions are either neurological abnormalities, when the basic metabolic defects have yet to be understood, or recognized inborn errors of metabolism, i.e.glucose 6-phosphatase deficiency (Sokal, Lowe, Mosoviich and Dorey 1961) and hepatic fructose 1,6-diphosphatase deficiency (Baker and Winegrad 1970). These forms of lactic acidosis have been extensively reviewed by Cohen and Woods (1976).

A single case of chronic recurrent lactic acidosis occurring in an otherwise normal adult female has been reported (Sussman, Alfrey, Kirsch, Zweig, Felig and Messener 1970). The patient had a chronically elevated blood lactate

level which increased further and was associated with a metabolic acidosis after exercise and the ingestion of ethanol. Study of her near relatives suggested that this may have been the result of a familial metabolic defect which was not precisely identified.

1.3.4.2 Clinical features

Type IIB lactic acidosis always develops against a background of illness or drug therapy. The condition is characteristically of sudden onset and develops rapidly. Hyperventilation is invariably present and abdominal pain and vomiting may occur. Deterioration in the state of consciousness, coma and death eventually supervene. In contrast to the clinical picture in Type IIA lactic acidosis, the blood pressure is well maintained, the peripheries warm and there is no evidence of cyanosis. Terminally, 'shock' may develop and the two types then coexist.

1.3.4.3 Diagnosis

The diagnosis of Type IIB lactic acidosis is initially suggested by the findings of a metabolic acidosis associated with an increased anion gap. In the absence of uraemia, ketosis and salicylate poisoning this strongly suggests a diagnosis of lactic acidosis. The diagnosis finally rests on the findings of an elevated blood lactate level.

Cohen and Woods (1976) reviewed the biochemical findings in 82 patients with lactic acidosis. The group included phenformin and non-phenformin associated cases and patients with evidence of circulatory failure. All of the

patients had a blood lactate level exceeding 2 mmol/l, this figure coinciding with the definition used by Oliva (1970). In the phenformin-associated cases the blood lactate levels and the lactate/pyruvate ratios were significantly higher than in the non-phenformin associated group.

Other abnormal biochemical findings have been reported in isolated cases. These include elevated blood acetoacetate and 3-hydroxybutyrate levels (Barnardo, Cohen and Iles 1970; Alberti, Corbett, Hockaday and Williamson 1971), raised levels of plasma inorganic phosphorus (Tranquada 1964) and raised plasma alanine levels (Marliss, Aoki, Toews, Felig, Cannon, Kyner, Huckabee and Cahill 1972).

1.3.4.4 Treatment and progress

A variety of therapeutic regimes have been used in the management of Type IIB lactic acidosis. Methylene blue, which acts as an electron acceptor, was first used by Tranquada, Bernstein and Grant (1964) but its efficacy has not been proved. Glucose and insulin have been administered simultaneously (Johnson and Waterhouse 1968; Alberti and Hockaday 1972) but again the value of this regime is unproven. The most frequently used and apparently the most successful method of treatment is the administration of sodium bicarbonate. Large quantities of this, sometimes exceeding 1000 mmol, may be necessary to achieve correction of the acidosis and lowering of the blood lactate. The mechanism of its lactate lowering effect is uncertain and will be considered in more detail in the discussion.

The mortality of Type IIB lactic acidosis is high.

In an early series Tranquada (1964) found a mortality of 90% in 58 cases in whom treatment was attempted. Review of more recent cases reveals a lower mortality of 70-80% for non-phenformin cases and 50-60% for cases associated with phenformin (Cohen and Woods 1976).

1.3.4.5 Pathogenesis

Accumulation of lactate may be due to its overproduction, failure of removal or to a combination of these factors. In patients with Type IIA lactic acidosis, evidence for overproduction of lactate has been provided by Sriussadaporn and Cohn (1968). In patients with Type IIB lactic acidosis clinical evidence of the underlying defect, be it overproduction of lactate or failure of its removal, is lacking. The following discussion of the pathogenesis of this type of lactic acidosis is therefore speculative.

1.3.4.5(a) Overproduction of lactate

Theoretically, this could be due to subclinical underperfusion of tissues, impaired release of oxygen from the erythrocytes, a defect of electron transport or uncoupling of oxidation and phosphorylation.

Huckabee (1961b) originally favoured the overproduction of lactate theory and considered that Type IIB lactic acidosis was due to subclinical underperfusion of tissues. The diagnosis of Type IIB lactic acidosis is made on the basis of the lack of clinical evidence of tissue underperfusion, the patients having no evidence of hypotension and warm peripheries. The problem is whether these signs indicate

adequate perfusion of all tissues. Huckabee (1961b) believed that they did not and demonstrated in dogs that progressive haemorrhage led to lactic acidosis before there was a measurable fall in cardiac output and blood pressure. Sriussadaporn and Cohn (1968) also found in dogs that progressive haemorrhage was followed by lactate accumulation before blood pressure fell and they demonstrated that hepatic and lower extremity lactate production accompanied moderate haemorrhage and that when haemorrhage was more severe the kidney also produced lactate. The same authors carried out the only reported study of regional lactate production in a patient with presumed Type IIB lactic acidosis and demonstrated renal production of lactate in this patient. They concluded that lactate production resulting from occult regional underperfusion at least contributed to the development of Type IIB lactic acidosis. In conclusion, it may be said that there is no conclusive evidence that Type IIB lactic acidosis is due to overproduction of lactate by subclinically under-perfused tissues. Indeed, the experimental findings of Huckabee and of Sriussadaporn and Cohn could be attributed to the failure of lactate removal as well as to overproduction by underperfused tissues.

Impaired release of oxygen from erythrocytes could theoretically give rise to lactate accumulation in the absence of clinical evidence of underperfusion. The oxygen dissociation curve has been studied in only two patients with Type IIB lactic acidosis (Huckabee 1961b),and it was found that in both patients the curve was moved slightly to the right which actually improved the release of oxygen to the tissues and

which could be attributed solely to the acidosis.

Lactate overproduction could also theoretically occur if there was a defect in the electron transport chain or an uncoupling of oxidation and phosphorylation. If the latter sequence of events occurred, lactate oxidation would continue but ATP levels would fall and this could lead to increased phosphofructokinase activity and hence to increased production of lactate. It has been suggested that phenformin blocks electron transport (Steiner and Williams 1958; Wick, Larson and Serif 1958) or interferes with the coupling of oxidation and phosphorylation (Falcone, Mao and Schrago 1962) and that this may be the underlying defect in phenformin-induced lactic acidosis.

1.3.4.5 (b) Failure of lactate removal

The evidence to support the hypothesis that Type IIB lactic acidosis is due to failure of lactate removal is once again indirect. It has been shown by Cohen and Woods (1976) that if the mechanisms of lactate removal suddenly failed completely, the rate of lactate production by skeletal muscle, brain and erythrocytes could account for the observed rate of rise of blood lactate in patients with Type IIB lactic acidosis.

Lactate is removed from the circulation by the liver and kidneys and the association of disease of these organs with Type IIB lactic acidosis is well documented. Mulhausen, Eichenholz and Blumentals (1967) described two patients with cirrhosis of the liver who developed lactic acidosis. Perrett,

Enrico, Montani and Pappalardo (1967) reported five patients with lactic acidosis who were found to have hepatic necrosis at post mortem. Tranquada (1964) collected 71 patients with lactic acidosis and of these 18 had hepatic disease. The association between phenformin induced lactic acidosis and hepatic disease has been noted on many occasions and will be discussed later.

The finding of a raised blood urea has been common in the reported cases of Type IIB lactic acidosis particularly in those associated with phenformin therapy. Sriussadaporn and Cohn (1968) produced evidence of failure of lactate removal, and actual lactate production by the kidneys in the patient with presumed Type IIB lactic acidosis whom they studied.

The other main piece of indirect evidence to support the failure of removal theory is the observed favourable response to treatment with large quantities of sodium bicarbonate. It has been demonstrated in both man and animals that metabolic and respiratory alkalosis is associated with a rise in blood lactate, presumably by stimulation of glycolysis at the step involving phosphofructokinase. The observed fall in blood lactate following alkalinisation of patients with Type IIB lactic acidosis would be difficult to explain if overproduction of lactate was the basic abnormality. If, however, lactate accumulation was the result of failure of lactate removal it can be postulated that bicarbonate lowers blood lactate by correcting the defect in the lactate removal mechanism. This hypothesis will be dealt with at greater length in the discussion.

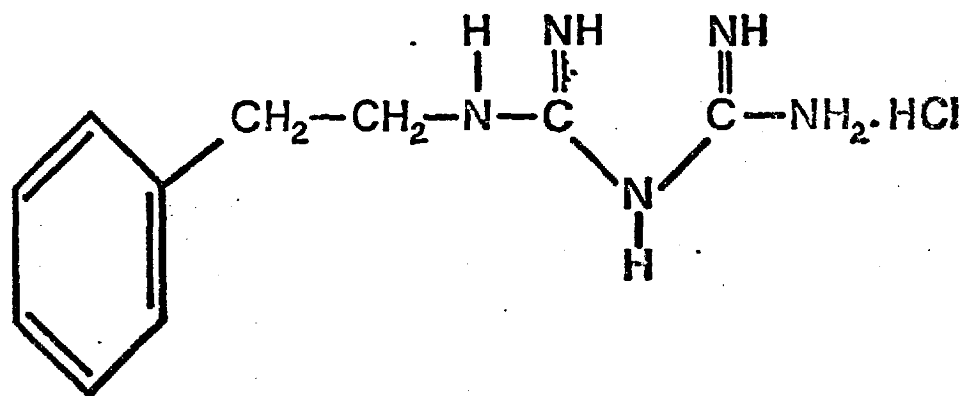
1.4 PHENFORMIN AND LACTATE METABOLISM

Phenformin (phenethylbiguanide hydrochloride) is a drug used in the treatment of diabetes; its formula is shown in Fig. 2. It is derived from guanidine, the hypoglycaemic properties of which were first suggested by Watanabe in 1918. Ungar, Freedman and Shapiro (1957) were the first to investigate the properties of phenformin and their work was followed by the introduction of phenformin into clinical medicine.

1.4.1 Mode of action of phenformin

Initial observations on the mode of action of phenformin indicated that, unlike the sulphonylureas its hypoglycaemic action was not due to stimulation of insulin secretion. It was also observed that it did not cause hypoglycaemia in normal subjects and that its use in diabetic subjects was often associated with a small rise in the resting blood lactate level. The hypotheses of its mode of action fall into three main groups:-

1. That it increases peripheral glucose utilization by stimulation of anaerobic glycolysis.
2. That it inhibits gluconeogenesis.
3. That it impairs intestinal absorption of glucose. This is unlikely to contribute significantly to its hypoglycaemic properties and will not be discussed further.



Phenethylbiguanide hydrochloride

Fig. 2 Chemical formula of phenformin

1.4.1.1 Effect on peripheral glucose utilization

The early observation that guanidine intoxication in dogs was associated with accumulation of lactic acid (Minot et al 1934) and that Synthalin A (another guanidine derivative) depressed oxygen consumption (Bodo and Marks 1928), suggested that guanidine compounds might cause hypoglycaemia by depressing cellular respiration. Inhibition of aerobic metabolism would cause accelerated anaerobic glycolysis by the Pasteur effect, and this would explain both the observed hypoglycaemia and raised blood lactate levels.

In vitro experiments have provided evidence that phenformin inhibits oxidative phosphorylation in respiring mitochondria. The actual site of action is unknown. It has been suggested that phenformin blocks electron transport between succinate and cytochrome c by inhibiting cytochrome oxidase (Steiner and Williams 1958), cytochrome b (Pressman 1963) and/or succinic oxidase (Wick, Larson and Serif 1958). Alternatively, Falcone et al (1962) claimed that phenformin does not block electron transport but interferes with the coupling of oxidation and phosphorylation.

Indirect evidence of the inhibitory effect of phenformin on oxidative processes is provided from studies on the isolated perfused guinea pig liver by Altschuld and Kruger (1968). They found a decrease in ATP levels when the liver was perfused with a medium containing phenformin.

Although this hypothesis is an attractive one there is evidence that it is not entirely satisfactory:-

1. The results of *in vitro* experiments discussed above

could be obtained only with high concentrations of phenformin equivalent to 20-30 times the blood level found in patients receiving the drug.

2. The longer chain alkyl biguanides, e.g. C_9-C_{12} are even more potent inhibitors of tissue respiration and yet they do not produce hypoglycaemia (Davidoff 1970).

1.4.1.2. Effect of phenformin on gluconeogenesis

Phenformin is concentrated and metabolized in the liver (Hall, Ramachander and Glassman 1968) which is the most important site of gluconeogenesis. It was demonstrated by Nielsen, Swanson, Tanner, Williams and O'Connell (1958) that phenformin decreased hepatic output of glucose in the intact guinea pig. However, they had to postulate that the liver was not the only site of action, as phenformin also produced hypoglycaemia in the eviscerated animal.

Evidence for the suppression of gluconeogenesis by phenformin has been provided by work on the isolated perfused guinea pig liver by Altschuld and Kruger (1968). They demonstrated that low doses of phenformin inhibited gluconeogenesis from lactate and glycerol. This effect was not observed when rat livers were used. Meyer, Ipaktchi and Clauser (1957), using starved intact guinea pigs found that phenformin suppressed glucose formation from $[^{14}C]$ pyruvate and $[^{14}C]$ alanine.

The mechanism of this observed suppression of

gluconeogenesis is uncertain. Altschuld and Kruger (1968) and Patrick (1966) considered that it was secondary to an inhibition of energy metabolism, having found decreased tissue levels of ATP. However, Toews, Kyner and Cannon (1970) and Haeckel and Haeckel (1972) did not confirm this observation and produced evidence, from cross-over studies, that phenformin inhibits either 3-phosphoglycerate kinase or glyceraldehyde phosphate dehydrogenase.

1.4.1.3 Clinical studies

Glucose kinetic studies in normal human subjects suggest that phenformin may affect both lactate production and gluconeogenesis from lactate. Kreisberg, Pennington and Boshell (1970) demonstrated, using ^{14}C labelled glucose, that in normal subjects given phenformin the conversion of glucose to lactate is accelerated but that this process is balanced by an accelerated recycling of lactate back to glucose and hence hypoglycaemia does not occur. In obese patients acceleration of glucose synthesis is less marked.

1.4.2 Effect of phenformin on lactate metabolism

There is substantial evidence that phenformin alters lactate metabolism in both animal and human subjects.

1.4.2.1 Animal studies

Phenformin produces a rise in fasting blood lactate levels in rats (Lacher and Lasagna 1966; Ramachander, Finkelman, Glassman and Sadow 1968). Lacher and Lasagna also found that the ability of the animal to metabolise an exogenous lactate

load was impaired. The latter observation was not confirmed by Ramachander et al (1968). Using the isolated perfused rat liver, Woods (1970) found that phenformin at a concentration of 0.1 mmol/l inhibited lactate uptake; at a concentration of 2 mmol/l glucose uptake and lactate production occurred.

1.4.2.2 Clinical studies

Diabetics treated with phenformin have been found to have a small but significant increase in resting blood lactate levels (Craig, Miller, Woodward and Merick 1960). Varma, Heaney, Whyte and Walker (1972) confirmed this observation and also found that blood lactate levels rose excessively during a glucose tolerance test. After the withdrawal of phenformin they found that both fasting lactate levels and those observed after the administration of glucose were similar to those seen in diabetics not treated by phenformin. Other workers (Güttler, Petersen and Kjeldsen 1963) have found no difference either in resting blood lactate levels or in levels observed during exercise in phenformin and non-phenformin treated diabetics. These discrepancies may be due to differences in the dose of phenformin employed and/or the degree of diabetic control in the patients studies (Walker, Linton and Thomson 1960).

1.4.2.3 Lactic acidosis

In view of the observed effects of phenformin on lactate metabolism it is not surprising that phenformin administration may be associated with lactic acidosis. Early reports doubted this association and suggested that the connection was between

diabetes and lactic acidosis, the treatment with phenformin being incidental. Evidence that refutes this is twofold:-

1. The majority of patients who develop lactic acidosis do so within two months of commencing phenformin. This would be an unexpected finding if lactic acidosis was associated only with their diabetic state (Cohen, Ward, Brain, Murray, Savege & Iles, 1973; Wise, Chapman, Thomas, Clarkson, Harding & Edwards, 1976).
2. Cases have been reported of non-diabetic people who have taken very large doses of phenformin in a suicide attempt and who have subsequently developed lactic acidosis (Bingle, Storey & Winter, 1970); (Cohen et al 1973).

Diabetic patients who develop lactic acidosis associated with phenformin almost invariably have impaired renal and/or hepatic function. It is of note that these are the two organs concerned both with gluconeogenesis and the metabolism and excretion of phenformin (Hall et al 1968). Wise et al (1976) also found a high incidence of cardiovascular disease in their series of fifteen diabetic patients with phenformin induced non-ketotic lactic acidosis. They suggested that hepatic congestion in these patients could have impaired lactate removal.

There are several observed differences between phenformin induced lactic acidosis and that associated with other conditions. When phenformin is the cause, the metabolic acidosis tends to be more severe and the lactate level and L/P ratio tend to be higher. However, response to treatment is often better and the mortality is lower than in other types (Cohen & Woods 1976).

1.5. INTRACELLULAR pH

The study of acid base changes in the extracellular compartment of an organism is relatively easy, but they do not necessarily represent the changes which are occurring in the tissues. It is in the intracellular compartment that acid-base changes exert their most important effects and, therefore, knowledge of intracellular pH is essential to an understanding of the inter-relationship of pH and cellular functions. Measurement of intracellular pH is an important element in the work reported in this thesis.

1.5.1. Measurement of intracellular pH

1.5.1.1. Historical background

Many experimental approaches have been used in the determination of intracellular pH. In the early years of this century methods of measurement of cell pH involved the determination of the pH of tissue extracts or expressed cell sap using pH sensitive colour indication or platinum/hydrogen electrodes. All these methods involved destruction of the cells and thus the validity of the results was questioned. Caldwell (1958) developed glass microelectrodes and used them to determine the pH of squid giant axons and individual crab muscle fibres. Since then further refinements have been made to the electrodes, but consideration of their use is not relevant to the present discussion.

Warburg (1922) studied the distribution of carbonic acid across the erythrocyte membrane and used it to determine a value for the intracellular pH of the erythrocyte. Since then methods based on the distribution of weak electrolytes have been widely used in the measurement of intracellular pH.

1.5.1.2. Methods based on the distribution of weak electrolytes

These methods depend on the assumption that at equilibrium the concentrations of the unionized form in the intracellular and extracellular compartments are equal. This assumption would hold either if the membrane was impermeable to the ionized form or if the hydrogen ions were distributed according to a Donnan equilibrium in which case the relative permeabilities of the two forms are irrelevant (Roos, 1965). The concentration of the ionized form will depend on the pH of the compartments. Thus the intracellular pH can be calculated if the total concentration of the indicator in the intracellular and extracellular compartments and the external pH are known.

The carbonic acid/bicarbonate system was the first indicator to be used in this way for the measurement of the cell pH of a variety of tissues (Robson, Bone and Lambie 1968). The use of the carbon dioxide method was criticised by Conway and Fearon (1944). They claimed that the carbon dioxide released by acid from the tissues was not all present in the form of bicarbonate and dissolved carbon dioxide, and thus falsely high values for pH_i were obtained. Although their criticisms have been refuted

(Butler, Waddell and Poole 1967), other indicators are now used which have several advantages over the carbon dioxide method.

1.5.1.3 The DMO method

5,5-dimethyloxazolidine-2,4-dione (DMO) is a weak organic acid; its formula is shown in Fig.3a. It is a metabolite of the anticonvulsant drug Trimethadione and was first used for the determination of intracellular pH by Waddell and Butler (1959). Its advantages over carbonic acid are that it is not metabolized and that it can be isotopically labelled, thus making analytical determination relatively easy and accurate. Another distinct advantage is that there is no doubt about the chemical identity of the substance determined by the analytical process. DMO has a dissociation constant, pK of 6.13.

1.5.1.3 (a) Theory of the DMO method

Fig. 3b illustrates intracellular and extracellular compartments separated by a membrane which is permeable only to the unionized form of DMO. Within each compartment DMO will ionize according to the equation:-

$$HDMO \rightleftharpoons H^+ + DMO^-$$

For simplicity let $[HDMO] = U$ i.e. the concentration of the unionized form

and $[DMO^-] = I$ i.e. the concentration of the ionized form

Let R = the total concentration of DMO i.e. [HDMO + DMO^-]

Let the subscripts e and i represent the extracellular and intracellular spaces respectively.

Let K represent the dissociation constant K_a of DMO at 37°C.

Let H represent the hydrogen ion activity.

From the law of Mass Action :-

$$K = \frac{HI}{U}$$

Assuming that the dissociation constant is the same for intracellular and extracellular compartments:-

$$K = \frac{H_e I_e}{U_e} = \frac{H_i I_i}{U_i} \qquad \ldots\ldots\ldots\ldots (1)$$

By definition $R_e = I_e + U_e$ . . . . (2)

and $R_i = I_i + U_i$

From equation (1) and (2)

$$U_e = \frac{H_e R_e}{K + H_e} \qquad U_i = \frac{H_i R_i}{K + H_i} \qquad \ldots\ldots\ldots (3)$$

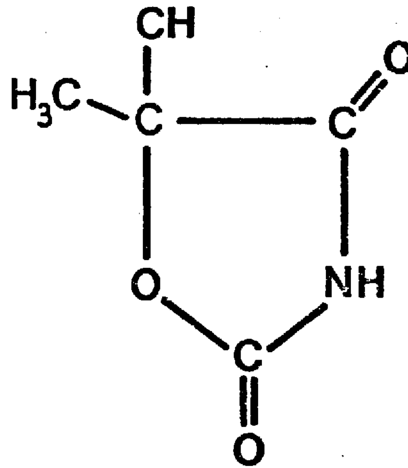
At equilibrium $U_e = U_i$

From equation (3)

$$\frac{H_e R_e}{K + H_e} = \frac{H_i R_i}{K + H_i} \qquad \ldots\ldots\ldots\ldots (4)$$

Re-arranging equation (4) :-

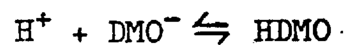
$$\frac{H_e}{H_i} \cdot \frac{H_i + K}{H_e + K} = \frac{R_i}{R_e} \qquad \ldots\ldots\ldots\ldots (5)$$



5·5-dimethyloxazolidine-2,4-dione (DMO)

Fig. 3a Chemical formula of DMO

Extracellular	Intracellular
H_e	H_i
DMO_e^-	DMO_i^-
$HDMO_e$ ⇌	$HDMO_i$
Total DMO = R_e	Total DMO = R_i



Assumption:- $HDMO_e = HDMO_i$

$$\frac{R_e}{R_i} = \frac{H_i}{H_e} \cdot \frac{H_e + K}{H_i + K}$$

Fig. 3b Two-compartmental model of DMO distribution

The dissociation constant, K, of DMO is known, and the extracellular hydrogen ion concentration (H_e), and the total concentration of DMO in the extracellular compartment (R_e) can be directly measured. The total intracellular concentration of DMO (R_i) can be calculated from the total tissue DMO, total tissue water, extracellular water and the plasma water concentration of DMO (R_e). Intracellular hydrogen ion concentration (H_i) can therefore be calculated from equation (5).

In the method described by Waddell and Butler (1959), chloride was used for the measurement of extracellular space. The chloride and DMO concentrations were determined by chemical methods which were laborious and time consuming. Schloerb and Grantham (1965) introduced a technically simpler method using isotopically labelled DMO, tritiated water and ^{36}Cl. Their method has been used in the present work, the only difference being that ^{14}C labelled inulin was used for the measurement of extracellular space instead of ^{36}Cl.

It has been shown by a number of authors that the use of a weak acid indicator measures a type of 'mean' intracellular pH which is more biased toward the more alkaline intracellular compartment than is a simple volume weighted arithmetic mean (Adler, Roy and Relman 1965; Robson et al 1968; Waddell and Bates 1969; Adler 1972). Conversely the use of a weak base indicator gives a value more biased towards the acid side. The difference in values for intracellular pH obtained by the use of a weak base and a

weak acid has been used as an index of intracellular heterogenicity of pH (Adler 1972).

1.5.1.3 (b) Validity of the DMO method

The basic assumption of the DMO method, that at equilibrium the concentrations of unionized DMO in the intracellular and extracellular compartments are equal, would be invalidated if there was active transport of DMO across the cell membrane or protein binding of DMO within the cell. Critics of the method have produced evidence that this occurs and their work will be considered in greater detail, and with particular reference to the hepatocyte, in the discussion. However, evidence that the DMO method does give valid measurements of intracellular pH has been obtained using several different experimental approaches. This evidence is summarised below:

1. Measurements of pH_i in skeletal muscle and brain under widely varying conditions using the DMO method have given similar results to those obtained when the carbon dioxide method was used (Roos 1965; Roos 1971; Butler, Waddell & Poole 1967). This would not be expected if active mechanisms were involved in the transport of DMO into the cell, since it would be very unlikely that DMO and carbonic acid were transported in a quantitatively identical fashion.
2. pH_i values obtained using the DMO method are independent of the total concentration of DMO

used (Miller, Tyson & Relman 1963; Zieve and Solomon 1966). This again argues against the active transport of protein binding of DMO. There is, however, some evidence for active transport of DMO across certain epithelial surfaces, e.g. gut (Dietschy and Carter 1965), and meninges (Rollins and Reed 1970).

3. Measurements of the pH of the plasma and coelomic fluids of the spiny dog fish, which differ by about 2 pH units, have been made using the DMO method and glass electrodes. The results agree very well, providing further evidence of the validity of the DMO method (Robin 1963).

4. Albers, Ludwig, Usingar and Spaich (1971) used the DMO method to measure whole body intracellular pH and used the value obtained to calculate intracellular bicarbonate concentration. Their results correlated well with values obtained by direct measurement by other workers, suggesting that the value for intracellular pH determined by the DMO method was valid.

1.5.2. Control of intracellular pH

1.5.2.1. Evidence that hydrogen ion distribution is not in electrochemical equilibrium

There is extensive evidence that the distribution of hydrogen ions across a cell membrane is not determined by the Donnan equilibrium. Studies on skeletal muscle, using both

the carbon dioxide method and microelectrodes have shown that, at an external pH of 7.4 the intracellular pH was higher (6.8-7.0) than 6.0 which is the value calculated from the Nernst equation (Wallace and Hastings 1942; Caldwell 1958) Conway and Fearon (1944) challenged these findings and criticised both the carbon dioxide method and the use of microelectrodes. Using the former method and making allowances for the non-barium-precipitable fraction of the acid labile carbon dioxide which they considered was derived from non-bicarbonate sources they obtained a value for the pH_i of skeletal muscle of 6.0 which is in keeping with the Donnan equilibrium. However, their work has been re-examined and criticised by Butler et al (1967). They considered that acid labile carbon dioxide is derived only from bicarbonate sources and that the higher pH_i value of 6.8-7.0 is valid.

A study of rat muscle pH_i using glass microelectrodes also suggested that hydrogen ions were in thermodynamic equilibrium across the cell membrane (Carter, Rector, Campion and Seldin 1970). But the work has been criticised and numerous other microelectrode studies in many species have revealed pH_i to be considerably higher than the value obtained from the Nernst equation at the prevailing membrane potential (Caldwell 1958; Paillard 1972; Thomas 1974).

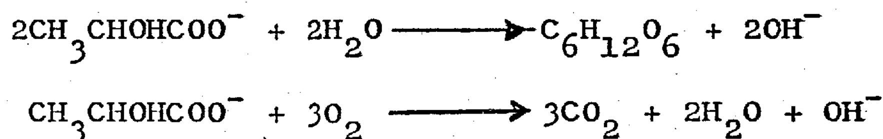
1.5.2.2 Factors controlling intracellular pH

Cellular metabolism and pH are inter-related; almost all enzymes are influenced by changes in pH and have pH optima and many metabolic processes release protons. There is a lot

of evidence to suggest that metabolic processes are involved in the control of intracellular pH.

Walker, Goodwin and Cohen (1969) found that the intracellular pH of the rat liver was 7.23 which was significantly higher than that of skeletal muscle ($pH_i = 6.93$) and whole body ($pH_i = 6.88$). It had previously been suggested (Cohen, Goodwin and Strunin 1967) that organs metabolizing lactate might tend to have high cell pH for the following reason.

Lactic acid has a pK value of 3.86 and is therefore completely dissociated at physiological pH. If part of the entry of lactate into the liver is as the lactate ion, the equation of the two metabolic pathways which it may follow may be written:



$$2CH_3CHOHCOO^- + 2H_2O \longrightarrow C_6H_{12}O_6 + 2OH^-$$

$$CH_3CHOHCOO^- + 3O_2 \longrightarrow 3CO_2 + 2H_2O + OH^-$$

In each case hydroxyl ions are produced which would be expected to cause a rise in intracellular pH. It is supposed that electrical neutrality is preserved in the short term by the movement of ions other than H^+, OH^- or buffer anions. Eventually most of the hydroxyl ions would leak out of the liver cell and this would increase the buffering capacity of the body. Support for this hypothesis comes from the work of Cohen et al (1971). Using the isolated perfused rat liver they demonstrated that an increase in the rate of lactate metabolism, induced by changing the lactate content of the perfusate, was associated with a rise in pH_i.

In their studies of rat brain exposed to different

concentrations (6 - 40 per cent) of CO_2 *in vivo* Folbergrova, MacMillan and Siesjö (1972a) found that hypercapnia resulted in a decrease in the tissue levels of lactate, pyruvate and other organic acids. They observed that the regulation of pH_i in the face of a rising CO_2 tension was better than would be expected if physiochemical buffering alone controlled pH_i (Siesjö, MacMillan and Folbergrova 1972a). They attributed this to the "consumption" of the metabolic acids removing hydrogen ions from the intracellular space. During hyperventilation, lactate and pyruvate levels in the brain increased and again this was considered to be important in the regulation of pH_i. Although tissue levels of fructose diphosphate were not measured, they considered that the observed metabolic changes were the result of phosphofructokinase activation and inhibition by rising and falling pH.

Siesjö and Messeter (1971) grouped the factors controlling pH under three headings:

(i) Physiochemical buffering.

(ii) Consumption or production of non-volatile acids.

(iii) Transmembrane fluxes of H^+ and HCO_3.

They considered, on the basis of the rat brain experiments discussed above, that metabolic processes occurring within the cell, i.e. (ii), were particularly important in the initial control of pH_i.

2. METHODOLOGY

2.1. THE TECHNIQUE OF ISOLATED LIVER PERFUSION

The perfused rat liver preparation provides a convenient means of investigating hepatic function and has been used extensively in a wide variety of studies since it was first described by Corey and Britton (1941). The technique used in the present study is a modified version of that described by Exton and Park (1967). The liver was perfused at constant flow rather than constant pressure, which was the technique employed by Hems, Ross, Berry and Krebs (1966).

2.1.1. The perfusion circuit (Figs. 4-6)

The perfusion was carried out in a perspex box measuring (42x42x42 cm), split horizontally into top and lower portions. The upper surface of the top portion had a removable section which formed a lid. The front face of the lower portion could be lowered on hinges. Removal of the top lid and lowering of the front face enabled manipulation of the preparation and circuit during the course of the perfusion. The preparation rested on a perspex tray placed on rails situated at the level of contact of the upper and lower portions: the tray could be moved laterally on the rails. The box was heated by a heating element and fan, which were mounted on a lateral wall of the box. The

internal temperature was controlled by a thermostat which was set at 37°C. The air within the box was humidified by means of a bowl of water containing several gauze wicks draped over the side, placed on the floor of the box.

The perfusion medium was contained within a reservoir which also functioned as an oxygenator. The cross section of this part of the perfusion circuit is shown in semi-diagrammatic form in Fig. 6.

The oxygenator/reservoir consisted of a wide tube of perspex (length 15 cm., internal diameter 14 cm.). The ends of the tube were held in place by screws and the joins were sealed by rubber O-rings. At one end of the reservoir there was a central hub which was rigidly fixed to a bracket attached to the floor of the box and around which the reservoir rotated. At the opposite end of the reservoir there was a central pin which was coupled to an electric motor placed outside the box and which rotated the reservoir at 60 rpm.

The lines carrying the perfusion medium to and from the reservoir were attached to tubes inserted into the central hub. Gases also entered and left the reservoir at the central hub. Before entering the reservoir the gases were humidified by bubbling them through water contained in a bottle placed on the floor of the box. The gas flow rate was 0.5 - 1l/minute.

The rest of the circuit was constructed of opalescent polyvinyl chloride tubing, O.D. 4.5 mm., I.D. 3.0 mm (Porter No. 6. Portex Plastics Ltd., Hythe, Kent). The permeability

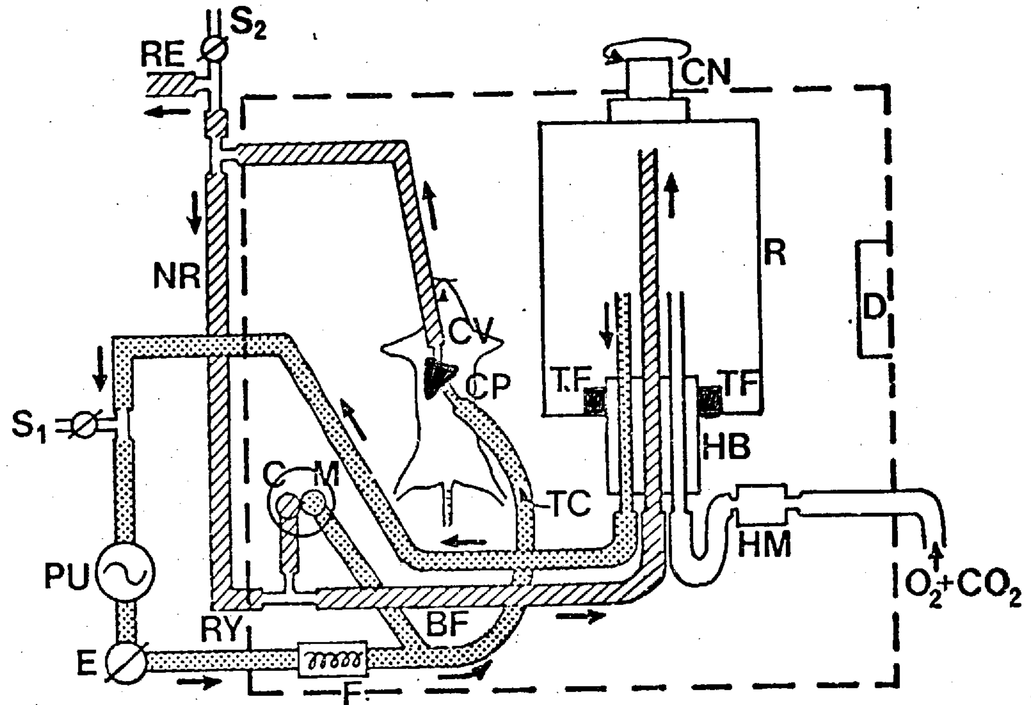


Fig. 4 Semidiagrammatic view of perfused liver preparation from above.

R - oxygenator/reservoir

HM - humidifier; D - heater; HB - hub; CN - centre pin

TF - Teflon seal; M - manometer

C - chimney; F - filter; BF - bubble trap

TC - thermocouple; S_1, S_2, E - sampling points

RE - medium exit; NR - position of clamp during non-recirculation.

PU - pump; RY - re-entry into box

CP - portal cannula; CV - vena caval cannula

— — — represents the perspex box

(hatched) represents venous circuit

(dotted) represents arterial circuit

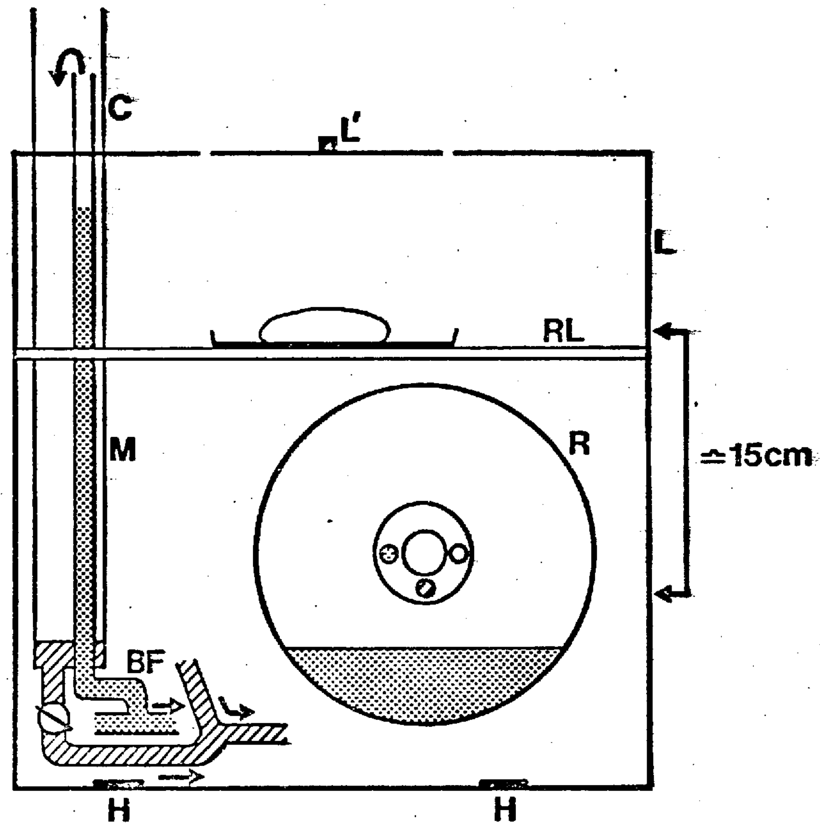


Fig. 5 Semidiagrammatic front view of some parts of the circuit.

L - lid; L' - subsidiary lid; T - tray

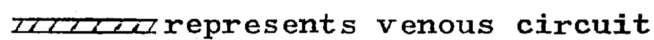
RL - rail; C - chimney; M - manometer

R - oxygenator; P - perspex box; BF - bubble trap

H - hinges.

1 - medium exit; 2 - medium entry

3 - gas entry

 represents venous circuit

represents arterial circuit

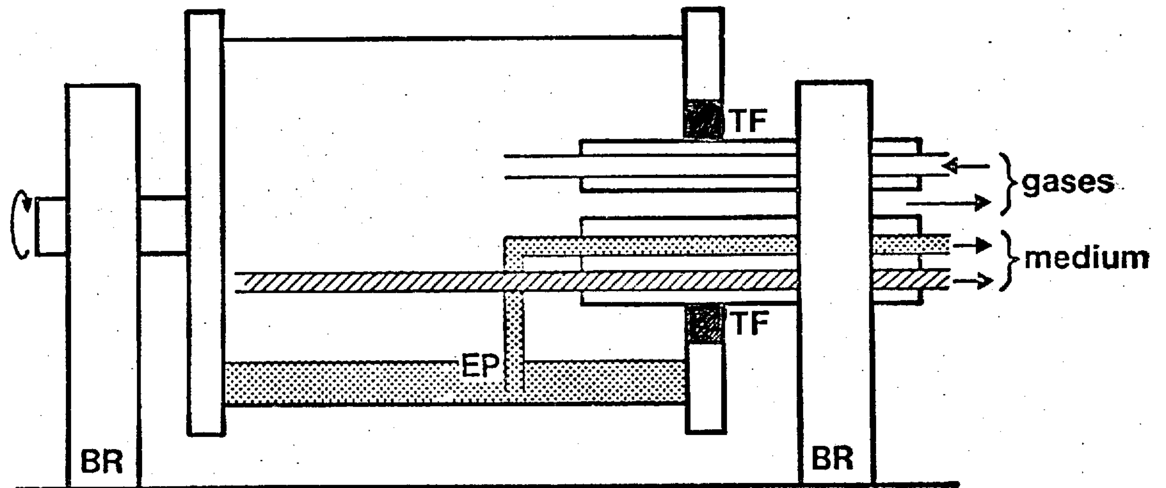


Fig. 6 Semidiagrammatic side view of oxygenator/reservoir

TF - Teflon seal; Br - bracket;

EP - Exit point

of the tubing to the gases was tested and negative results were obtained. The circuit also contained T junctions and a bubble trap which were made of glass and supplied for Technicon autoanalyser equipment (Technicon Instruments Co. Ltd., Basingstoke, Hants.). The three-way taps included in the circuit were supplied by Pharmoseal Laboratories, Glendale, California, U.S.A.

The medium was pumped from the reservoir to the liver via the portal vein by a Watson-Marlow MHRE pump (Watson-Marlow Ltd., Falmouth, Cornwall). Flexible silicone rubber pump tubing (Gallenkamp, London E.C.2) O.D. 9.2 mm., I.D. 6.0 mm was used. Before entering the pump the medium passed to a three-way junction S1, from which samples of medium were obtained. Another three-way tap, E, was placed at the opposite side of the pump and from this any air which inadvertently entered the circuit could be removed. After re-entering the box the medium passed through a filter, F, which was constructed of approximately 2.5 cm of tightly packed glass wool. This removed any fragmented red cells or other debris present in the medium and its presence was essential to the successful perfusion of the liver. The medium then passed to a bubble trap (BF) which was also a side arm to the manometer (M) and thence to the portal vein cannula. The temperature of the medium entering the liver was monitored by a thermocouple (TC).

The medium left the liver through the inferior vena cava cannula and then passed outside the box to a three-way junction, S2, from which samples of hepatic venous blood were taken. The medium re-entered the box at RY1 and then

passed back to the reservoir. At the point of entry to the reservoir the venous line was 12-15 cm below the preparation; this prevented swelling of the liver during perfusion.

The flow rate of the medium was measured by collecting effluent from RE in a graduated cylinder over timed intervals of one minute. During each experiment there were periods of recirculation of the medium after passing through the liver and this was achieved by placing a clamp at point RE. When periods of non-recirculation were required, a clamp was placed at NR and the medium collected at point RE.

The liver perfusion pressure was measured by a manometer system M which was contained within a perspex chimney (C) which protruded through the lid. An estimate of portal pressure was made by measuring the height of the column of blood contained in the manometer above the top surface of the perfusion box. The circuit was filled via the chimney and this also served to collect any medium which overflowed from the portal venous line and return it to the venous line.

2.1.2. The perfusion medium

The medium contained equine erythrocytes suspended in physiological buffer solution (Krebs and Henseleit 1932) containing bovine albumin (see Table 1).

The erythrocytes were obtained from equine blood which was preserved in acid-citrate-dextrose solution and was supplied by Wellcome Reagents Ltd., Beckenham, Kent. The

Table 1. Composition of bicarbonate diluent.

	Concentration(g/l)	Volume(ml)	
Potassium dihydrogen phosphate	16.4	5	Made up to
Potassium chloride	17.85	10	approx.429 ml
Calcium chloride	12.60	10	with distilled
Magnesium sulphate. $7H_2O$	14.80	10	water.

blood was stored at 4°C and was used approximately one week after collection. On the day of perfusion the cells were spun down, the plasma removed and the cells washed twice with normal saline and then with two volumes of buffer. The cells were then added to a 4% w/v solution of albumin in the proportion of 3 volumes of cells to 8 volumes of albumin solution. This was prepared using bovine albumin (Fraction V, Armour Pharmaceuticals, Eastbourne, Sussex) dissolved in buffer. The albumin solution was dialysed against several changes of buffer for 48 hours before use. Failure to dialyse the albumin solution often resulted in an unsatisfactory appearance of the liver and reduced rates of lactate uptake; the reason for this is unknown.

After preparation, the medium was placed within the reservoir and allowed to equilibrate at the intended P_{CO_2} and temperature of the experiment. When equilibration had been achieved, the pH of the medium, which always was low due to the acidity of the albumin, was adjusted to the desired value by the addition of hypertonic sodium bicarbonate solution (1 mmol/l) mixed with a bicarbonate diluent in the proportion of 6:1. The diluent consisted of a solution containing all the constituent salts of the Krebs buffer except sodium bicarbonate and sodium chloride (Table 1). The haematocrit of the medium ranged from 0.15 - 0.20. During equilibration the portal venous and inferior vena cava (IVC) lines were joined and the medium was allowed to circulate. Immediately before the preparation of the animal, the lines were separated and a clamp placed at VY. The IVC

line was filled with saline which was injected at RE and a second clamp was placed at RE.

2.1.3. The cannulae

The liver was perfused by the insertion of cannulae into the portal vein and inferior vena cava of the animal. The cannulae were made from stainless steel tubing and had bevelled tips and a shallow circular groove ground about 1.5 mm from the tip. The vena cava cannula was of larger bore than the portal vein cannula. The dimensions of the cannulae were as follows:-

Vena cava cannula	I.D. 1.75 mm	O.D. 2.4 mm
	length 2.6 cm	
Portal vein cannula	I.D. 0.75 mm	O.D. 1.25 mm
	length 3.4 cm	

2.1.4. Operative techniques

The animals were anaesthetised by an intraperitoneal injection of a 60 mg/ml solution of sodium pentobarbitone ('Nembutal', Abbott Laboratories Ltd., Queenborough, Kent), 0.15 ml/120g rat. and then weighed. The upper portion of the perfusion box was removed and the preparation tray was moved as far to the right as possible. The lower portion of the box was enclosed by placing the lid on the rails to the left of the tray. By these procedures fall of temperature of the medium during the setting up of the preparation was reduced to a minimum.

The anaesthetised animal was placed supine on the tray and the limbs were secured to the tray by adhesive tape.

A cruciate incision was made, extending from the xiphisternum to the symphysis pubis and laterally into the flanks. The inferior vena cava was exposed by displacement of the intestine. A cotton ligature was placed around the IVC just above the right renal vein, and a single loop was loosely tied. Heparin 100 units (Weddel Pharmaceuticals Ltd., London E.C.1) was then injected into the IVC. Two ligatures were similarly placed around the portal vein, one just above the point of entry of the first major tributary and the other about 1 cm lower down. After the careful expulsion of air from the portal venous line, the cannula, bevel face upwards, was inserted into the portal vein through a small incision. The cannula was secured by the ligature which was tied tightly around the shallow groove in the cannula. Immediately after the insertion of the cannula, the pump was switched on and the IVC was cut just above its division into the iliac veins, to allow the escape of the perfusion medium. The liver was then being perfused and was independent of the functioning of the animal's heart. The ischaemic time of the liver during the insertion of the cannula was approximately 15 seconds but was not complete as the hepatic artery was not ligated.

The rib cage of the animal was then opened and the point of entry of the IVC into the right atrium was exposed. The IVC cannula, bevel facing downwards, was then inserted through the right atrium into the supradiaphragmatic portion of the IVC. The cannula was held in position by the negative pressure in the line. The clamp at RE was then removed and

medium flowed into the venous line. The preparation was completed by the tying of the ligature around the IVC. The liver was then left in situ but was functionally isolated from the rest of the animal. The tray on which the preparation rested was then moved back to its central position and the upper half of the box was replaced. After the removal of the saline in the venous line, the medium was recirculated by transferring the clamp from NR to RE. The flow rate was adjusted to between 6 and 7 ml/100g animal. Observations of the pressure, temperature and flow rate were made at frequent intervals during the course of each experiment. The preparation was judged to be satisfactory if the appearance of the liver did not differ from that of the liver *in vivo*. If this criterion did not hold the preparation was rejected.

2.1.5 Preparation of isotopes

At the beginning of each perfusion $[^{14}C]$ 5.5-dimethyloxazolidine-2,4-dione (DMO), hydroxy $[^{14}C]$ methyl inulin and tritiated water were added to the medium for the measurement of intracellular pH. The isotopes were prepared as follows.

$[^{14}C]$ DMO was obtained from New England Corporation, Boston, Mass., U.S.A. Hydroxy $[^{14}C]$ methyl inulin was obtained from the Radiochemical Centre, Amersham, Bucks.

$[^{14}C]$ DMO was supplied in aliquots of 50 µCi dissolved in 0.5 ml of ethyl acetate (specific activity 9.15 mCi/mmol). A further 4.5 ml of ethyl acetate was added to this to give a solution of 10 µCi/ml 0.7 ml of this (7 µCi) was taken for

each experiment and evaporated to dryness under nitrogen. To this was added 10 ml of a solution of non-isotopic DMO (Baxter Laboratories, Inc., Morton Grove, Illinois, U.S.A.) in physiological saline (2.5 mg/ml). This was added to the perfusion medium, giving a final specific activity of 7.6 μCi/mmol.

Hydroxy [^{14}C] methyl inulin had a specific activity of 12.6 mCi/mmol. It was dissolved in phosphate buffer, pH 7.4 to give a solution of 20 μCi/ml. Aliquots of 1.25 ml (25 μCi) were taken periodically and diluted with 10 ml of a 1% stable inulin solution (in physiological saline) to give a solution of approximately 2-3 μCi/ml. 3 ml aliquots of this final solution (i.e. 7 μCi) were added to the medium giving a final specific activity of approximately 1.2 mCi/mmol.

Tritiated water had a specific activity of approximately 100 μCi/ml; 0.3 ml (30 μCi) was diluted in 7-10 ml of physiological saline and added to the medium.

2.2 METABOLIC ACIDOSIS EXPERIMENTS

The aim of this group of experiments was to investigate the effect of simulated metabolic acidosis on hepatic intracellular pH and lactate uptake.

Inbred Wistar rats (weight 100-150g) which had been starved for 20-24 hours were used. They had previously been fed on a diet of Dixon's rat cake (E. Dixon & Sons Ltd., Ware, Herts.), supplemented by mashed white bread and milk. Isolated, perfused liver preparations were set up as previously described. Experiments were performed over the external pH range 6.7 - 7.4.

The medium was prepared as described previously. L(+) lactic acid (Sigma Chemical Co.Ltd., London, S.W.6) was added to produce an initial concentration of 2.5 mmol/l. A large volume of medium (about 500 ml) was used in order to minimise the changes in the composition of the medium during the course of the perfusion. Each batch of medium was used for two or three successive perfusions at the same external pH. After the addition of lactate the medium was equilibrated in the perfusion circuit at 37°C and a P_{CO_2} of approximately 40 mm Hg. The pH of the medium was adjusted to the required level by the addition of modified Krebs bicarbonate buffer, as previously described.

The experimental protocol is illustrated in Fig. 7. The medium was non-recirculated for the last 30 minutes of the experiment in order to ensure that the conditions were

constant during the sampling period. Samples of hepatic venous blood were used for measurements of extracellular pH since they were considered to be closer to the pH of the interstitial fluid than that of the arterial blood. Pairs of arterial and venous blood samples were taken for lactate estimation, and from the results lactate uptake was calculated as described later. Acid-base measurements were made on the same blood samples. The flow rate and pressure were checked after sampling. At the end of the fifty minute perfusion period, 10 ml of hepatic venous blood was taken and the right lobe of the liver was removed, lightly blotted and placed in 5 ml of distilled water in a stoppered tube. The distribution of isotopes in the samples of tissue and medium was measured and the intracellular pH was calculated as described later.

PROTOCOL OF METABOLIC ACIDOSIS EXPERIMENTS

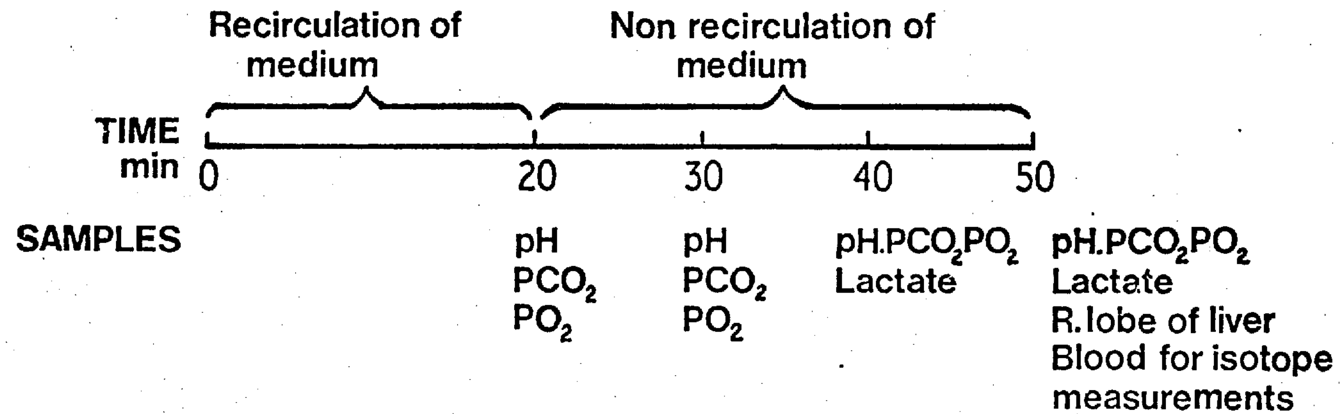


Fig. 7

2.3 PHENFORMIN EXPERIMENTS

The effect of phenformin on intracellular pH, lactate uptake and glucose production was studied using the isolated, perfused guinea pig liver preparation. The guinea pig was used in preference to the rat because of its greater sensitivity to the hypoglycaemic action of phenformin (Altschuld and Kruger 1968). Inbred guinea pigs (weight 130-200 g) which had been starved for 40-48 hours were used. There were 33 animals in the phenformin group and 18 animals in the control group.

The medium contained L(+) lactic acid (Sigma) at a concentration of approximately 14 mmol/l. It was equilibrated in the circuit at a P_{CO_2} of 40 mm Hg and the pH was adjusted to be within the physiological range by the addition of modified Krebs buffer. 150 ml of medium was used for each experiment and it was recirculated throughout the sixty-minute perfusion time.

The experimental protocol is illustrated in Fig. 8. Arterial blood samples were taken for lactate and glucose estimations at the times indicated. At the same times, samples of arterial and venous blood were taken for measurement of pH, P_{CO_2} and P_{O_2}. After the twenty-minute samples had been taken, phenformin hydrochloride (supplied by Winthrop Laboratories) was added to the reservoir to produce a concentration of 0.05, 0.1 or 0.25 mmol/l. In the control group a similar volume of 154 mmol/l sodium chloride was added to the

reservoir. In some experiments in the phenformin group a second dose of phenformin was added at forty minutes. An hepatic venous blood sample and the right lobe of the liver were taken at the conclusion of the sixty-minute perfusion period for the determination of the intracellular pH.

At the end of each experiment the volume of medium remaining in the circuit and reservoir was measured. By adding to this final volume the quantity of medium removed for the purpose of estimation, the volume of the medium at 20, 40, 50 and 60 minutes was calculated. From these values and the measured arterial lactate and glucose concentrations the lactate and glucose content of the reservoir at the time of sampling was calculated. Glucose production and lactate uptake during the 20-40 minute, 40-50 minute, and 50-60 minute perfusion periods were determined from the calculated reservoir content of glucose and lactate at the relevant times.

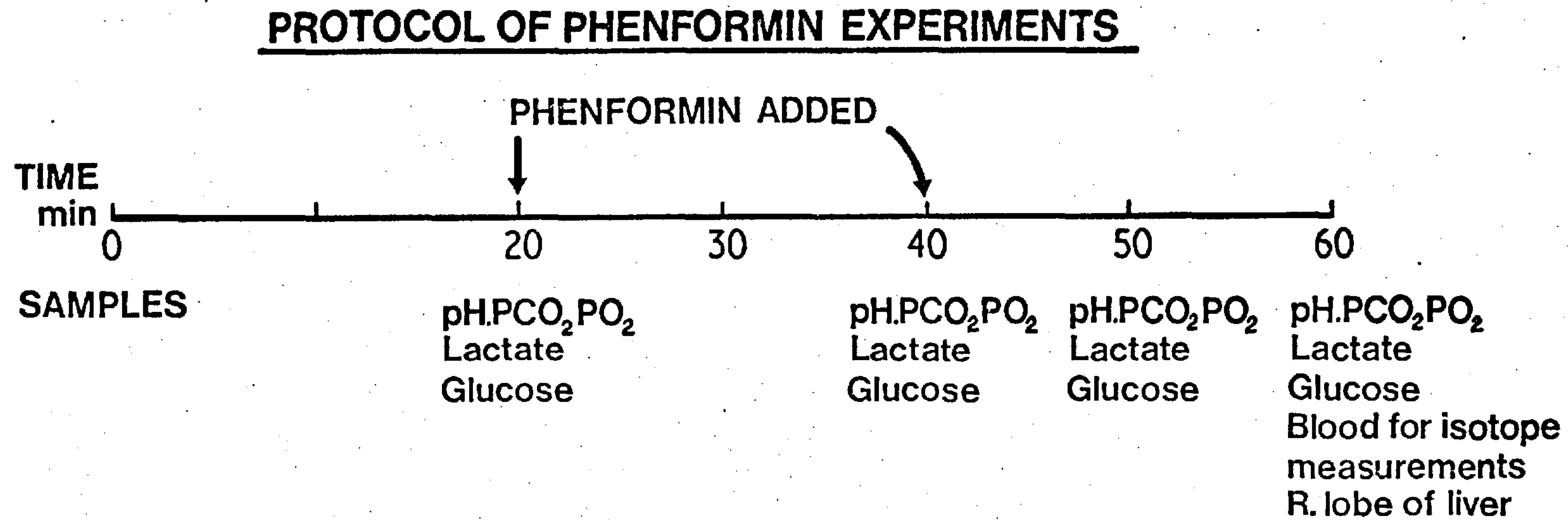


Fig. 8

2.4. ADDITIONAL EXPERIMENTS

In relation to the phenformin group of experiments described above two additional experiments were performed.

2.4.1. Determination of glucose production in the absence of added lactate

In order to assess the relative contribution of glycogenolysis and gluconeogenesis to hepatic glucose production, the experiments were repeated in the absence of added lactate. There were four animals in the control group and six animals in the phenformin group.

2.4.2. Assessment of the effect of phenformin on glucose consumption by erythrocytes

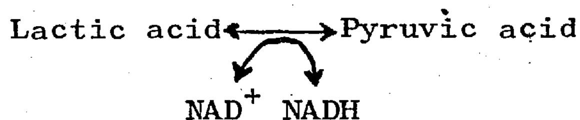
In this small series of experiments the change in perfusate glucose concentration in the absence of a liver preparation and added lactate was determined.

Glucose was initially added to the perfusate to produce a concentration of 4-6 mmol/l. In some experiments phenformin was added to the perfusate to produce a concentration of 0.25 mmol/l.

2.5. CHEMICAL METHODS

2.5.1. Lactate determination

1 ml of the medium was immediately added to previously weighed tubes containing 2 ml of 6% perchloric acid at 4°C and the tubes were reweighed and centrifuged. The supernatant was filtered and the lactate concentration was determined by an AutoAnalyzer adaptation of the method of Hohorst (1962). This method involves the measurement of NADH produced by the reaction:



The conversion of lactate to pyruvate was driven to completion by the removal of pyruvate as the hydrazone and by the provision of excess NAD. Rabbit muscle L-lactate dehydrogenase (Boehringer Corporation Ltd., London W5) was used to catalyse the reaction. The assay was carried out on a Technicon AutoAnalyzer and samples were analysed at a rate of 40/hour. Standard solutions were prepared using L+ lactic acid (Sigma Chemical Co.Ltd.). The mean recovery of lactate added to blood was 99.8% (SD 7.3% n=18). The within assay coefficient of variation of repeated measurements of lactate concentration or lactate uptake on the same sample (or pair of samples) was estimated as 2.2% and 15% respectively.

Calculation of lactate uptake

Lactate concentration in thc sample of medium was

calculated from the measured concentration in the perchloric acid supernatant, the weight of added medium and the haematocrit, assuming an erythrocyte water content of 65% of erythrocyte volume. The details of the calculation are in Appendix A2 p153.

In the metabolic acidosis experiments lactate uptake was calculated by multiplying the difference between portal and hepatic venous lactate concentrations by the flow rate. In the phenformin experiments the lactate content of the reservoir was calculated from the lactate concentration of the medium and the volume of medium remaining in the reservoir at the time of sampling. From these values lactate uptake between sampling periods was calculated.

In both groups of experiments the lactate uptake was multiplied by a factor of $\frac{100}{\text{Wt. of animal}}$ so that results from animals of different weights could be compared. Lactate uptake values were expressed in units μmol min^{-1}(100 g animal)$^{-1}$.

2.5.2. Glucose determination

For the determination of glucose concentration, 1 ml aliquots of portal venous (i.e. arterial) blood were placed in tubes containing sodium fluoride. The tubes were centrifuged and the plasma removed. The glucose content was determined using a Technicon AutoAnalyzer employing a glucose oxidase method (GOD-Pernid Method, Boehringer Corporation Ltd., London W5).

Calculation of glucose uptake

Whole blood glucose content was calculated from the

perfusate plasma glucose and the packed cell volume, assuming an erythrocyte water content of 65% and erythrocyte water glucose concentration equal to that of perfusate plasma water.

Glucose uptake by the liver was calculated from the change in reservoir content of glucose over a timed interval and was expressed as μmol min^{-1} (100 g guinea $pig)^{-1}$.

2.5.3. Acid-base measurements

(a) pH, PCO_2 and P_{O2}

1 ml samples of portal and hepatic venous blood were taken anaerobically into 1 ml syringes. pH was determined using a Radiometer BMS3 microelectrode system at 38°C with standard buffers of nominal pH values 6.84 and 7.381. Arterial and venous P_{CO2} was measured using a Severinghaus type of electrode calibrated with 5% and 10% CO_2. Arterial P_{O2} was measured by means of a Clark electrode maintained at 38°C and calibrated with 0% and 15% O_2. No corrections were made for the small difference between the temperature of the perfusate and that of the electrode.

2.5.4. Determination of intracellular pH

Intracellular pH was determined by measuring the distribution of ^{14}C labelled DMO between plasma water and hepatic tissue water. The extracellular space was measured using ^{14}C labelled inulin and tritiated water was used to measure tissue water.

The right lobe of the liver was used in all the

experiments. Iles (1974) showed that there was excellent correlation between pH_i measurements on the simultaneously excised right and left lobes and on duplicate samples of the same lobe.

2.5.4.1. Preparation of samples for counting

The liver samples were each homogenized in 5 ml of distilled water with an Ultra-Turrax homogenizer (Janke and Kunkel, K.G. Staufen, B.R. Germany) for 15 seconds. 4 ml of the homogenate was taken and the protein precipitated with 4 ml of 16.7% trichloracetic acid (TCA) and the supernatant was retained after centrifugation. 4 ml aliquots of plasma were similarly deproteinised with 4 ml of TCA.

2.5.4.2. Separation of $[^{14}C]$ DMO and $[^{14}C]$ inulin

This was done chemically by a modification of the method of Longmore, Niethe and McDaniel (1969). 2 ml of the supernatant remaining after the TCA deproteinisation of the plasma and tissue homogenates was shaken with 10 ml of toluene-ethyl acetate (1:1 v/v). The solvent phase was discarded and the process repeated three times. This procedure extracts $[^{14}C]$ DMO but does not remove $[^{14}C]$ inulin.

2.5.4.3. Counting of the samples

Duplicate aliquots of 0.5 ml of the extracted and unextracted supernatant were added to 10 ml of a dioxan based phosphor contained in a counting vial. The phosphor was made by mixing 390 ml of 1,4-dioxan, redistilled from

'AR' dioxan (B.D.H.) with 390 ml of xylene and 235 ml of ethanol (absolute alcohol B.P.C., James Burrough Ltd., London). This solution was poured into a litre flask containing 50mg of 1,4-bis 2-(methyl-5-phenyloxaxolyl) - benzene (dimethyl-POPOP) (scintillation grade, Packard Instrument Co., London), 5 g of 2,4-diphenyloxazole (PPO) and 80g of naphthalene (scintillation grade, Koch-Light Laboratories Ltd., Colnbrook, Bucks.) and made up to the mark. In the phenformin group of experiments Unisolve I (Koch Light Laboratories) was used as the phosphor.

The vials were counted in a three channel Packard 3003 liquid scintillation spectrometer with gate settings to provide optimal discrimination between ^{14}C and ^{3}H. Sufficient counts were recorded to obtain 1-2% statistical accuracy for ^{14}C and ^{3}H. Quenching was measured by successive addition of 25 µl internal standards (Hexadecane ^{3}H and Hexadecane ^{14}C) followed by recounting of the vials.

2.5.4.4. Calculation of intracellular pH

This is described in Appendix A2 p147.

2.6. STATISTICAL METHODS

In the metabolic acidosis experiments a two tailed t-test (Snedecor and Cochran, 1967) was used to assess the significance of differences between means and of the difference of a mean from zero. The Mann-Whitney U-test (Siegel, 1956) was used when the distribution in the groups under comparison was not appropriate for the t-test.

In the phenformin experiments, parametric and non-parametric methods were used to assess the significance of differences between the control and phenformin groups and they were found to give similar results. One tailed t-tests were used throughout.

In both groups of experiments linear regression and correlation analyses were made (Snedecor and Cochran, 1967). Means are expressed throughout as $\pm$ SEM.

3. RESULTS

3.1 METABOLIC ACIDOSIS EXPERIMENTS

3.1.1. pH and P_{CO_2} of the perfusate

Experiments were performed over the pH_e range 7.4-6.8. In each experiment the pH and the P_{CO_2} of the portal venous blood did not vary by more than 0.05 units or 9 mm Hg respectively during the course of the experiment. The mean portal venous P_{CO_2} at the end of the perfusion was 38.3 mm Hg (SD 4.7 n=42).

3.1.2. Lactate concentration of the perfusate

The experiments were arranged into five groups according to pH_i values. The pH_i ranges of these groups were: 7.4-7.2; 7.2-7.1; 7.1-7.0; 7.0-6.9 and 6.9-6.8. The mean lactate concentration in the perfusate at the time of pH_i measurement were 2.13 $\pm$ SEM 0.219; 2.02 $\pm$ 0.140; 2.19 $\pm$ 0.290; 2.36 $\pm$ 0.124 and 1.98 $\pm$ 0.241 mmol/l respectively. These mean values do not differ significantly from each other.

3.1.3. Relationship between pH_e and pH_i (Fig. 9)

As pH_e decreased from 7.4-6.85 mean pH_i fell only from 7.2 to 7.07. In contrast to this, further lowering of the pH_e from 6.85 to 6.70 resulted in almost as great a fall of mean pH_i and the difference is significant ($p < 0.05$).

The linear regression of the individual values of pH_i and pH_e is:

$$pH_i = 0.366\ (\pm \text{SEM } 0.058)\ pH_e + 4.506$$

The regression coefficient is significantly different from zero ($p < 0.001$).

3.1.4 Relationship between pH_e and lactate uptake (Fig. 10)

When pH_e lay between 7.4 and 7.1 the mean lactate uptake was between 0.9 and 2.0 μmol min^{-1} (100 g $rat)^{-1}$. When pH_e fell below 7.1-7.0 the mean lactate uptake was negative. However, the mean value of the lactate outputs from the two groups of perfusions with the two lowest pH_e levels are not significantly less than zero ($0.1 < p < 0.2$) when combined.

3.1.5 Relationship between pH_i and lactate uptake (Fig. 11)

The experiments were grouped into pH_i ranges 7.4-7.2; 7.2-7.1; 7.1-7.0; 7.0-6.9 and 6.9-6.8. For the first three groups the mean lactate uptakes were 1.73, 0.62 and 1.46 μmol min^{-1} (100 g $rat)^{-1}$ respectively. These values are not significantly different from each other. Below pH_i 7.0 there was a change from lactate uptake by the liver to lactate production, the values being -1.31 and -3.30 μmol min^{-1} (100 g $rat)^{-1}$ for the pH_i ranges 7.0-6.9 and 6.9-6.8 respectively. The mean lactate uptake for these two groups was -1.86 μmol min^{-1} (100 g $rat)^{-1}$. This value is significantly less than zero ($p < 0.05$) and also significantly

less ($p < 0.02$, < 0.05 and < 0.01 respectively) than the individual mean values in those groups with pH_i 7.0 (1.73, 0.62 and 1.46 μmol min^{-1} (100 g $rat)^{-1}$ respectively). The individual results are given in Table 2.

Table 2. Relationship between pH_e, pH_i and lactate uptake by the rat liver

Rat No.	pH_e	pH_i	Lactate uptake * μmol min^{-1} (100 g rat)$^{-1}$
1	7.42	7.17	4.3
2	7.40	7.17	-0.2
3	7.40	7.05	-2.2
4	7.37	7.15	1.3
5	7.37	7.32	4.1
6	7.36	7.27	0.7
7	7.13	7.06	1.8
8	7.11	7.22	1.3
9	7.11	7.15	4.5
10	7.14	7.13	-3.5
11	7.12	7.14	0.1
12	7.19	7.04	6.3
13	6.60	6.83	-0.9
14	6.70	6.91	-1.3
15	6.91	6.91	-4.5

Table 2. Relationship between pH_e, pH_i and lactate uptake by the rat liver

Rat No.	pH_e	pH_i	Lactate uptake* µmol min^{-1} (100 g rat)$^{-1}$
16	6.85	6.88	-6.0
17	7.39	7.24	0.5
18	7.39	7.26	0.3
19	7.42	7.14	-0.6
20	7.20	7.22	0.1
21	6.93	7.06	-0.5
22	6.96	7.16	2.2
23	6.97	7.24	2.3
24	6.93	7.06	2.0
25	7.22	7.22	2.4
26	7.20	7.08	3.1
27	7.15	7.06	3.5
28	6.86	7.11	0
29	6.86	7.12	-2.6
30	6.92	7.10	0

Table 2. Relationship between pH_e, pH_i and lactate uptake by the rat liver

Rat No.	pH_e	pH_i	Lactate uptake* μmol min^{-1} $(100\ g\ rat)^{-1}$
31	6.93	7.04	-1.6
32	6.89	6.96	1.7
33	6.78	6.98	-3.3
34	6.78	6.86	-3.1
35	6.76	6.98	-2.1
36	6.78	6.98	3.1
37	6.80	7.16	0.4
38	6.80	6.95	0
39	6.90	6.96	-4.1
40	6.91	7.15	4.7
41	6.86	7.06	0
42	6.85	7.10	0

* The lactate uptake is calculated from the arterial and venous samples taken at the end of the 50 minute perfusion period.
A negative sign indicates lactate <u>output</u> by the liver

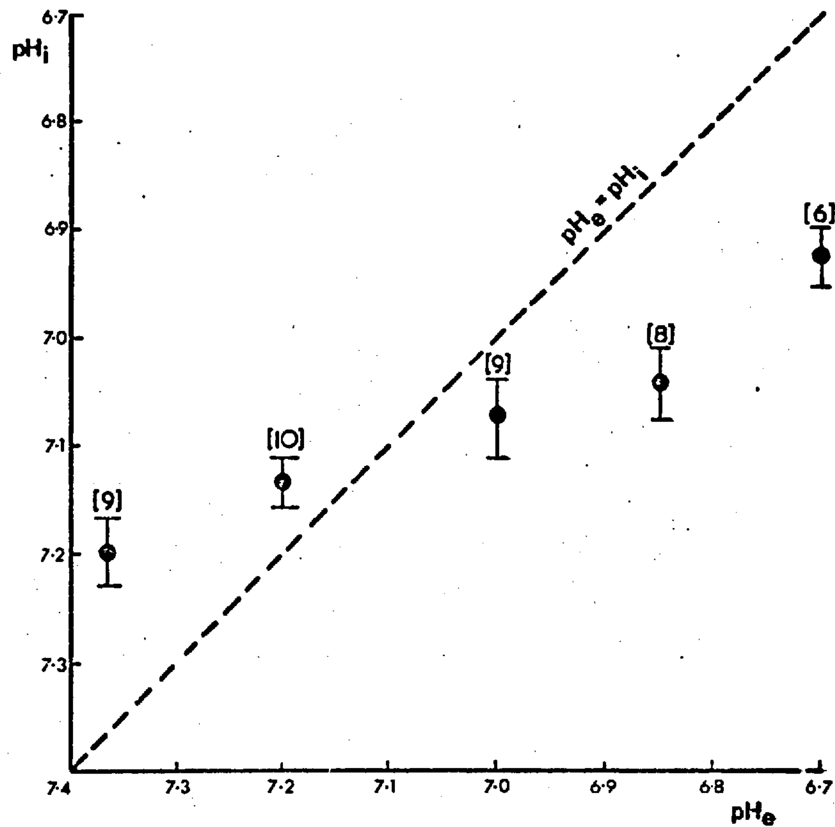


Fig. 9 Relationship between pHe and pH_i. The pHe values represent the mid-points of the pH ranges 7.44-7.3; 7.3-7.1; 7.1-6.9; 6.9-6.8 and 6.8-6.6. pH_i is represented as mean ± SEM. The numbers in each group are given in parentheses.

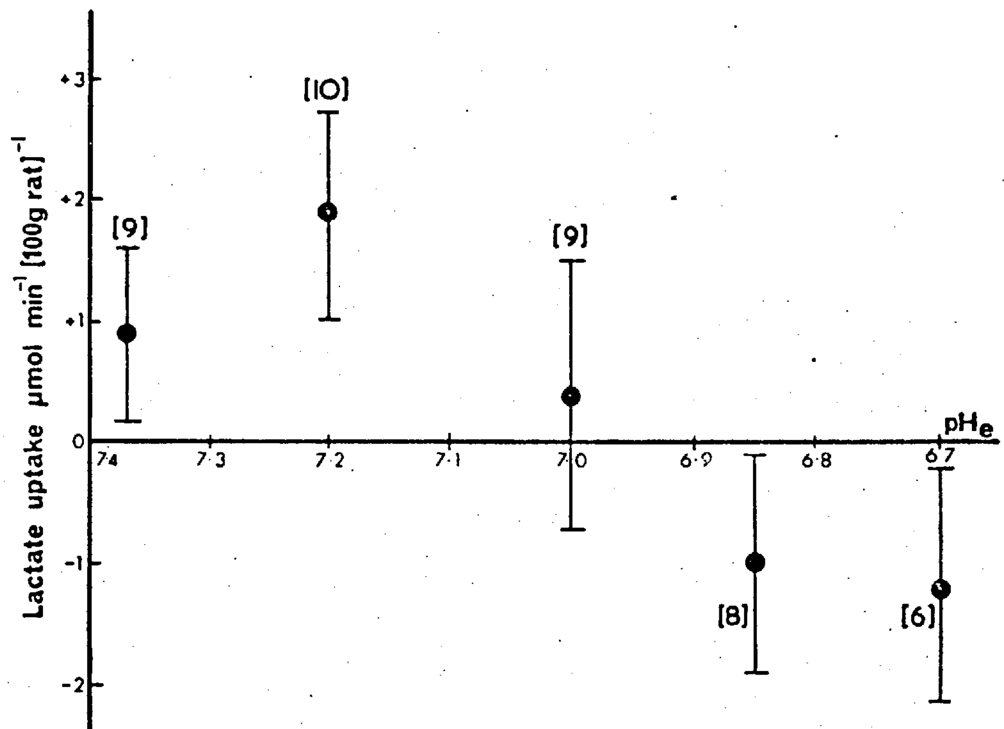


Fig. 10 Relationship between pHe and lactate uptake. The pHe values represent the mid-points of the pH ranges 7.44-7.3, 7.3-7.1, 7.1-6.9, 6.9-6.8 and 6.8-6.6. Lactate uptake is represented as mean $\pm$ SEM

Points above the zero line represent lactate uptake by the liver and those below represent lactate output by the liver. The numbers in each group are given in parentheses.

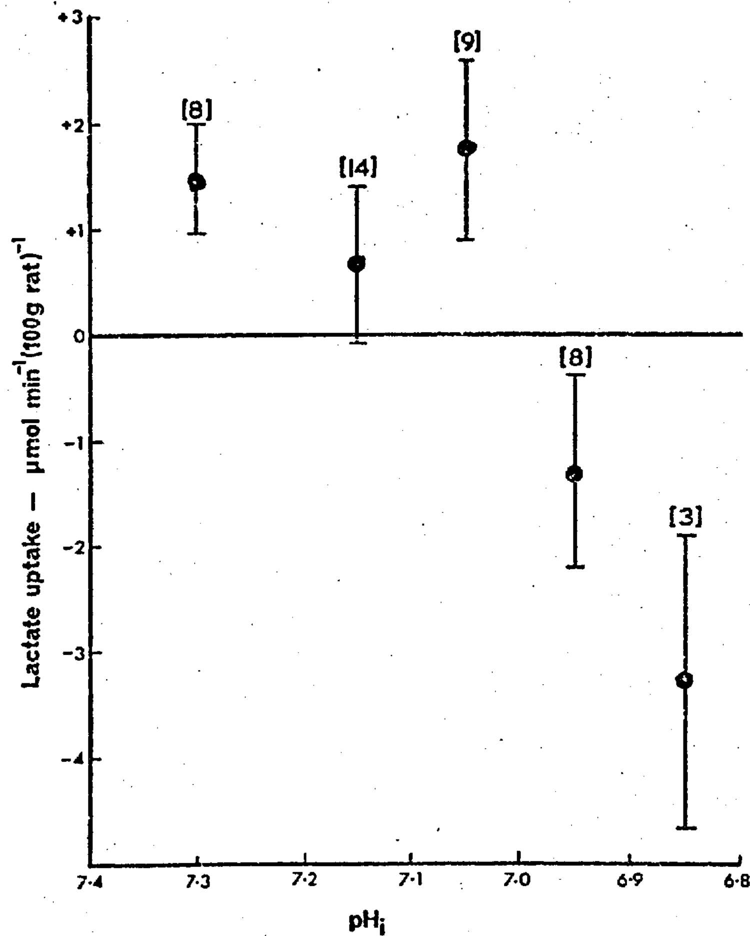


Fig. 11 Relationship between lactate uptake and pH_i. The pH_i values represent the mid-points of the pH ranges 7.4-7.2, 7.2-7.1, 7.1-7.0, 7.0-6.9 and 6.9-6.8.

Points above the zero line represent lactate uptake by the liver and points below represent lactate output by the liver. The numbers in each group are given in parentheses.

3.2 PHENFORMIN EXPERIMENTS

3.2.1. pH, P_{CO2} and P_{O2} of the perfusate

At the time of pH_i measurement the mean portal venous pH and P_{CO2} were 7.40 ± 0.007 and 42.5 ± 0.10 mm Hg respectively in the control group and 7.38 ± 0.007 and 41.4 ± 0.73 mm Hg in the phenformin group. These differences are not significant.

The mean hepatic venous P_{CO2} at the time of pH_i measurement was 42.7 ± 0.93 mm Hg in the control group and 44.4 ± 0.95 mm Hg in the phenformin group. The mean hepatic venous pH measured at the same time was 7.40 ± 0.008 for the control group and 7.36 ± 0.006 for the phenformin group. This difference is significant $p < 0.01$.

The P_{O2} of the medium in the reservoir was always about 400 mm Hg. The mean hepatic venous P_{O2} was 32.2 ± 2.28 mm Hg at 20 minutes and 33.7 ± 2.27 mm Hg at 60 minutes in the control group. This difference is insignificant. The corresponding values in the phenformin group were 32.6 ± 1.43 at 20 minutes and 42.1 ± 2.23 mm Hg at 60 minutes. This difference (9.45 mm Hg) is highly significant ($p < 0.01$ Mann-Whitney U-test).

3.2.2. Lactate concentration of the perfusate

The mean lactate concentration at the beginning of each experiment was 13.59 ± 0.40 mmol/l for the phenformin group and 14.37 ± 0.41 for the control group. The difference is not significant.

3.2.3. Lactate uptake

There was a variable response to phenformin and there

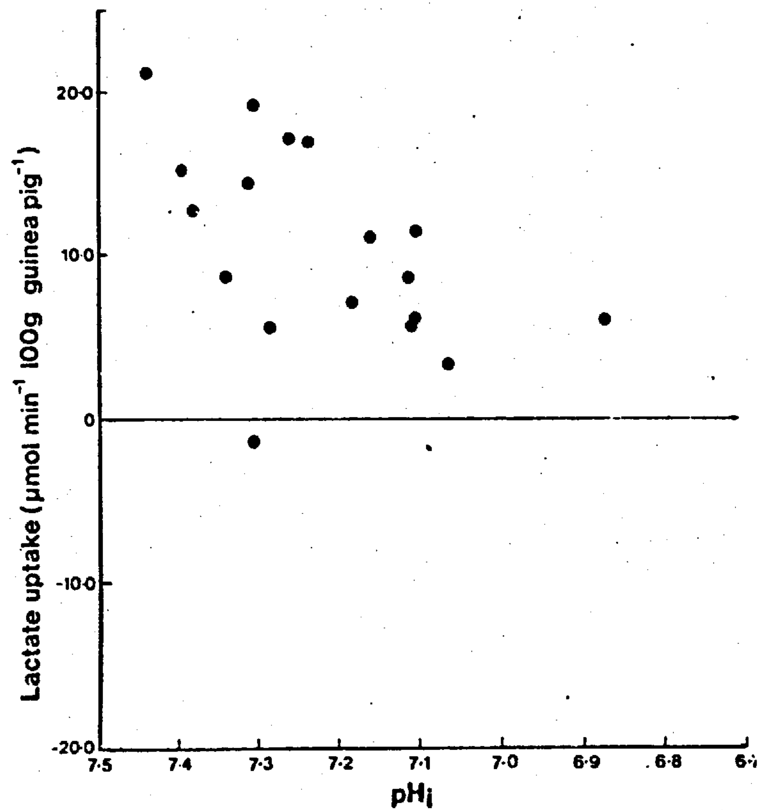


Fig. 12 Relationship between hepatic intracellular pH (pH_i) and lactate uptake in the control group.

Points above the zero line represent lactate uptake and those below represent lactate output by the liver.

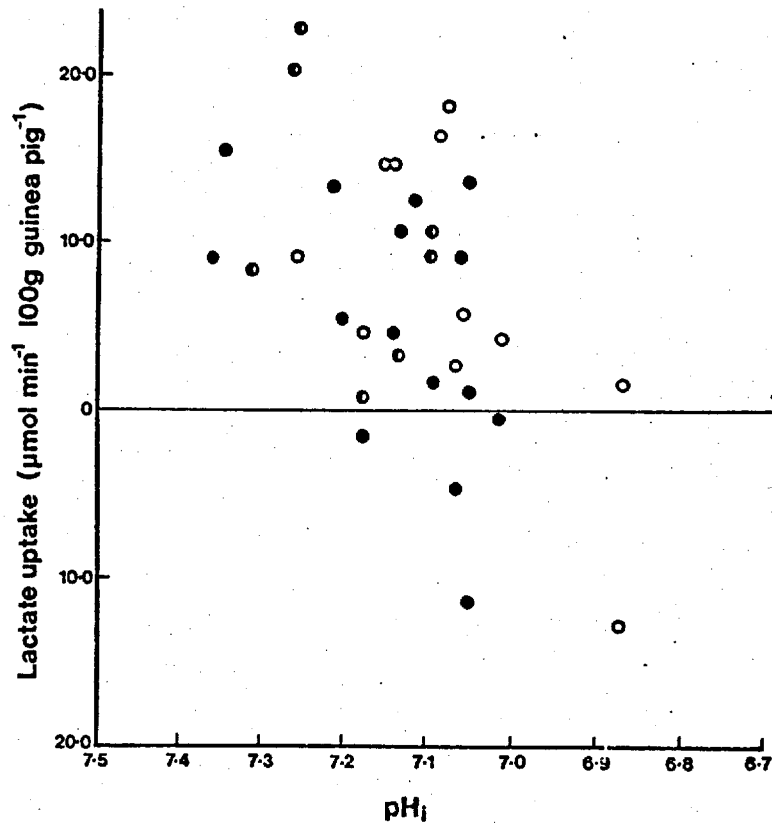


Fig. 13 Relationship between hepatic intracellular pH (pH_i) and lactate uptake in the phenformin group.

Points above the zero line represent lactate uptake and those below represent lactate output by the liver. The symbols indicate the concentration of phenformin used:- ● 0.05-0.1 mM at zero time; ◉ 0.1 mM at zero time and a similar increment at 20 mins; ○ 0.025 mM at zero time and a similar increment at 20 mins.

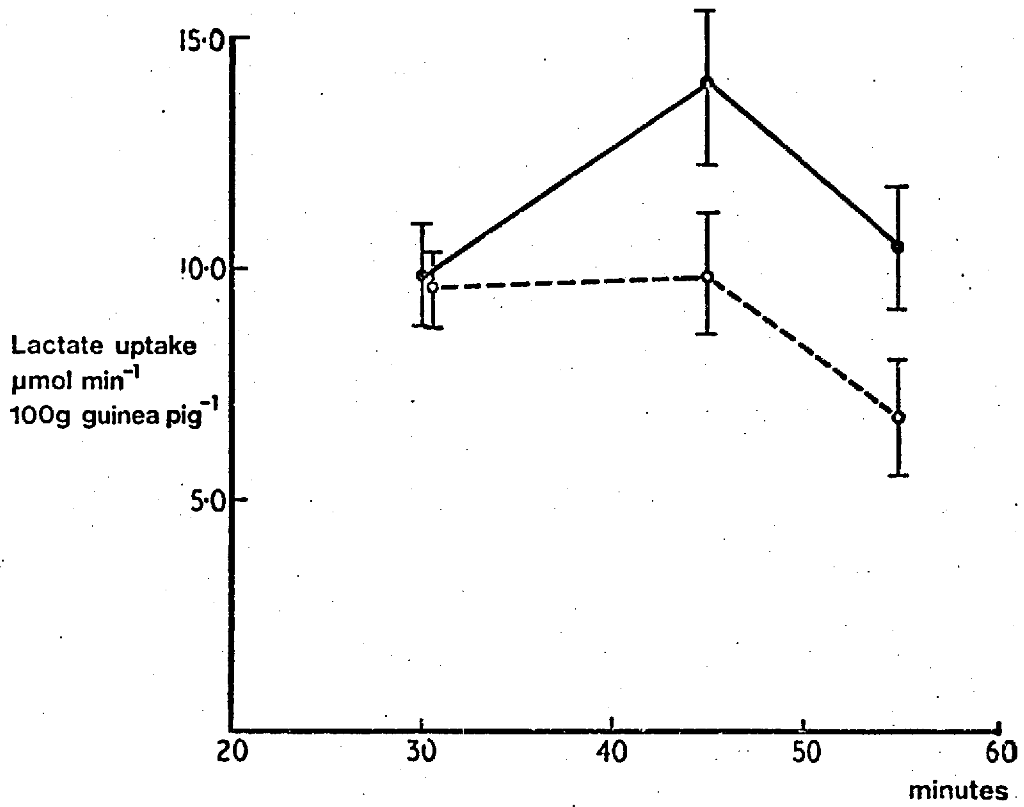


Fig. 14 Mean lactate uptake (± SEM) during the 20-40 min., 40-50 min., and 50-60 min., perfusion periods.

● control animals

○ phenformin animals

was no obvious relationship between lactate uptake and phenformin dose (see Fig. 13). Because of this, all the phenformin dose groups were considered together.

Fig. 14 shows the mean lactate uptake during the course of the perfusion in the two groups. There was no difference between the two groups during the 20-40 minute perfusion period. During the 40-50 minute period the mean lactate uptake was 10.51 ± 1.35 µmol min^{-1} (100 g guinea $pig)^{-1}$ in the control group and 6.83 ± 1.39 µmol min^{-1} (100 g guinea $pig)^{-1}$ in the phenformin group. This difference is significant ($p = 0.033$).

3.2.4. Relationship between lactate uptake and pH_i

When the lactate uptake in the 50-60 minute perfusion period is considered, there is a significant correlation between lactate uptake and pH_i (Figs. 12 & 13). In the control group the regression equation is:

$$pH_i = 7.098 + 0.0116 \quad : \quad r = 0.492 \quad 0.01 < p < 0.05$$

In the phenformin group the equation is:

$$pH_i = 7.072 + 0.0078 \quad r = 0.551 \quad p < 0.001$$

These two regression lines are not significantly different either in slope or intercept.

When pH_i was particularly low in the phenformin series, lactate output occurred. The results are shown in tabulated form in Tables 3 and 4.

3.2.5. Glucose production

Mean glucose production fell in both groups during the

course of the perfusion but the fall was much greater in the phenformin treated series (Fig. 15).

Mean glucose production was similar in the two groups during the 20-40 minute perfusion period. Thereafter it fell in both groups. During the 40-50 minute perfusion period the mean glucose production was 4.33 ± 1.22 µmol min^{-1} (100 g guinea pig)$^{-1}$ in the control group and 0.73 ± 0.52 µmol min^{-1} (100 g guinea pig)$^{-1}$ in the phenformin group; this difference is highly significant ($p < 0.01$). During the 50-60 minute perfusion period the glucose production in the control group was 1.38 ± 0.58 µmol min^{-1} (100 g guinea pig)$^{-1}$, whilst in the phenformin group glucose production had actually ceased and the glucose content of the reservoir was falling at a rate of 1.69 ± 0.46 µmol min^{-1} (100 g guinea pig)$^{-1}$. The difference between the two groups is significant ($p < 0.01$).

There is a significant relationship ($p < 0.01$) between cell pH and glucose production during the final 10-minute perfusion period.

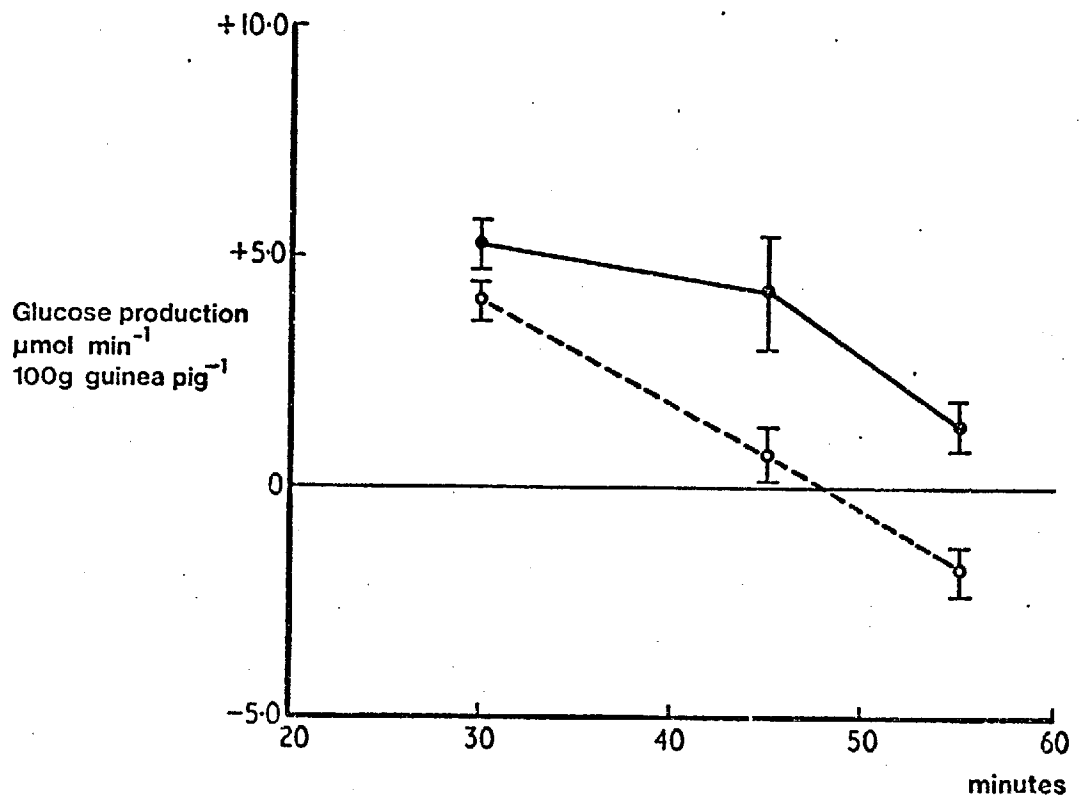


Fig. 15 Mean glucose production (± SEM) during the 20-40 min., 40-50 min. and 50-60 min. perfusion periods.

Points above the zero line represent glucose output by the liver, and points below represent glucose uptake.

● Control animals

o Phenformin animals

Table 3. Intracellular pH, lactate uptake and

Guinea Pig No.	pHi	Lactate uptake µmol min^{-1} (100 g guinea pig)$^{-1}$ 20-40 mins	40-50 mins	50-60 mins
1	7.38	9.81	24.44	12.70
2	7.44	22.96	25.92	21.06
3	7.16	27.39	11.66	11.21
4	7.19	14.67	8.39	7.28
5	7.11	21.27	20.03	6.27
6	7.11	36.24	7.41	11.69
7	7.28	21.06	19.32	5.73
8	7.12	-	16.88	8.90
9	7.24	15.15	8.32	17.04
10	7.30	19.64	8.93	- 1.37
11	7.30	21.32	19.37	19.37
12	7.39	-	7.73	15.30
13	7.34	17.08	19.66	8.82
14	7.31	30.62	2.63	14.57
15	6.87	- 2.50	11.63	6.34
16	7.07	26.90	11.56	3.65
17	7.26	14.63	5.04	17.47
18	7.11	-	10.33	5.71

glucose production in control series

Glucose production $\mu mol\ min^{-1}$ (100 g guinea pig)$^{-1}$		
20-40 mins	40-50 mins	50-60 mins
6.65	14.36	1.93
7.93	- 4.86	-2.95
5.12	9.71	-2.96
16.43	0.83	-1.29
8.04	3.72	-1.22
9.45	0.20	3.70
18.92	5.61	3.60
-	6.18	-0.176
11.22	1.06	3.85
4.51	1.01	1.46
9.00	4.88	2.46
11.12	4.86	2.06
14.45	-0.02	3.10
8.59	7.66	-0.30
18.17	15.71	-0.796
12.79	1.65	4.79
6.51	0.36	0.76
6.43	-	5.84

Table 4. Intracellular pH, lactate uptake and

Guinea Pig No.	pHi	Lactate uptake µmol min^{-1} (100 g guinea pig)$^{-1}$ 20-40 mins	40-50 mins	50-60 mins
1	7.09	25.21	3.04	1.71
2	7.02	23.86	5.16	-0.66
3	7.11	24.38	11.28	12.64
4	7.13	25.53	0.05	10.75
5	7.05	-	12.79	1.12
6	7.36	12.80	20.24	9.01
7	7.21	32.02	5.91	13.37
8	7.35	20.16	26.58	15.49
9	7.05	23.12	15.09	-11.28
10	7.20	33.42	- 0.23	5.61
11	7.17	- 1.49	17.37	- 1.59
12	7.05	30.74	6.45	13.72
13	7.26	20.88	12.00	9.26
14	7.06	14.72	3.17	- 4.98
15	7.06	13.43	15.10	9.41
16	7.14	9.84	8.22	4.84
17	7.31	30.08	13.57	8.35
18	7.26	12.35	22.63	22.96

glucose production in phenformin series

Glucose production $\mu mol\ min^{-1}$ (100 g guinea pig)$^{-1}$		
20-40 mins	40-50 mins	50-60 mins
15.11	-0.45	-2.29
7.19	0.87	-6.55
4.85	-2.98	-2.94
0.42	-1.52	-3.79
-	2.15	5.70
9.31	-1.85	-0.26
11.69	2.47	5.70
14.84	-5.01	-0.37
3.59	0.83	-2.13
14.87	0.73	1.48
12.30	2.32	0.31
10.03	4.69	0.69
6.72	0.67	0.34
4.92	2.08	-2.62
19.56	9.35	-8.06
3.71	-1.27	-1.54
6.90	-2.12	-1.74
10.56	6.68	-1.75

Table 4. (cont'd) Intracellular pH, lactate uptake and

Guinea Pig No.	pHi	Lactate uptake $\mu mol\ min^{-1}$ (100 g guinea pig)$^{-1}$ 20-40 mins	40-50 mins	50-60 mins
19	7.26	31.18	9.88	20.26
20	7.09	9.44	30.73	8.21
21	7.09	29.03	3.94	10.77
22	7.18	27.08	4.43	1.14
23	7.13	21.43	2.82	3.42
24	7.01	13.95	0.99	4.45
25	7.08	23.28	3.69	16.53
26	6.87	27.84	5.89	-12.65
27	7.06	13.52	1.63	5.86
28	7.14	6.54	13.15	14.78
29	7.17	11.90	15.63	4.66
30	7.15	-9.49	21.03	14.70
31	7.09	16.66	2.00	9.37
32	6.87	16.58	16.40	1.63
33	7.06	13.52	1.63	5.86

glucose production in phenformin series

Glucose production $\mu mol\ min^{-1}$ (100 g guinea pig)$^{-1}$		
20-40 mins	40-50 mins	50-60 mins
6.27	1.88	1.73
3.12	-5.85	-1.99
10.64	2.91	-2.53
8.94	-0.09	-3.25
5.70	-0.14	-2.65
12.73	-1.12	-2.55
9.99	3.50	-1.36
11.62	1.09	-1.90
10.09	1.31	1.43
1.71	-1.95	-3.67
5.52	-0.28	-2.43
5.71	0.33	-0.17
13.01	1.95	-4.67
4.77	3.31	-4.08
3.80	-2.25	-4.22

3.3. ADDITIONAL EXPERIMENTS

3.3.1. Glucose production in the absence of added lactate

In the absence of added lactate mean glucose production was 1.44 ± 0.084 μmol min^{-1} $(100\ g\ guinea\ pig)^{-1}$ in the control group (n=4) and 0.967 ± 0.507 μmol min^{-1} $(100\ g)^{-1}$ in the phenformin group (n=6) in the 20-40 minute perfusion period. These values represent 27% and 23% respectively of the glucose produced during the same period when lactate was added to the perfusate. By 50-60 minutes glucose production had apparently ceased.

3.3.2. Effect of phenformin on perfusate glucose consumption and lactate production

The mean glucose consumption by the perfusate erythrocytes was 0.40 ± 0.08 μmol min^{-1} 100 ml^{-1} (n=4) in the absence of phenformin and the lactate production 0.67 ± 0.06 μmol min^{-1} 100 ml^{-1}. In the presence of 0.25 mmol/l phenformin glucose consumption was 0.45 ± 0.18 μmol min^{-1} 100 ml^{-1} and lactate production 0.71 ± 0.21 μmol min^{-1} 100 ml^{-1} (n=4). Phenformin thus made no significant difference to glucose production and lactate consumption by perfusate erythrocytes.

4. DISCUSSION

4.1 DETERMINATION OF HEPATIC INTRACELLULAR pH BY THE DMO METHOD

4.1.1 pH of the liver cell

The first measurement of hepatic intracellular pH was made in the rat by Kahler and Robertson (1943) using electrodes and they obtained a value of 7.39. All subsequent studies have used the DMO method of measuring intracellular pH and have been carried out in the rat either in vivo (Wilson and Simmons 1970; Walker et al 1969; Williams and Woodbury 1971) or in the isolated perfused liver (Longmore, Niethe and McDaniel 1969; Cohen et al 1971; Iles and Cohen 1974). Most of the values obtained lie between 7.0 and 7.30. Wilson and Simmons (1970) obtained a value of 6.32 but they used sulphate to measure the extracellular space, a substance which is known to be taken up and rapidly excreted by the liver, thus making it very unsuitable as an extracellular space marker.

Walker et al (1969) measured the whole body pH_i and the pH_i of the liver, heart and skeletal muscle of the rat. They found that the hepatic pH_i was 7.23 which was higher than whole body pH_i (6.88), heart muscle pH_i (7.04) and skeletal muscle pH_i (6.93). Cohen et al (1971) suggested that the higher hepatic pH might be partly attributed to hepatic lactate metabolism.

4.1.2 Validity of the DMO method with particular reference to the liver cell

4.1.2.1 Equilibration of DMO

One of the basic assumptions of the DMO method is that at equilibrium the concentration of undissociated DMO in the extracellular and intracellular compartments is the same. It is, therefore, essential that sufficient time for the achievement of equilibrium is allowed between addition of the labelled DMO and the taking of the tissue specimens. In the metabolic acidosis experiments this time interval was fifty minutes.

Studies of whole body pH_i measurement have shown that up to two hours is necessary for DMO equilibration (Cohen, Simpson, Goodwin and Strunin 1967; Schloerb and Grantham 1965). In isolated organs the equilibration time is less than this; Roos (1965) produced evidence that thirty minutes was sufficient in the brain and Waddell and Butler (1959) showed that equilibrium was achieved within an hour in skeletal muscle. In cell suspensions, i.e. platelets, the equilibration time is less than fifteen minutes (Zieve and Solomon 1966). As the liver is a highly vascular organ, it is to be expected that equilibrium would be achieved in a time comparable to that found in cell suspensions. Cohen et al (1971) obtained evidence suggesting that, in the isolated perfused rat liver, equilibration of DMO was reached in less than twenty-five minutes. Iles and Cohen (1975) studied DMO equilibration in the isolated perfused guinea pig liver and calculated an approximate half-time of equilibration of 1.9 minutes. Therefore, it can be assumed that in the metabolic acidosis experiments, equilibration of DMO between the extracellular and intracellular compartments was complete at fifty minutes.

The distribution of DMO across a membrane is determined by the pH_i of the extracellular and intracellular compartments and it is essential that this remains constant for a sufficient time to allow equilibration of DMO to take place. If this criterion is not fulfilled, the calculated values of pH_i cannot be considered valid. Cohen et al (1967) described, in quantitative terms, the determination of the whole body pH_i under non-steady state conditions, but for technical reasons this cannot be applied to the isolated perfused organ. In the phenformin experiments, lactate uptake in the 50-60 minute period was less than that in the 40-50 minute period in both the control and phenformin experiments. This raises the possibility that a steady state was not present when pH_i was determined at the end of the sixty minute perfusion period and that the significant relationship between pH_i and lactate uptake during the 50-60 minute perfusion period could be due to failure of DMO equilibration. The evidence against this is twofold. Firstly, the absolute decrease in lactate uptake over the 40-50 minute and 50-60 minute perfusion periods was similar in the phenformin and control groups so the degree of non-equilibrium was similar in the two groups. Secondly, no relationship could be demonstrated between pH_i and the change in lactate uptake between the 40-50 and 50-60 minute perfusion periods, suggesting that there was little influence of the lactate uptake in the penultimate period on the pH_i determined at the end of the final period. The short half-time of DMO equilibration (vide supra) would be consistent with this finding.

4.1.2.2 Choice of the extracellular space marker

The choice of the extracellular marker substance influences the calculated value of intracellular pH. Cohen et al (1971) found that when inulin was used the calculated pH of the hepatic cell was 0.2 units higher than the value obtained when chloride was used as the marker. This can be accounted for by the finding of Williams and Woodbury (1971) that the chloride space is larger than the inulin space in the rat liver. This has been attributed to the low membrane potential of the liver cell which allows the passive entry of chloride ions into the cells. The same workers studying the distribution of $[^{14}C]$ inulin in the liver of the nephrectomized rat, obtained a value for the extracellular space of 10% of tissue water at one hour. After this time there was a slow increase in the size of the space due to tissue binding and/or phagocytosis of inulin. They concluded that the inulin space at one hour was the most accurate measurement of extracellular space in the rat liver because the chloride distribution calculated from this space was Donnan in type. Because of these considerations inulin was used as the extracellular marker substance in the present work.

4.1.2.3 Validity of the assumptions on which the DMO method is based

The DMO method is based on two important assumptions:

a) That the membrane is selectively permeable to the undissociated indicator.

b) That there is no active transport of the indicator into the cell or binding of the indicator within the cell.

The validity of these assumptions with particular reference to the liver cell will now be discussed.

4.1.2.3 (a) Selective permeability of the membrane

It is assumed that DMO crosses membrane by non-ionic diffusion and therefore the ratio of the permeability of the cell membrane to the unionized and ionized fractions of DMO is very high. Irvine, Saunders, Milne and Crawford (1960) studied the steady state concentration gradient of DMO between plasma water and skeletal muscle water. They concluded that as the permeability ratio exceeded 1000 no error would result from its use. Roos (1965) studied the intracellular pH of cat brain and criticized their work on the basis that they failed to take account of the membrane potential. He was able to show that if the membrane potential was within the range 0 to -100 mV there would be negligible error in the calculated pH_i if the permeability ratio of unionized to ionized DMO was between 500 and 1000. In the liver, where the membrane potential is lower (-40 mV) than it is in excitable tissues (-70 to -100 mV) the error in the calculated pH_i would be lower than it is in the brain and skeletal muscle. Cohen and Iles (1976) showed that if the membrane potential of the isolated perfused liver lies within the range -30 to -40 mV then there would be little error if the permeability ratio exceeded 400 : 1 and that at this value the calculated error would be +0.02 pH unit. They also showed that for a given permeability ratio the error in calculated pH_i was greater the higher the external pH.

Though these considerations give insight into the

nature and possible magnitude of the errors which would occur if there was significant permeability to the ionized form of DMO, and show that in one respect the liver cell is less sensitive to such errors, there is as yet no direct proof that the assumptions under discussion are valid in the liver. The argument for the use of DMO in liver rests mainly on analogy with the better substantiated situation in brain (Roos 1971), skeletal muscle (Miller, Tyson and Relman 1963) and with the observed distribution of DMO between the blood and the coelomic spaces in the spiny dogfish and semi-aquatic turtle.

4.1.2.3 (b) Is there active transport or protein binding of DMO within the liver ?

Measurements of pH_i made by the DMO method can only be considered valid if the distribution of DMO at equilibrium is determined only by the extracellular and intracellular hydrogen ion concentrations. For this relationship to hold there must be no active transport or binding of the indicator and it must be assumed that the membrane is very much more permeable to the undissociated form. Evidence that these criteria are fulfilled and therefore values of pH_i obtained by the DMO method are valid has been discussed previously. However, it is possible that cell membranes of different tissues vary in their handling of DMO and that protein binding of DMO may occur in some tissues. Two groups of workers have produced evidence of the active transport of DMO across epithelial surfaces. Dietschy and Carter (1965) found that DMO accumulated in higher concentrations on the serosal side than on the mucosal

side of everted rat intestine *in vitro* and this could not be predicted from the pH gradient. Rollins and Reed (1970) studied the effect of an increasing DMO load on the $[^{14}C]$ DMO space in the CSF, brain and muscle of the rat. They found that DMO loading had no effect on the $[^{14}C]$ DMO space of the brain or of muscle but did result in an increased CSF space. They explained this observation on the basis of the saturation of a DMO transport system.

In view of the epithelial nature of the hepatocyte and its embryological relationship to intestinal epithelium, it is important to consider the possible existence of an active DMO transport system across the membrane of the hepatocyte or binding of DMO within the cell. Iles and Cohen (1974) investigated these possibilities by measuring hepatic intracellular pH in the intact rat using $[^{14}C]$ DMO and a range of doses of unlabelled DMO which was neutralized to pH 7.4 with sodium hydroxide. They found that an increase in the dose of unlabelled DMO from zero to 0.19 mmol/kg rat body weight, was associated with a fall in calculated pH_i from 7.27 to 7.17. Further increase in the dose of 'carrier' DMO had no effect on calculated pH_i. They postulated that this could be an effect of the sodium load associated with the unlabelled DMO and in fact showed that if a sodium load was administered in the form of sodium chloride rather than neutralized 'carrier' DMO, the calculated pH_i was 7.17, significantly lower than the pH_i in the 'no carrier' group which was not given sodium chloride.

These results suggest that the variation in pH_i can be attributed to the variations in sodium load rather than to the saturation of a saturable process. However, the presence

within the liver of a DMO transport mechanism or protein binding of DMO which is saturated by excess DMO cannot be entirely excluded. For this reason 'carrier' DMO in concentrations exceeding 0.2 mmol/l was used in the experiments described in this thesis.

4.2 METABOLIC ACIDOSIS EXPERIMENTS

4.2.1 Relationship between pH_e and pH_i

This has been studied in a number of tissues. Using the isolated rat diaphragm Adler et al (1965) showed that the pH of the external medium (pH_e) could be decreased from 7.39 to 6.92 by varying the concentration of bicarbonate without a significant fall in the intracellular pH (pH_i). However, the cell pH was less resistant to change when external acidosis was induced by CO_2 or when the cells were exposed to an alkalotic medium. In their original work Adler et al (1965) found a range of pH_e over which pH_i did not change at all. In later work Adler (1972) repeated these studies using both DMO and the weak base nicotine; for reasons which are not clear there were certain differences from the earlier studies in the results using DMO. Firstly, the mean pH_i at 'normal' external acid base conditions was 7.1 rather than 6.9 and secondly, the plateau of pH_i as pH_e fell was less well defined. Nevertheless, the cells were clearly partially resistant to changes in pH_e.

This resistance of cells to metabolic acidosis has been demonstrated in other tissues including human platelets (Zieve and Solomon 1966), dog brain (Kibler, O'Neill and Robin 1964) and in man <u>in vivo</u> by measurement of mean whole body pH_i (Lambie, Anderton, Cowie, Simpson, Tothill and Robson 1965). In all these studies DMO was used in the determination of pH_i. Paillard (1972) obtained similar results using pH

sensitive microelectrodes to measure the intracellular pH of rat and crab muscle.

The relationship between pH_e and pH_i in the intact liver has not previously been investigated. The only reported study, which was made up of only four observations on rat liver slices, showed that pH_i and $[HCO_3^-]_i$ closely followed those of the external medium (Hastings and Longmore 1965). The results of the present work do not confirm these observations but do show that, as in other tissues, the liver cells are partially protected against external acidosis except when this becomes very severe, i.e. $pH_e < 6.85$. There is no clear region of constant pH_i as pH_e is lowered by decreasing $[HCO_3^-]_e$ (Fig. 9) as Adler et al (1965) originally found in skeletal muscle.

The regulation of pH_i in the face of external pH changes involves active mechanisms. As previously discussed, the distribution of hydrogen ions across cell membranes is not in accordance with the Donnan equilibrium. Adler et al (1965) demonstrated, using the isolated rat diaphragm, that pH_i control in the presence of external acidosis was abolished by anoxia and metabolic poisons and concluded that active mechanisms were involved in the maintenance of pH_i. The nature of these mechanisms is not completely understood. Active transport of H^+ or HCO_3^- across the cell membrane could take place or changes in the metabolic production of H^+ within the cell could occur, as suggested by Siesjo and Messeter (1971) with particular reference to the brain. Additional control mechanisms may be involved within the hepatocyte where there appears to be a close relationship

between pH_i and lactate metabolism; lactate uptake has been shown to be accompanied by a rise in cell pH (Cohen et al 1971).

4.2.2 Relationship between pH and lactate uptake in the perfused rat liver

Very little effect of lowering the external pH from 7.4 to 7.0 on hepatic lactate uptake was observed. However, when pH fell below 7.0 lactate uptake by the liver ceased and lactate was actually produced by the liver (Fig. 10). Related observations were made by Hems et al (1966). Using 48hr starved rats, a higher lactate concentration (10 mmol/l) and measuring glucose production rather than lactate uptake, they demonstrated that glucose production remained relatively constant as the pH of the perfusate was reduced from 7.4 to 7.1 but then fell to a tenth of its value as the pH was further reduced to 7.0. In their work pH was not reduced below 7.0 and intracellular pH was not measured.

In the present work, the relationship between intracellular pH and lactate uptake was similar to that observed with pH_e. Lactate uptake by the liver remained relatively constant when pH_i was above 7.0. When the cells became more acid they changed from lactate uptake to lactate production (Fig. 11).

Lactate uptake by the liver is influenced by the concentration of lactate in the perfusate. The lactate level for half-maximal gluconeogenesis by the isolated perfused rat liver is 2 mmol/l (Exton et al 1970). This was the perfusate

lactate concentration used in the metabolic acidosis group of experiments. This raises the possibility that the observed relationship between pH and lactate uptake could be explained on the basis of different perfusate lactate concentrations in the five pH groups. This, however, can be discounted as the mean lactate concentrations in the perfusate at the time of pH_i measurement in the five groups were not significantly different from each other.

4.2.3 The mechanism of suppression of lactate uptake by acidosis

The main pathway of lactate metabolism in the liver is gluconeogenesis and the results of these experiments indicate that this pathway is inhibited by acidosis. The rate limiting step in the gluconeogenic sequence lies between pyruvate and phosphoenolpyruvate and in liver the rate limiting enzyme may be pyruvate carboxylase (Exton and Park 1969).

Recently it has been shown from cross-over studies that acidosis probably exerts its inhibitory effect at a point between pyruvate and phosphoenolpyruvate (Iles, Cronje, Holman, Richards and Cohen 1975). These workers studied the intermediates of the gluconeogeneic pathway in isolated rat liver perfused with either normal or acidotic media (pH 6.71, P_{CO_2} 41 mm Hg) containing either low levels of lactate, i.e. 2-4 mmol/l, or high levels, i.e. 7-12 mmol/l. They confirmed that acidosis inhibited lactate uptake. Measurement of the concentration of some of the intermediate metabolites demonstrated a 'cross-over' point between pyruvate

and phosphoenolpyruvate.

The conversion of pyruvate to phosphoenolpyruvate involves several steps, some of which take place within the mitochondria. Pyruvate is transported from the cytosol to the mitochondrion where it is converted to oxaloacetate by pyruvate carboxylase. Oxaloacetate is then converted to either aspartate (if lactate is the substrate) or malate (if pyruvate is the substrate). The aspartate or malate is then transported out of the mitochondrion into the cytosol where it is converted back to oxaloacetate which is then converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK). A low pH could affect any of these steps. In view of its _in vitro_ pH characteristics (see below), pyruvate carboxylase is the most likely enzyme to be influenced by pH. It is now known that there is a specific mechanism for the transport of pyruvate into the mitochondrion and this could also be pH dependent.

4.2.3.1 The pH characteristics of pyruvate carboxylase

The original observations on the pH characteristics of this enzyme were made by Scrutton and Utter (1968) using purified enzyme from chicken liver. They showed that the activity of pyruvate carboxylase is influenced by pH in two ways. Firstly, the activation of the enzyme by acetyl Co A (for which it has an absolute requirement) is pH dependent; the activation constant falls steeply between pH 6.9 and pH 7.6. Secondly, there is evidence that the enzyme itself

is intrinsically pH sensitive in that the maximal velocity of the reaction in the presence of high concentrations of acetyl CoA rises steeply over the same pH range. Below pH 7.0 the enzyme is virtually inactive _in vitro_ (Scrutton and Utter 1968).

Subsequent studies have shown that pyruvate carboxylase from rat, guinea pig and human liver have pH characteristics which closely resemble those of the avian enzyme (Kleineke and Söling 1971; Scrutton and White 1974). For the guinea pig and rat enzyme the Ka value at pH 7.88 is approximately 24 µmol/l, whereas ar pH 7.0 it is about 90-150 µmol/l.

This inhibition of pyruvate carboxylase activity under acidotic conditions _in vitro_ could explain the observed cessation of lactate uptake by the liver at low pH. For this to be so, it is necessary to suppose that the measured values of pH_i reflect the pH at the site of pyruvate carboxylase activity.

The exact intracellular location of pyruvate carboxylase activity has been a subject of controversy. Some workers have demonstrated pyruvate carboxylase activity both within the mitochondria and cytosol of the cell (Ballard and Hanson 1967). Seubert, Henning, Schoner and L'Age (1968) showed that it was the cytosolic component which adapted to changing physiological conditions. However, it is now generally agreed that in the chicken, rat and human liver, pyruvate carboxylase activity is confined to the mitochondria (Böttger et al 1969; Keech and Utter 1962; Scrutton and Utter 1974). It has been suggested that the appearance of pyruvate

carboxylase activity in the cystolic fraction of the cell is related to the method of separation used, the enzyme being reversibly bound to the outer mitochondrial membrane. Böttger et al (1969) considered that the enzyme was located in the matrix space of the mitochondrion, which is also thought to be the site of the enzymes of the citric acid cycle.

It is impossible to be certain that the calculated value for hepatic pH obtained using the DMO method reflects the pH at the site of pyruvate carboxylase activity, i.e. the mitochondrion. Addanki et al (1967) determined the pH_i of beef heart mitochondria and found them to be alkaline (pH 8.31). However, the conditions under which these experiments were performed were artificial, particularly with respect to the absence of permeant ions. When the latter are present it is unlikely that large pH gradients exist between the cytosol and mitochondria and it seems likely that the measured value of hepatic pH_i approximates to the pH at the site of pyruvate carboxylase activity. However, no studies have been made of the variation of mitochondrial pH with various acid-base states and it is not possible to comment further upon the pH variation at the site of pyruvate carboxylase activity.

4.2.3.2 Pyruvate transport into the mitochondrion

Pyruvate is probably transported from the cytosol into the mitochondrion by a specific carrier mechanism. Evidence for this comes from experiments using α-cyano-4-hydroxycinnamate

which has been shown to be a specific inhibitor of pyruvate transport across the membranes of mitochondria. This has been demonstrated using isolated rat liver and heart mitochondria and in kidney cortex slices, the isolated perfused rat heart and human erythrocytes (Halestrap and Denton 1975). It has been suggested that under certain conditions pyruvate transport might be a rate limiting step in pyruvate carboxylation and therefore in gluconeogenesis (Halestrap 1975). Nothing is yet known about the effect of acid-base changes on the pyruvate carrier mechanism; theoretically acidosis could produce the observed inhibition of lactate uptake by interfering with the transport of pyruvate into the mitochondria.

4.2.3.3 Redox status and pyruvate carboxylation

Intracellular acidosis has been shown to increase tissue lactate/pyruvate ratio (Folbergrova et al 1972b). A fall in pH_i might therefore inhibit pyruvate carboxylation by the limitation of substrate availability (Scrutton and Utter 1967).

The effect of acidosis on the redox status of the cell could also explain the pattern of metabolites in the livers perfused with acidotic media found by Iles et al (1975). In subsequent similar experiments the level of oxaloacetate was reduced by 40% when compared with livers perfused with media of physiological pH (Iles and Cohen, personal communication). This could be explained either by decreased synthesis of oxaloacetate, the result of inhibition of pyruvate carboxylase, or by the known effect of acidosis on redox status, the lactate/pyruvate

and malate/oxaloacetate ratios rising as cell pH falls.

4.2.4 Lactate production by the liver

In the experiments described in this thesis, cessation of lactate uptake by the liver and actual lactate production was observed under severely acidotic conditions.

The ability of the liver to produce lactate has been demonstrated by Woods and Krebs (1971). Using the isolated perfused rat liver preparation and using very low initial perfusate concentrations of lactate (0.26 µmol/ml), they showed that under aerobic conditions livers from well fed rats produced lactate from endogenous glycogen at an initial rate of 0.78 µmol/min/g wet wt. but that lactate production subsequently decreased and ceased altogether when the lactate concentration in the medium reached 2 mmol/l. This approximates to the blood lactate concentration in the whole animal and they considered that this was regulated by control mechanisms within the liver itself.

Livers from 24hr and 48hr starved animals also produced lactate from endogenous glycogen but at a much lower rate. After 24 hours of starvation the liver produced lactate until a steady state perfusate concentration of 0.61 mmol/l was reached; after 48 hours of starvation the concentration was 0.32 mmol/l. As in the fed rats, these concentrations are similar to the blood lactate concentrations in starved whole animals.

In the present work, the rats had been starved for

24 hours and when the pH of the medium and the intracellular pH fell below 7.0, the mean lactate production by the isolated perfused livers was 1.86 µmol/min^{-1} (100 g rat)$^{-1}$. The most likely source of lactate is endogenous glycogen, although the possibility that the lactate was derived from alanine cannot be ruled out.

The rate limiting enzyme in the glycolytic sequence is phosphofructokinase (PFK) which is pH sensitive. The activity of the enzyme is stimulated by alkalosis and inhibited by acidosis. This has been demonstrated in a number of tissues, including liver slices (Gevers and Dowdle 1963), PFK is inhibited by ATP and it is the inhibitory concentration which is pH dependent. Using the intact rat diaphragm, Ui (1966) demonstrated that in the presence of an ATP concentration below 3 mmol/l, lactate production was stimulated by increasing the external pH from 7.4 to 8.0.

In order to explain the observed lactate production by the liver under acidotic conditions, it is necessary to postulate that inhibition of pyruvate carboxylase by a falling pH occurs before inhibition of PFK and therefore lactate production becomes apparent when lactate uptake has been completely inhibited.

4. PHENFORMIN EXPERIMENTS

4.3.1 Choice of experimental animal

The guinea pig was chosen in preference to the rat because of reports that the rat is insensitive to phenformin. Altschuld and Kruger (1968) showed that phenformin failed to produce hypoglycaemia in the intact rat and inhibition of gluconeogenesis from lactate in the perfused rat liver. In contrast to this a much smaller dose of phenformin than was used in the rat experiments produced hypoglycaemia and inhibition of gluconeogenesis in the guinea pig. Subsequent reports have, however, shown that the rat does respond to high doses of phenformin (Toews et al 1970; Ogata, Jomain-Baum and Hanson 1974).

This species difference in the response to phenformin has been attributed to the ability of the animal to convert phenformin to the metabolically inactive p-hydroxy form in the liver. The relatively insensitive rat is more efficient than the sensitive guinea pig in hydroxylating phenformin. Sensitivity of the rat to phenformin can be increased when the drug is given with a compound which inhibits hepatic microsomal hydroxylation (Cook, Blair, Gilfillan and Lardy 1973).

4.3.2 Effect of phenformin on lactate uptake

Phenformin inhibited lactate uptake by the perfused liver and this was associated with a fall in glucose production (Figs. 14 and 15).

4.3.2.1 Dose-response relationship

In the present work, no relationship between phenformin dose (which ranged from 0.05 mmol/l to 0.5 mmol/l) and the degree of inhibition of lactate uptake was demonstrated (Fig. 13). This is contrary to the work of others who have demonstrated a dose-response relationship (Altschuld and Kruger 1968; Ogata et al 1974). Ogata et al showed that the dose-response relationship became increasingly apparent during the course of the experiment and was not obvious until 30-40 minutes after the addition of phenformin to the perfusate. This may explain the failure to demonstrate a dose-response relationship in the present work, particularly as the phenformin was usually added to the perfusate in two doses, thirty and ten minutes before the final ten minute perfusion period when measurements of lactate uptake were made.

4.3.2.2 Time course of response to phenformin

The inhibitory action of phenformin did not become apparent until the forty to fifty minute perfusion period (Fig. 14). This delay in action has been observed by other workers (Toews et al 1970; Connon 1973; Ogata et al 1974). Cook, Blair and Lardy (1973), who considered that phenformin inhibited gluconeogenesis by depressing mitochondrial function, suggested that delayed action of phenformin represented that time taken for mitochondria to concentrate the drug. It is interesting to note that Ogata et al (1974) found that the time-course of the inhibitory effect of phenformin was similar in the isolated perfused guinea pig liver and isolated guinea pig liver mitochondria.

4.3.2.3 Mechanism of lactate uptake inhibition

Phenformin could theoretically inhibit lactate uptake by the liver either by interfering with the transport of lactate into the cell or by inhibiting gluconeogenesis from lactate within the cell. It would have been possible in this work to distinguish between these two suppositions by measuring tissue lactate levels but this was not done. There is substantial evidence that phenformin inhibits hepatic gluconeogenesis by a direct action on the pathway (Altschuld and Kruger 1968; Meyer et al 1967; Haeckel and Haeckel 1972; Cook et al 1973(b); Ogata et al 1974). The actual mechanism of this inhibition is uncertain. Haeckel and Haeckel (1971) and Toews et al (1970) produced evidence from cross-over studies which suggested that phenformin has a direct inhibitory effect on the triose phosphate dehydrogenase step of the gluconeogenic pathway. Cook et al (1973b) failed to find evidence that phenformin had a direct inhibitory effect on any enzymes of the pathway, basing this conclusion on their studies of gluconeogenic intermediates in the perfused guinea pig liver and on *in vitro* enzyme studies.

An alternative hypothesis is that the primary effect of phenformin is on mitochondrial function and that inhibition of gluconeogenesis is secondary to this. Early studies on the action of phenformin showed that it inhibited energy transfer in respiring mitochondria, although the actual site of action was controversial (Steiner and Williams 1958; Wick et al 1958; Falcone et al 1962). Ogata et al (1974) studied isolated guinea pig liver mitochondria and demonstrated that at low concentration (0.1 mmol/l) phenformin blocked energy transfer at

NADH dehydrogenase (Site I) of the electron transport chain. It may be significant that other compounds (i.e. cycloheximide and diphenyleneiodonium) which are known to have an inhibitory action at Site I are also inhibitors of gluconeogenesis. (Jomain-Baum, Garber, Farber and Hanson 1973; Garber, Jomain-Baum, Salganicoff, Farber and Hanson 1973; Holland, Clark, Bloxham and Lardy 1973).

The relationship between phenformin-induced depression of mitochondrial function and inhibition of gluconeogenesis is probably a complicated one (Ogata et al 1974; Cook et al 1973b). One effect of a block of energy transfer would be a reduced ATP/ADP ratio. Altschuld and Kruger (1968) demonstrated that ATP levels were lowered by phenformin in the isolated perfused guinea pig liver and attributed inhibition of gluconeogenesis from lactate to this. Cook et al (1973b) produced evidence that phenformin reduced the level of ATP in the perfused rat liver but that it was unlikely that inhibition of gluconeogenesis was directly related to this. It must be pointed out that the level to which ATP must fall to produce inhibition of gluconeogenesis is unknown.

In the experiments described in this thesis, oxygen consumption fell significantly during the course of the perfusion in the phenformin group but no such fall was observed in the control group. Ogata et al (1974) also observed a fall in oxygen consumption in the presence of phenformin in both isolated perfused guinea pig liver preparations and isolated pig and rat liver mitochondria. In the experiments on the isolated liver preparation the observed fall in oxygen consumption could be explained by either phenformin induced inhibition of mitochondrial function or by a direct inhibitory

action of phenformin on the gluconeogenic pathway.

It is of interest that the concentrations of phenformin found by Assan, Heuclin, Girard, Lemaire and Attali (1975) in the plasma of patients with phenformin induced lactic acidosis was 0.2 mmol/l - very similar to that employed in the experiments reported in this thesis.

4.3.3 Lactate uptake and intracellular pH

In these experiments there was a positive correlation between lactate uptake and cell pH (Figs. 12 & 13). The results are compatible with one of the hypotheses being tested in this thesis, namely that lactate uptake and cell pH are interrelated. Alteration of the rate of lactate uptake should therefore be accompanied by corresponding changes in cell pH and should be independent of the method used to alter lactate uptake. Initial support for this hypothesis came from the work of Cohen et al (1971) who increased the concentration of lactate in the perfusate and observed a rise in cell pH. Further support comes from the experiments described in this thesis in which lactate uptake was reduced by inhibiting gluconeogenesis with phenformin and was accompanied by a fall in cell pH.

The validity of the intracellular pH measurements in these experiments could be questioned on two counts:

(1) The lactate uptake in the control and phenformin groups was lower in the 50-60 minute perfusion period than it was in the 40-50 minute period. The non-steady state could have resulted in failure of full equilibration of DMO and

thus the cell pH measurements could not be considered valid. However, for reasons discussed elsewhere (see 4.1.2.1) it is unlikely that the correlation between lactate uptake and cell pH was due to failure of DMO equilibration.

(2) The hepatic venous pH was significantly lower in the phenformin group than it was in the control group. This is presumably due to decreased lactate uptake resulting in a lower bicarbonate production. The lower hepatic venous pH could have influenced cell pH. However, the differences in pH were very small and the results obtained in the metabolic acidosis experiments indicate the difference could have made only a slight contribution to the observed changes in cell pH (see Fig. 9).

Whilst there is considerable evidence that phenformin has a direct inhibitory action on the gluconeogenic pathway and its effect on cell pH would be secondary to this, the possibility remains that its primary action is on cell pH itself. It could be postulated that phenformin poisons the membrane pump responsible for active extrusion of H^+ thereby causing a fall in cell pH and a reduction in lactate uptake (see Fig. 16). It is impossible from these experiments to differentiate between these alternative modes of action of phenformin.

4.3.4 Effect of phenformin on glucose production

Glucose production fell during the course of the perfusion in both the control and phenformin groups. The fall off was greater in the phenformin group and during the final

ten minute period the livers were taking up glucose from the perfusate rather than producing it (Fig. 15). Part of the observed fall off during the 40-50 minute perfusion period was due to the fact that a proportion of the glucose produced during the 20-40 minute perfusion period came from a non-gluconeogenic source. When livers were perfused with a medium which did not contain added lactate, glucose was produced during the 20-40 minute perfusion period which represented 27% and 23% of glucose produced in the control and phenformin groups respectively when lactate was added to the perfusate. No glucose was being produced by the livers in the absence of added lactate during the 40-60 minute period.

The fall off in glucose production was accompanied by a lesser decrease in lactate uptake and this lack of the expected stoichiometric relationship in the control group and to a greater extent in the phenformin group is difficult to explain. In the control group, 80% of the lactate taken up during the initial perfusion period could be accounted for by glucose production, but during the final period this proportion had fallen to about 30%, although there was no change in oxygen consumption as judged by lack of change in hepatic venous P_{O2}. It has been shown from the study of other metabolites that the discrepancy cannot be explained by the diversion of lactate along other metabolic pathways to the synthesis of pyruvate, alanine, free fatty acids or glycogen (Lloyd, Iles, Walton, Hamilton and Cohen 1975). An alternative suggestion is that a proportion of the lactate was converted to acetyl Co A which entered the tricarboxylic acid cycle. As this

would be expected to cause a rise in hepatic oxygen consumption it is necessary to postulate, particularly in the phenformin series where oxygen consumption fell, that lactate replaced other substrates for oxidation. This lack of a stoichiometric relationship between lactate and glucose has been observed in two other published studies using the isolated perfused rat liver (Thurman and Scholz 1971; Krebs 1971b). Krebs suggested that a proportion of lactate could be directed along the pathway of ribose phosphate synthesis.

4.4 THE PHYSIOLOGICAL AND PATHOLOGICAL SIGNIFICANCE OF THIS WORK

The results of experiments described in this thesis provide further evidence that hepatic cell pH and lactate metabolism are inter-related. Three broad conclusions can be drawn from this work:

1) That the cells of the liver are partially protected against changes in the external pH (pH_e). Hepatic lactate metabolism may play a role in the protection of the cells against external acidosis.

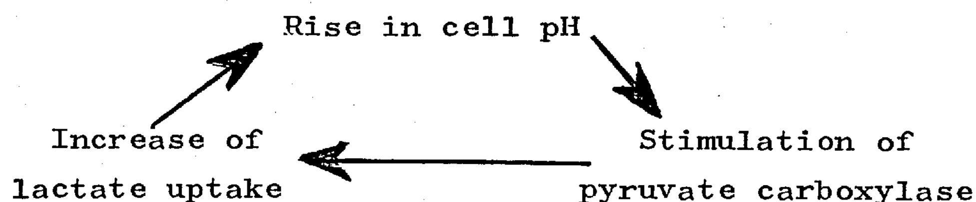
2) That simulated metabolic acidosis of severe degree (i.e. $pH_i < 7.0$) produces an inhibition of lactate uptake and a change to lactate production by the liver. Above 7.0 change in pH_i has little effect on lactate uptake.

3) That suppression of lactate uptake by the liver, caused by phenformin induced inhibition of gluconeogenesis is accompanied by a fall in pH_i.

These conclusions are based on work on animal models, the isolated perfused liver of the rat and guinea pig. Do they have any physiological or pathological relevance in man ?

It has been suggested that in the liver there is positive feedback between lactate metabolism and cell pH (Cohen et al 1971). This relationship is illustrated by

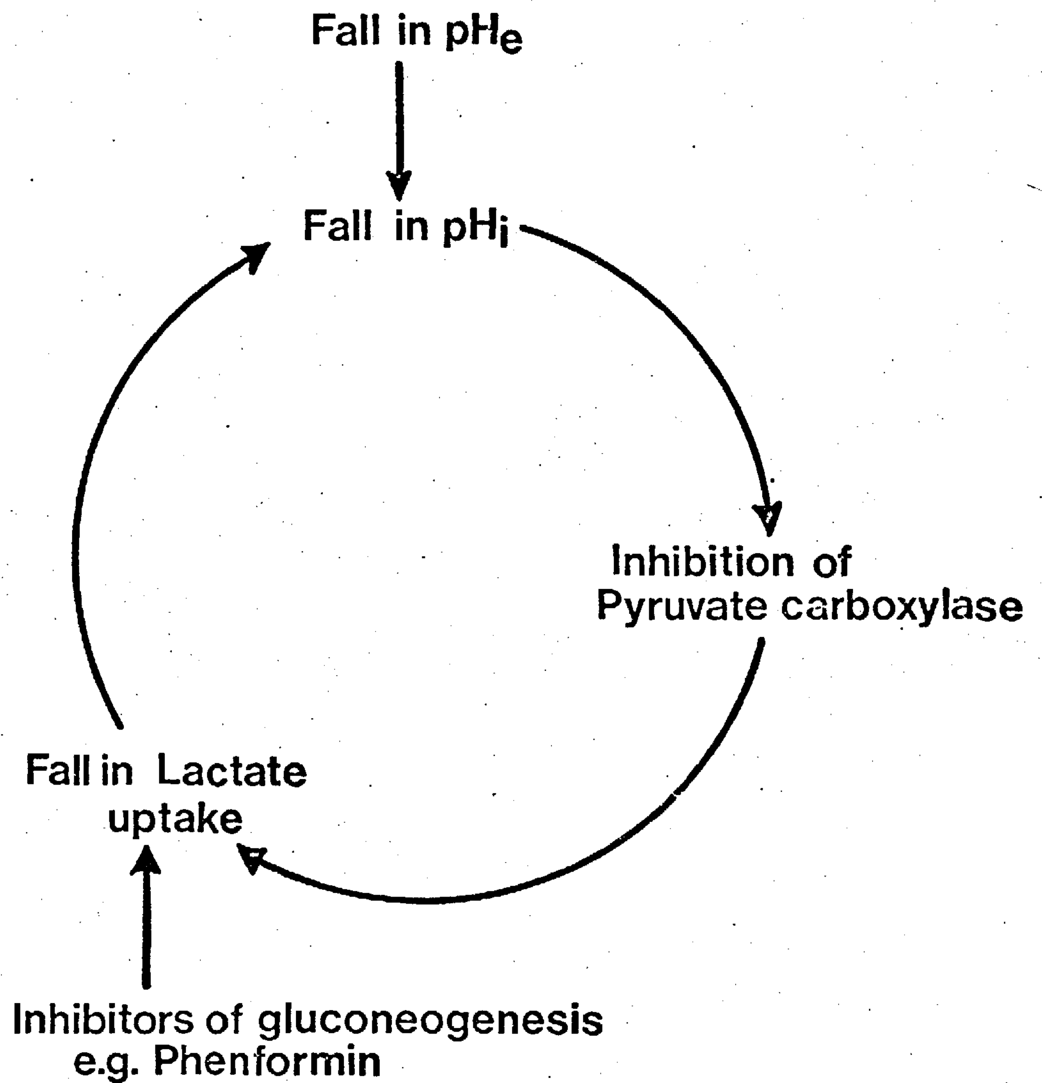
the following diagram:



The site at which hepatic cell pH exerts its influence on lactate metabolism may be pyruvate carboxylase. Evidence suggests that human pyruvate carboxylase has the same cellular location and pH characteristics as rat and guinea pig pyruvate carboxylase (Scrutton and White 1974).

The work described in this thesis, by demonstrating that cellular acidosis results in a fall in lactate uptake and that inhibition of lactate metabolism by phenformin results in a fall in cell pH, gives further support to the proposed positive feedback control mechanism of hepatic lactate metabolism. It also establishes that an hepatic cell pH of 7.0 is the critical point at which lactate uptake may cease and lactate production begin; if this point was reached self-perpetuating lactic acidosis could result. Therefore, this work may be relevant to an understanding of certain aspects of lactic acidosis and to homeostatic mechanisms operating during exercise.

The sequence of events which could lead to lactic acidosis is illustrated in Fig. 16. The factor initiating the sequence could either be an inhibitor of gluconeogenesis, e.g. phenformin, or a condition producing extracellular and intracellular acidosis, e.g. 'shock'. In either case the



POSSIBLE MECHANISM OF THE PATHOGENESIS OF LACTIC ACIDOSIS

Fig. 16

fall in cell pH, once it had passed the critical value, would inhibit lactate uptake which in turn would lead to a further fall in pH_i. Eventually the critical pH would be reached when failure of lactate uptake by the liver occurred followed by hepatic lactate production.

This sequence may be important in the development of both Type IIA and Type IIB lactic acidosis. In phenformin-associated Type IIB lactic acidosis it could account for: (i) the fulminant course of the condition; (ii) the observed therapeutic efficacy of large quantities of sodium bicarbonate. Any therapeutic measure, if it is to be successful must break the 'vicious circle' shown in Fig. 16. Alkalinization of the hepatic cell by the administration of large quantities of bicarbonate would promote lactate uptake by the liver and thus lead to the correction of the hyperlactaemia and acidosis. These considerations would apply whether the primary action of phenformin was on gluconeogenesis or on cell pH.

Self-perpetuating lactic acidosis would result if hepatic cell pH fell below 7.0. The question arises of the nature of the mechanisms which protect the liver from reaching that critical point, particularly under conditions of increased lactate production, e.g. exercise. A number of possible mechanisms exist. Firstly, during exercise hepatic cell pH may be kept well above the critical pH by the increased production of hydroxyl ions because of increased lactate uptake produced by the high level of circulating lactate. Though the marked falls in hepatic blood flow that occur during exercise might at first sight seem to offset

this effect; there is evidence both in dog and man (Tashkin, Goldstein and Simmons 1972; Rowell et al 1966) that this may not be so. Thus Tashkin et al (1972) found in the dog that even at normal levels of blood lactate a greater than 70% fall in hepatic blood flow had to occur before absolute lactate uptake by the liver fell. However, very severe reductions in hepatic blood flow as in shock may result in hepatic output of lactate (Schröder, Eltringham, Gumpert, Jenny, Pluthand, Zollinger 1969; Sriussadaporn and Cohn 1968).

The kidney is the second most important organ of lactate removal. If uptake of lactate is accompanied by production of hydroxyl ions as is suggested in the liver, the kidney may play an important role in acid-base homeostasis by virtue of its metabolic activities. There is evidence that renal uptake of lactate is stimulated under acidotic conditions. Using conscious fed rats, Yudkin and Cohen (1975) compared the renal clearance of a lactate load in nephrectomized and sham-operated animals. Under conditions of metabolic acidosis, when hepatic lactate uptake would be expected to be impaired, they found that the renal contribution to lactate removal increased from 16% to 44% as the pH fell from 7.45 to 6.75. They attributed this to increased uptake and metabolism of lactate by the kidney. Further evidence for this was obtained from experiments on the isolated perfused rat kidney (Yudkin and Cohen 1976). These experiments suggested that the metabolic activities of the kidney were important in acid-base homeostasis *in vivo* during acute as

well as chronic acidosis. The mechanism of this stimulatory effect of acidosis on the rate of lactate removal is uncertain. Alleyne (1970) demonstrated an increase in the rate of gluconeogenesis from glutamate and succinate in renal cortical slices from rats made acidotic with ammonium chloride. He attributed this to increased phosphoenolpyruvate carboxykinase (the rate limiting enzyme in the gluconeogenic pathway in the kidney) activity which became apparent four hours after making the animals acidotic. Whilst this may be the explanation in chronic acidosis, it is unlikely to be the explanation of increased lactate removal in acute acidosis and some unknown factor is probably responsible (Yudkin and Cohen 1976).

Thus under acidotic conditions increased renal uptake of lactate may partly compensate for impaired hepatic uptake. However, this protective mechanism probably does not operate under certain conditions which lead to lactic acidosis. (i) During 'shock', reduced renal blood flow would interfere with renal lactate uptake. (ii) In phenformin-associated lactic acidosis renal function is usually impaired and in addition to this there is evidence that *in vitro* phenformin inhibits renal as well as hepatic gluconeogenesis (Meyer et al 1967; Alleyne, Besterman and Flores 1971).

During severe exercise increased renal uptake of lactate may play an important role in the correction of the hyperlactaemia and acidosis. Two additional compensatory mechanisms may be operating.

Firstly, skeletal muscle itself may utilize lactate as a source of energy and therefore contribute to the removal of the lactate load. Hermansen and Stensvold (1973) showed

in man that following a period of severe exercise the fall in blood lactate was greater when the subject continued to exercise sub-maximally than when the subject rested. Other workers have found an arterio-venous difference in lactate concentration across non-exercising muscles following a period of exercise of other muscle groups and attributed this to the raised blood lactate stimulating lactate uptake by the resting muscles (Freyschuss and Strandell 1967; Harris, Bateman and Gloster 1962). However, it is impossible to assess what proportion of the apparent lactate uptake was metabolized and what proportion was due to equilibration of the raised blood lactate level with the resting tissues. Kreisberg (1974) considered that removal oflactate by skeletal muscle would compensate for isolated hepatic failure of lactate uptake and concluded that the latter alone would not result in clinical lactic acidosis. However, Yudkin and Cohen (1975) found in nephrectomized rats that loss of the renal capacity for lactate removal was not compensated for by the liver and skeletal muscle, making it unlikely that the kidneys and skeletal muscle would compensate for the loss of hepatic lactate removal.

Secondly, hepatic uptake of lactate would be stimulated by the raised arterial lactate accompanying severe exercise. This in turn would be expected to raise hepatic cell pH which would further stimulate lactate uptake by the mechanism discussed previously. This would have two effects: a) correction of the systemic acidosis either by excess hydroxyl ions "escaping" from the liver cells or by entry of hydrogen ions into the cell; b) resynthesis of glucose from lactate within the liver and thus the conservation of energy supplies. A criticism

of this theory would be the failure to demonstrate a rise in lactate uptake as pH_i values rose above 7.1 (see Fig.11). However, the results of work on the isolated liver probably underestimate the metabolic capabilities of the organ *in vivo* (Yudkin and Cohen 1975).

APPENDICES

A1. EXPERIMENT TO ASSESS THE EFFICIENCY OF [^{14}C] DMO EXTRACTION USING TOLUENE ETHYL ACETATE

[^{14}C] DMO and [^{14}C] inulin in tissue and plasma samples were separated chemically using toluene/ethyl acetate. This experiment was designed to ascertain the efficiency of [^{14}C] DMO extraction and to see if varying the weight of the tissue sample had any effect on the efficiency of extraction.

Method

Four samples of rat liver of different weights were taken and homogenised and deproteinised using trichloracetic acid as described in 2.5.4.1. After centrifugation, the supernatants were removed and [^{14}C] DMO added to each sample. Aliquots were taken for counting. A second aliquot of each sample was taken and extracted twice by shaking with 10 ml toluene/ethyl acetate (1:1 v/v). Each extracted sample was counted. Quenching corrections were made by addition of internal standard (Hexadecane ^{14}C) and recounting the vials. By comparing the corrected ^{14}C counts before and after extraction, the efficiency of extraction was calculated for each sample.

Results

wt of tissue g	[^{14}C] DMO counts extracted %
1.06	98.68
1.32	98.47
2.97	98.51
4.65	99.01

Conclusion

The mean percentage of $[^{14}C]$DMO extracted was 98.7 and the amount extracted was independent of the weight of tissue.

A2. CALCULATIONS

1. CALCULATION OF INTRACELLULAR pH

1.1 Theoretical considerations

The method of determination of intracellular pH used in these experiments was based on the technique described by Schloerb and Grantham (1965). Their technique was modified by using ^{14}C labelled inulin in place of ^{35}Cl as the extracellular marker. $[^{14}C]$ DMO and $[^{14}C]$ inulin were separated by extraction of the latter with toluene/ethyl acetate.

It has been shown previously (1.5.1.3 p52) that intracellular pH can be calculated from the DMO distribution ratio:

$$\text{i.e. } \frac{Ri}{Re} = \frac{He}{Hi} \cdot \frac{Hi+K}{He+K} \qquad \text{(Equation 5)}$$

where Ri represents total intracellular concentration of DMO

Re represents total extracellular concentration of DMO

The ratio $\frac{Ri}{Re}$ was calculated by substituting ^{14}C and ^{3}H counts in plasma and tissue before and after extraction into the following formula:

$$\frac{Ri}{Re} = \frac{\left\{\dfrac{\text{Tissue } ^{14}C \text{ cts(unex)} - \text{Tissue } ^{14}C \text{ cts(ex)}}{\text{Plasma } ^{14}C \text{ cts(unex)} - \text{Plasma } ^{14}C \text{ cts(ex)}}\right\} - \dfrac{\text{Tissue } ^{14}C \text{ cts (ex)}}{\text{Plasma } ^{14}C \text{ (ex)}}}{\dfrac{\text{Total tissue } ^{3}H \text{ cts}}{\text{Plasma } ^{3}H \text{ cts}} - \dfrac{\text{Tissue } ^{14}C \text{ cts (ex)}}{\text{Plasma } ^{14}C \text{ cts (ex)}}}$$

where ^{14}C cts (unex) represents unextracted ^{14}C counts

^{14}C cts (ex) represents ^{14}C counts after extraction

Plasma and tissue samples were potted in duplicate before and after extraction. They were counted in a scintillation counter using channels for ^{14}C (channel A) and ^{3}H (channel B). Quenching was measured by recounting after addition of ^{14}C and ^{3}H internal standards. The computer programme outlined below was used to calculate intracellular pH from this data. All counts were corrected for quenching, background and cross-over to give 'true mean counts' (where 'mean' refers to the mean of duplicates).

1.2 Computer programme for the calculation of intracellular pH

This programme was written in JEAN which is a conversational language for use on the Queen Mary College multi-access on-line computer system (MINIMOP).

Input

A(1) = Counting time for each pot

A(2)-A(5) = Counts recorded for pots (and 2 channels A (carbon) and B (tritium)

A(6)-A(9) = Counts recorded for liver pots 1 and 2, channels A and B

A(10)-A(17) = Counts recorded for plasma and liver pots 1 and 2 after extraction with toluene/ethyl acetate

A(18) and A(19) = Background counts, channels A and B

A(20) = Counting time after addition of internal standard

A(21)-A(36) = Counts recorded after addition of internal standard for plasma and liver samples corresponding to A(2)-A(17)

A(37) = Extracellular hydrogen ion activity (nmol/litre)

Description of programme

Steps 2.1-2.5 Correction for time and background

Steps 3.1-3.96 Calculation of quenching factors

Steps 4.1-4.91 Calculation of cross-over factors

Steps 5.1-5.53 Solution of simultaneous equations to obtain 'true' carbon and tritium counts, corrected for cross-over and quenching

Steps 6.1-6.3 Calculation of counts due to tritium, inulin and DMO

Steps 7.1-7.9 Calculation of intracellular pH and inulin space/tissue water ratio

Output

B(2)-B(16) = Mean crude ^{14}C counts in 'carbon' channel, corrected for time and background

C(3)-C(17) = Mean crude ^{3}H counts in 'tritium' channel corrected for time and background

Q(1)-Q(6) = Quenching factors for ^{14}C and ^{3}H (plasma taken as 1.0)

X(1)-X(8) = Cross-over factors for ^{14}C and ^{3}H

P(1)-P(8) = 'True' plasma (P) and liver (T) ^{3}H counts

R(1)-R(8) = 'True' plasma (P) and liver (T) ^{14}C counts

Aq(P) = Plasma ^{3}H counts

Aq(T) = Tissue ^{3}H counts

In(P) = Plasma ^{14}C counts after extraction

In(T) = Tissue ^{14}C counts after extraction

i.e. ^{14}C counts in tissue and plasma due solely to inulin

DMO(P) = Plasma ^{14}C counts (unextracted) - plasma ^{14}C counts (extracted)

DMO(T) = Tissue ^{14}C counts (unextrated) - tissue ^{14}C counts (extracted)

i.e. ^{14}C counts in tissue and plasma due solely to DMO

DMO distribution ratio = $\frac{Ri}{Re}$

HE = Extracellular pH HI = Intracellular pH

```
1.1PAGE
1.2DEMAND A(I) FOR I=1(1)37
1.3DO PART 2
1.4TO STEP 1.1
2.1B(I)=(A(I)-A(18))/A(1) FOR I=2(2)16
2.2C(I)=(A(I)-A(19))/A(1) FOR I=3(2)17
2.3TYPE B
2.4TYPE C
2.5DO PART 3
3.1J=A(21)/A(20)-A(2)/A(1)
3.2Q(1)=(A(25)/A(20)-A(6)/A(1))/J
3.3TYPE Q(1) IN FORM 1
3.4Q(2)=(A(29)/A(20)-A(10)/A(1))/J
3.5TYPE Q(2) IN FORM 2
3.6Q(3)=(A(33)/A(20)-A(14)/A(1))/J
3.7TYPE Q(3) IN FORM 3
3.8K=A(24)/A(20)-A(5)/A(1)
3.9Q(4)=(A(28)/A(20)-A(9)/A(1))/K
3.91TYPE Q(4) IN FORM 4
3.92Q(5)=(A(32)/A(20)-A(13)/A(1))/K
3.93TYPE Q(5) IN FORM 5
3.94Q(6)=(A(36)/A(20)-A(17)/A(1))/K
3.95TYPE Q(6) IN FORM 6
3.96DO PART 4
4.1X(1)=(A(22)/A(20)-A(3)/A(1))/J
4.2X(2)=(A(26)/A(20)-A(7)/A(1))/(A(25)/A(20)-A(6)/A(1))
4.3X(3)=(A(30)/A(20)-A(11)/A(1))/(A(29)/A(20)-A(10)/A(1))
4.4X(4)=(A(34)/A(20)-A(15)/A(1))/(A(33)/A(20)-A(14)/A(1))
4.5X(5)=(A(23)/A(20)-A(4)/A(1))/K
4.6X(6)=(A(27)/A(20)-A(8)/A(1))/(A(28)/A(20)-A(9)/A(1))
4.7X(7)=(A(31)/A(20)-A(12)/A(1))/(A(32)/A(20)-A(13)/A(1))
4.8X(8)=(A(35)/A(20)-A(16)/A(1))/(A(36)/A(20)-A(17)/A(1))
4.9TYPE X
4.91DO PART 5
5.1R(1)=(B(2)-X(5)*C(3))/(1-X(1)*X(5))
5.2P(1)=C(3)-X(1)*R(1)
5.21R(2)=(B(4)-X(5)*C(5))/(1-X(1)*X(5))
5.22P(2)=C(5)-X(1)*R(2)
5.23R(3)=(B(6)-X(6)*C(7))/((1-X(2)*X(6))*Q(1))
5.24P(3)=(C(7)-X(2)*R(3))/Q(4)
5.25R(4)=(B(8)-X(6)*C(9))/((1-X(2)*X(6))*Q(1))
5.26P(4)=(C(9)-X(2)*R(4))/Q(4)
5.3R(5)=(B(10)-X(7)*C(11))/((1-X(3)*X(7))*Q(2))
5.31P(5)=(C(11)-R(5)*X(3))/Q(5)
5.32R(6)=(B(12)-X(7)*C(13))/((1-X(3)*X(7))*Q(2))
5.33P(6)=(C(13)-X(3)*R(6))/Q(5)
5.4R(7)=(B(14)-X(8)*C(15))/((1-X(4)*X(8))*Q(3))
5.41P(7)=(C(15)-X(4)*R(7))/Q(6)
5.42R(8)=(B(16)-X(8)*C(17))/((1-X(4)*X(8))*Q(3))
5.43P(8)=(C(17)-X(4)*R(8))/Q(6)
5.5TYPE £,"PC",R(1),R(2),£,"PEXC",R(5),R(6)
5.51TYPE £,"TC",R(3),R(4),£,"TEXC",R(7),R(8)
5.52TYPE £,"PH",P(1),P(2),£,"PEXH",P(5),P(6)
5.53TYPE £,"TH",P(3),P(4),£,"TEXH",P(7),P(8)
5.6DO PART 6
6.1W(1)=(P(1)+P(2))/2
6.2W(2)=(P(3)+P(4))/2
6.3W(3)=(P(5)+P(6))/2
6.4W(4)=(P(7)+P(8))/2
6.5U(1)=(R(5)+R(6))/2
6.51U(2)=(R(7)+R(8))/2
6.52V(1)=((R(1)+R(2))/2)-U(1)
6.53V(2)=((R(3)+R(4))/2)-U(2)
6.6TYPE £,"TRUE MEAN COUNTS IN PLASMA, TISSUE"
6.61TYPE FORM 7,£
6.62TYPE U(1),V(1),W(1),U(2),V(2),W(2) IN FORM 8
6.63DO PART 7
7.1T=(V(2)/V(1)-U(2)/U(1))/(W(2)/W(1)-U(2)/U(1))
7.2TYPE T IN FORM 9
7.3H=(741.3*A(37))/(T*(A(37)+741.3)-A(37))
7.31 Z = -(LOG(H*(10↑(-9)))/LOG(10))
7.32 TYPE Z IN FORM 12
7.4TYPE A(37) IN FORM 10
7.5TYPE H IN FORM 11
7.6L=(U(2)/U(1))/(W(2)/W(1))
7.7 TYPE L IN FORM 13
7.8TYPE "CHECK W(1)=W(3),W(2)=W(4), APPROX",£,£,£,£,£,£,£
7.9TYPE W(1),W(3),W(2),W(4)
FORM1:
QCT=£.££££
FORM2:
QCPEX=£.££££
FORM3:
QCTEX=£.££££
FORM4:
QHT=£.££££
FORM5:
QHPEX=£.££££
FORM6:
QHTEX=£.££££
FORM7:
   IN(P)       DMO(P)       AQ(P)      IN(T)      DMO(T)       AQ(T)
FORM8:
  £££££       £££££       £££££££    £££££       £££££       ££££££
FORM9:
DMO DISTRIBUTION RATIO = £.£££££
FORM10:
HE=£££.£££
FORM11:
HI=£££.£££
FORM12:
PHI=£££.£££
FORM13:
INULIN SPACE/WATER SPACE = £.££££
```

```
A(1) = 20
A(2) = 127674
A(3) = 79849
A(4) = 128002
A(5) = 80228
A(6) = 7490
A(7) = 7723
A(8) = 7298
A(9) = 7870
A(10) = 86778
A(11) = 69941
A(12) = 87818
A(13) = 70005
A(14) = 3026
A(15) = 6990
A(16) = 3032
A(17) = 7092
A(18) = 606
A(19) = 479
A(20) = 2
A(21) = 35954
A(22) = 14532
A(23) = 12878
A(24) = 22762
A(25) = 24080
A(26) = 7684
A(27) = 972
A(28) = 15270
A(29) = 34200
A(30) = 12865
A(31) = 9368
A(32) = 24197
A(33) = 25235
A(34) = 6751
A(35) = 667
A(36) = 17420
A(37) = 50.12

B(2) = 6353.4
B(4) = 6369.8
B(6) = 344.2
B(8) = 334.6
B(10) = 4308.6
B(12) = 4360.6
B(14) = 121
B(16) = 121.3

C(3) = 3968.5
C(5) = 3987.45
C(7) = 362.2
C(9) = 369.55
C(11) = 3473.1
C(13) = 3476.3
C(15) = 325.55
C(17) = 330.65

QCT=1.0062
QCPEX=1.1007
QCTEX=1.0753
QHT= .9826
QHPEX=1.1667
QHTEX=1.1338

X(1) = .2823656767
X(2) = .2962453388
X(3) = .2300311102
X(4) = .2427363591
X(5) = 5.278441168-3
X(6) = 1.672305462E-2
X(7) = 3.408833193E-2
X(8) = 2.177035211E-2

PC
B(1) = 6341.904802
B(2) = 6358.229106

PEXC
B(5) = 3836.837565
B(6) = 3884.352397

TC
B(3) = 337.7232024
B(4) = 328.0123562

TEXC
B(7) = 106.4991349
B(8) = 106.6758022

PH
P(1) = 2177.763759
P(2) = 2192.104336

PEXH
P(5) = 2220.336432
P(6) = 2213.711107

TH
P(3) = 266.7884507
P(4) = 277.1961526

TEXH
P(7) = 264.339252
P(8) = 268.7997105

TRUE MEAN COUNTS IN PLASMA, TISSUE
  IN(P)     DMO(P)     AQ(P)     IN(T)     DMO(T)     AQ(T)

  3861      2489       2185      107       226        272
DMO DISTRIBUTION RATIO = .65326
PHI= -7.099
HE= 50.120
HI= 79.578
INULIN SPACE/WATER SPACE = .2218
CHECK W(1)=W(3),W(2)=W(4), APPROX

W(1) = 2184.934047
W(3) = 2217.023769
W(2) = 271.9923017
W(4) = 266.5694813
```

2. CALCULATION OF LACTATE CONCENTRATION AND UPTAKE

The AutoAnalyzer method used gives a value for lactate concentration in µmol/ml of perchloric acid (PCA) - precipitated whole blood supernatant.

Total water in sample (blood + PCA) = water content of plasma + PCA volume + water content of red cells.

Let T_{wa} = Total water (ml)

A = Weight of sample tube + PCA (g)

B = Weight of sample tube + blood (g)

HCT = Haematocrit

Volume of PCA = 2 ml

D = Lactate concentration from AutoAnalyzer measurement (µmol/ml)

F = Flow rate (ml /min)

W = Weight of rat (g)

Assuming plasma water to be approximately 97% of total plasma volume and water in red cells to be 65% of red cell volume -

$$\text{Then } T_{wa} = 2+\left[(1-HCT)\cdot(B-A)\cdot 0.97\right] + \left[HCT\ (B-A)\cdot 0.65\right]$$

$$T_{wa} = 2+\left[(B-A)\right]\cdot\left[0.97\right] - \left[HCT\cdot (B-A\cdot 0.32\right] \text{ (ml)}$$

$$\text{Lactate in (B-A)g blood} = D.T_{wa} \ (\mu mol)$$

$$\text{Lactate/g blood} = \frac{D.T_{wa}}{(B-A)} \ (\mu mol)$$

Using the subscripts A and V to denote arterial and venous blood respectively

(A-V) Lactate concentration difference

$$= A\left[\frac{D.T_{wa}}{(B-A)}\right] - V\left[\frac{D.T_{wa}}{(B-A)}\right] (\mu mol/min)$$

Liver lactate consumption/output per 100 g rat =

$$\frac{D.T_{wa}\ \ (A-V)}{W\ (B-A)} .F.100\ (\mu mol/min)$$

The specific gravity of the perfusion medium was assumed to be 1.0.

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PUBLICATIONS ARISING FROM THIS THESIS

The experimental work presented in this thesis formed the basis of the following two publications:

LLOYD, M.H., ILES, R.A., SIMPSON, B.R., STRUNIN, J.M., LAYTON, J.M., COHEN, R.D. (1973) The effect of simulated metabolic acidosis on intracellular pH and lactate metabolism in the isolated perfused rat liver. Clinical Science and Molecular Medicine, 45, 543-549

LLOYD, M.H., ILES, R.A., WALTON, B., HAMILTON, C.A., COHEN, R.D. (1975) Effect of phenformin on gluconeogenesis from lactate and intracellular pH in the isolated perfused guinea pig liver. Diabetes, 24, 618-624

SUMMARY

1. The relationship between intracellular pH (pH_i) and lactate uptake by the isolated perfused rat and guinea pig livers has been studied during simulated metabolic acidosis and in the presence of phenformin.

2. In the metabolic acidosis experiments, as extracellular pH (pH_e) was decreased from 7.4 to 6.85 the fall in pH_i was considerably less (from 7.2 to 7.07). A further decrease in pH_e from 6.85 to 6.70 resulted in a corresponding fall in pH_i.

3. When pH_i was greater than 7.0 the liver took up lactate but when pH_i fell below 7.0 lactate output occurred.

4. In the phenformin experiments lactate uptake, glucose production and pH_i were measured in the isolated perfused livers of starved guinea pigs in the presence and absence of phenformin.

5. Phenformin inhibited lactate uptake and glucose production and this was associated with a fall in pH_i.

6. The relevance of these observations to the pathogenesis of clinical lactic acidosis and its treatment is discussed.