PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN SHEEP

A Thesis submitted by

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SUMMARY

The activities of two key gluconeogenic enzymes, pyruvate carboxylase and PEP carboxykinase, have been measured in the livers and kidneys of sheep under conditions of physiological perturbation. When normal, pregnant or phlorhizinised sheep were fasted for six days there was a marked increase in the hepatic activity of pyruvate carboxylase. Diabetes, induced either by pancreatectomy or by an injection of alloxan, caused a similar increase in activity, while administration of glucocorticoid hormones led to a significant reduction in the activity. Hepatic PEP carboxykinase increased only in diabetic sheep. The changes in the activities of the kidney enzymes were generally similar, except in diabetes, to those observed in the liver, but were less marked. In the diabetic animals neither enzyme increased in activity in the kidney.

Glucose entry rates were also estimated after a single injection of ^{14}C-glucose. Glucocorticoids caused no change in the entry rate, fasting of normal animals caused a slight decrease and there was an increase in alloxan diabetes. A very pronounced increase was observed in phlorhizinised animals when either fed or fasted. These results are discussed in relation to the corresponding measurements of the activities of the two enzymes.

The intramitochondrial and subcellular distributions of pyruvate carboxylase and PEP carboxykinase were determined in the livers of normal sheep prior to an investigation of the increased activity of pyruvate carboxylase in fasting and of both enzymes in diabetes. Digitonin fractionation of isolated mitochondria revealed that these enzymes were localised in the matrix. In the normal liver approximately 30% of the PEP carboxykinase activity was found in the mitochondria, the remainder being in the cytosol. The activity increased to a similar extent in both cell fractions in diabetes. The proportion of pyruvate carboxylase activity in the cytosol fraction increased from about 10% in the normal liver to 30 - 40% in fasting and diabetes. However, comparable distributions were also observed for mitochondrial matrix marker enzymes, glutamate dehydrogenase and citrate synthase, which suggested that the mitochondria were more fragile in these physiological conditions. This hypothesis was substantiated by electron micrographs which showed that in liver sections from fasted and diabetic sheep a high proportion of mitochondria were several times the normal size, but that in preparations of mitochondria isolated from these livers there were only normal sized mitochondria and a large amount of membrane fragments. Further evidence for the common identity of the pyruvate carboxylase activities of the cytosol and mitochondria was provided by immunochemical techniques, including antibody titration experiments and Ouchterlony double diffusion analysis. Pyruvate carboxylase therefore appears to be exclusively mitochondrial *in vivo* under the conditions examined.

In a study of the time course of the induction of pyruvate carboxylase and PEP carboxykinase in diabetes it was found that PEP carboxykinase reached the usual diabetic level within 24 hr, but that the response of pyruvate carboxylase was considerably slower. The increased activity of pyruvate carboxylase in fasted and diabetic sheep was shown to be associated with a proportionate increase in the amount of immunochemically-reactive enzyme protein.

The increased amount of enzyme protein present in fasting and diabetes represents long term control of pyruvate carboxylase activity. Previous work has established that $MgATP^{2-}$ and acetyl-CoA are involved in the acute control of the enzymic activity and the following evidence, obtained with purified sheep liver pyruvate carboxylase, supports the conclusion that there is negative cooperativity in the binding of pyruvate:

(a) the double reciprocal plot is biphasic

(b) the R_s value is greater than 81

(c) in the Hill plot there is a region of slope less than one

(d) two distinct K_d values have been obtained from the stimulation of avidin inhibition by pyruvate, thus indicating that at least two molecules of pyruvate are bound.

The physiological significance of these observations is discussed.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material which has previously been published or written by any other person, except where due reference is made in the text.

P.H. TAYLOR

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PUBLICATIONS

1. O.H. Filsell, I.G. Jarrett, P.H. Taylor and D.B. Keech. Effects of Fasting, Diabetes and Glucocorticoids on Gluconeogenic Enzymes in the Sheep. Biochim. Biophys. Acta, 184, 54 (1969).

2. H. Taylor, J. Nielsen and D.B. Keech. Substrate Activation of Pyruvate Carboxylase by Pyruvate. Biochem. Biophys. Res. Commun., 37, 723 (1969).

3. P.H. Taylor, J.C. Wallace and D.B. Keech. Gluconeogenic Enzymes in Sheep Liver. Intracellular Localisation of Pyruvate Carboxylase and PEP Carboxykinase in Normal, Fasted and Diabetic Sheep. Biochim. Biophys. Acta (In press).

ABBREVIATIONS

The following abbreviations have been used in this thesis:

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
CoA	Coenzyme A
Cyclic AMP	cyclic adenosine-3',5'-monophosphate
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
IDP	inosine-5'-diphosphate
GSH	reduced glutathione
PEP	phosphoenolpyruvate
S.E.M.	Standard Error of the Mean.

ENZYMES

Throughout the text of this thesis, enzymes have been referred to by their trivial names. The following is a list of these enzymes with their systematic names and numbers designated in the Report of the Commission on Enzymes of the International Union of Biochemistry, 1961.

Number	Trivial Name	Systematic Name
1.1.1.27	Lactate dehydrogenase	L-lactate:NAD oxidoreductase
1.1.1.37	Malate dehydrogenase	L-malate:NAD oxidoreductase
1.1.1.40	Malic enzyme	L-malate:NADP oxidoreductase (decarboxylating)
1.3.99.1	Succinate dehydrogenase	Succinate:(acceptor) oxidoreductase
1.4.1.3	Glutamate dehydrogenase	L-glutamate:NAD(P) oxidoreductase (deaminating)
1.4.3.4	Monoamine oxidase	Monoamine:O_2 oxidoreductase (deaminating)
2.6.1.1	Glutamate-oxaloacetate transaminase	L-aspartate:2-oxoglutarate aminotransferase
2.7.1.1	Hexokinase	ATP:D-hexose 6-phosphotransferase
2.7.1.11	Phosphofructokinase	ATP:D-fructose-6-phosphatase 1-phosphotransferase
2.7.1.40	Pyruvate kinase	ATP:pyruvate phosphotransferase
3.1.3.9	Glucose-6-phosphatase	D-glucose-6-phosphate phosphohydrolase
3.1.3.11	Fructose-1,6-diphosphatase	D-fructose-1,6-diphosphate 1-phosphohydrolase
4.1.1.32	PEP carboxykinase	GTP:oxaloacetate carboxy-lyase (transphosphorylating)
4.1.3.7	Citrate synthase	Citrate oxaloacetate-lyase (CoA-acetylating)
6.4.1.1	Pyruvate carboxylase	Pyruvate:CO_2 ligase (ADP)

CHAPTER 1. GENERAL INTRODUCTION

1.1. IMPORTANCE OF GLUCONEOGENESIS

Glucose is a major energy source for most vertebrate tissues, particularly the central nervous system, muscle and erythrocytes. It also serves as a precursor for the synthesis of amino sugars, uronic acids, pentoses and lipids. In spite of considerable fluctuations in the rate at which glucose is derived from the diet and utilised by the tissues, the blood glucose concentration is maintained within limits which are fairly well defined for a given species. Control of the blood glucose concentration depends largely on regulated alterations in the rates of glucose production by glycogenolysis or gluconeogenesis. Gluconeogenesis occurs in the liver and kidney cortex and is the process by which glucose is synthesised from non-carbohydrate precursors such as lactate, amino acids and glycerol. Gluconeogenesis is important when the dietary supply of glucose is inadequate for the metabolic requirements of the animal, and is also involved in the removal of excessive quantities of glucose precursors from the blood, *e.g.*, lactate after vigorous exercise.

Extensive studies with perfused rat liver preparations and with kidney cortex slices have indicated that the gluconeogenic capacities of both tissues are similar. Although little is known about the relative contributions of the liver and kidney to glucose production in the whole animal, it is believed that hepatic gluconeogenesis is of greater significance on a quantitative basis because of the larger mass of the liver and availability of substrates in the portal

circulation. It has been calculated from arterio-venous differences and from renal blood flow that the kidney in man (Cahill, 1964) and dog (McCann and Jude, 1958) may contribute about 15% of the inflow of glucose into the circulating blood.

The quantitative aspects of hepatic and renal gluconeogenesis in the whole animal are probably best illustrated by studies undertaken in man by Cahill and coworkers, who have found that under conditions of prolonged fasting the glucose production by the kidney is almost as great as that of the liver. In an adult human (about 70 kg) under normal conditions, the glucose consumption per day of the various tissues has been estimated as follows: brain, 120 - 130 g; blood constituents, 34 g; muscle, 30 g (see Cahill and Owen, 1968). Values obtained for other species are comparable with those obtained for man when body size is taken into account. The net splanchnic glucose production in man after an overnight fast varies from 180 to 350 g per day (Bondy, James and Farrar, 1949; Myers, 1950), an amount which corresponds to approximately half of the basic caloric turnover. After 36 to 48 hours of fasting the arterio-hepatic venous difference for glucose in man (Felig et al., 1969) is in the same range as for the post-absorptive state. The process of gluconeogenesis must be largely responsible for this production of glucose because the liver glycogen stores are depleted rapidly (although temporarily) early in starvation (Haro, Blum and Falcon, 1965). During prolonged starvation in man the brain gradually diminishes its rate of glucose

utilisation, and acetoacetate and β-(OH)butyrate become the most important sources of energy for this tissue. At the same time, there is marked attenuation of gluconeogenesis, with the glucose production after 5-6 weeks of starvation reduced to 86 g/24 hr, of which the liver contributes about 55% and the kidney 45% (Owen et al., 1969). Half of the glucose synthesised is derived from recycled lactate and pyruvate while the remainder is from glycerol and amino acids.

1.2. GLYCOLYTIC AND GLUCONEOGENIC PATHWAYS

Liver and kidney cortex are unique among tissues in higher animals in that they are capable of both synthesising and degrading glucose by the processes of gluconeogenesis and glycolysis, respectively. Although related anabolic and catabolic processes do not usually share common pathways, some enzymes of the glycolytic pathway are common to both the synthesis and the breakdown of carbohydrates.

A. Enzymes unique to each pathway

The gluconeogenic and glycolytic pathways are shown in Fig. 1.1. The reversal of some of the steps of glycolysis is thermodynamically unfavourable and hence to circumvent this difficulty each of the pathways possesses some unique enzymes:

(a) hexokinase and glucokinase catalyse the conversion of glucose to glucose-6-phosphate, while glucose-6-phosphatase catalyses the formation of glucose from glucose-6-phosphate.

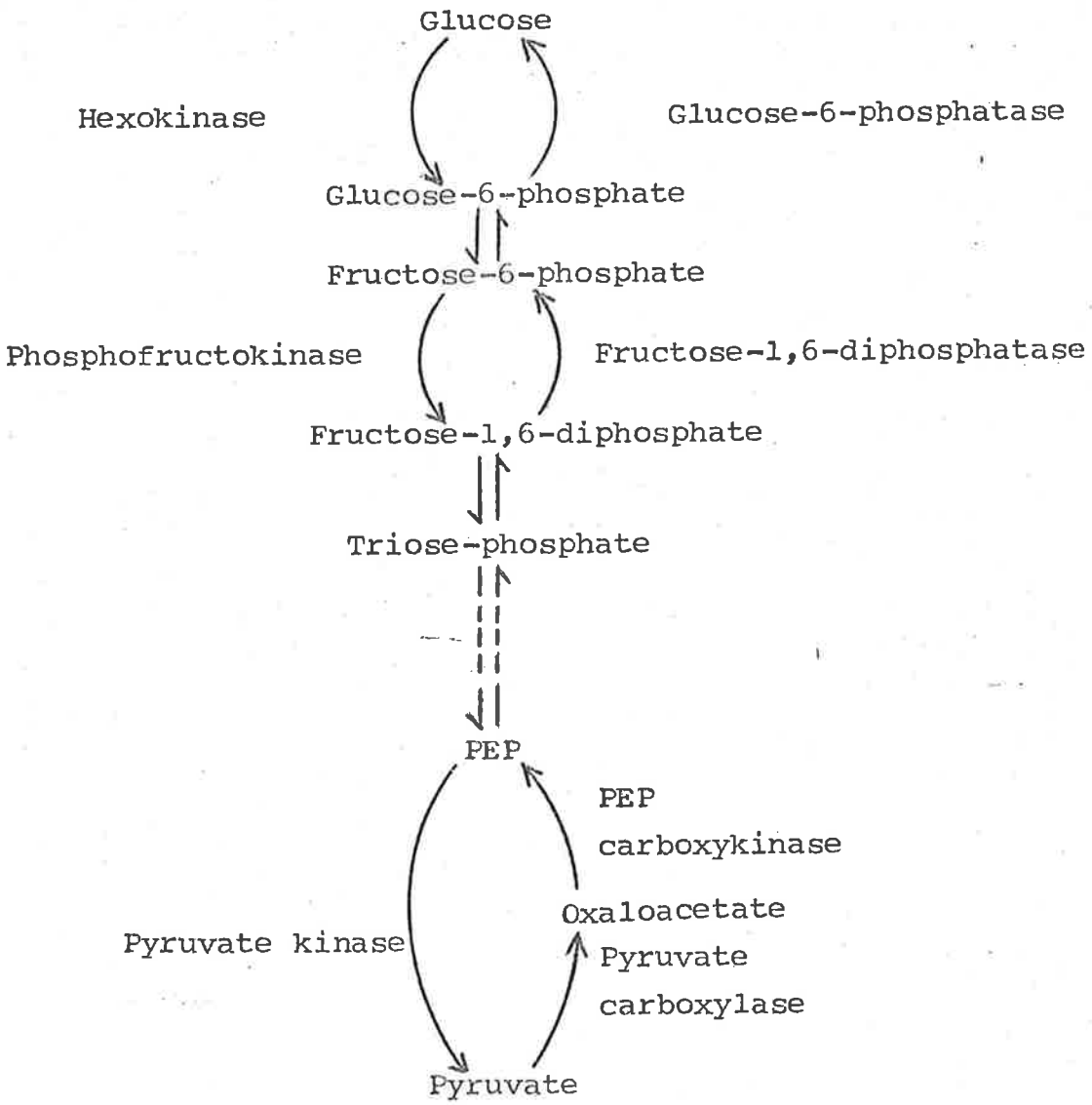


FIG. 1.1. The pathways of glycolysis and gluconeogenesis. The enzymes unique to glycolysis are hexokinase, phosphofructokinase and pyruvate kinase. The enzymes unique to gluconeogenesis are pyruvate carboxylase, PEP carboxykinase, fructose-1,6-diphosphatase and glucose-6-phosphatase.

(b) phosphofructokinase functions in glycolysis with fructose-1,6-diphosphatase as the corresponding gluconeogenic enzyme.

(c) pyruvate kinase is a glycolytic enzyme and it is proposed that the conversion of pyruvate to phosphoenolpyruvate (PEP) is carried out in two steps by the enzymes pyruvate carboxylase and PEP carboxykinase.

B. Conversion of pyruvate to PEP.

Although the reversibility of the pyruvate kinase reaction has been demonstrated (Lardy and Ziegler, 1945), the physiological significance of this reaction in the conversion of pyruvate to PEP has been questioned on energetic grounds by Krebs (1954). Solomon et al. (1941) first suggested a role for CO_2 fixation in the pathway of gluconeogenesis when they found that rats could incorporate $^{11}CO_2$ into liver glycogen. Labelling patterns observed in the glucosyl residues of glycogen derived from pyruvate (Topper and Hastings, 1949) and lactate (Lorber et al., 1950) indicated the presence of a symmetrical four-carbon intermediate in the pathway of CO_2 incorporation. These observations, together with the discovery of L-malate enzyme (Ochoa et al., 1947) and PEP carboxykinase (Utter and Kurahashi, 1954) led to the proposal that PEP was formed by a dicarboxylic acid shuttle (Fig. 1.2) with malate and oxaloacetate as intermediates. However, the equilibrium of the overall system, involving malate enzyme and PEP carboxykinase, was no more favourable to PEP synthesis than that of the pyruvate kinase reaction (Utter,

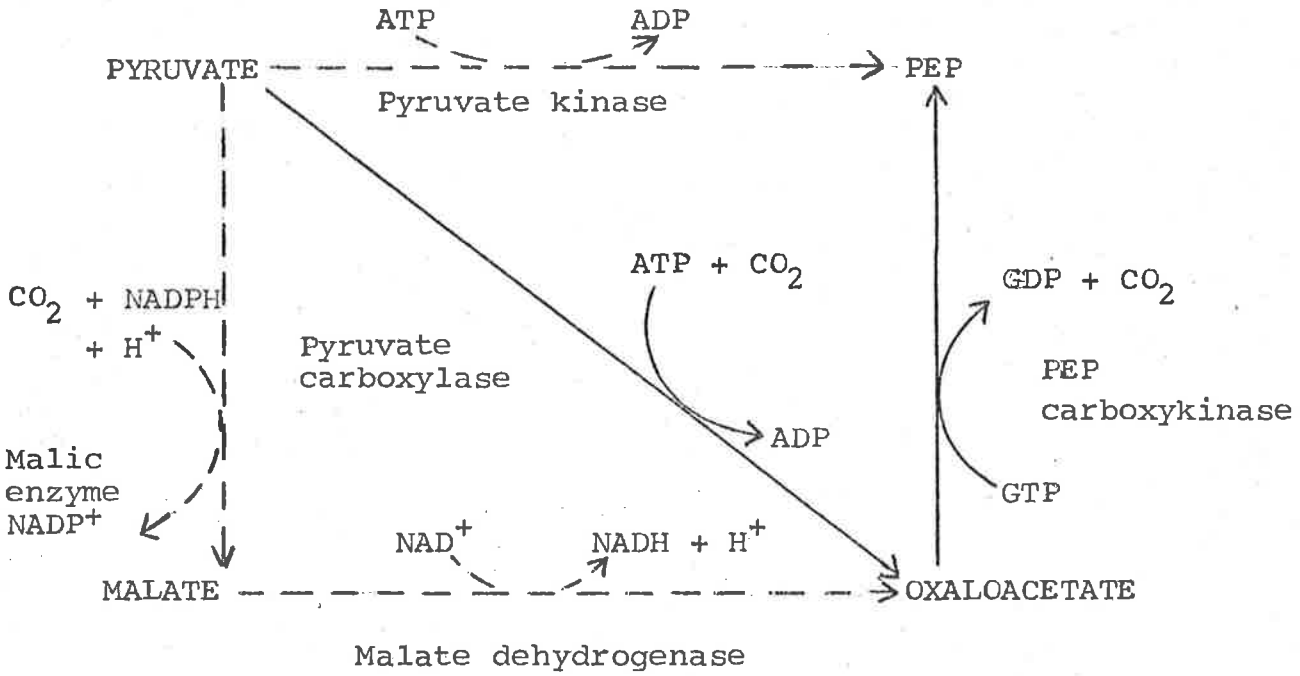


FIG. 1.2. Postulated pathways for the conversion of pyruvate to PEP.

1959). Furthermore, examination of the kinetic properties, tissue and intracellular distributions and maximal capacities of these enzymesrevealed that the properties of PEP carboxykinase, but not of malate enzyme were consistent with a gluconeogenic role. The activity of malate enzyme appears to be unrelated to gluconeogenic activity (Shrago et al., 1963) since conditions leading to an increase in gluconeogenesis either do not affect malate enzyme or else depress its activity. Chicken liver mitochondria, which are devoid of malate enzyme, can form PEP from pyruvate (Utter, 1963) and further evidence for the non-participation of malate enzyme is the observation that this enzyme is virtually absent from the liver of ruminants (Hanson and Ballard, 1967) which rely almost entirely on gluconeogenesis for their glucose requirements. The discovery of pyruvate carboxylase (Utter and Keech, 1960) led to the postulate that PEP is formed from pyruvate by an abbreviated dicarboxylic acid shuttle involving pyruvate carboxylase and PEP carboxykinase, the equilibrium of the overall reaction lying far in the direction of PEP synthesis as a result of the utilisation of two high energy phosphate equivalents. The distribution and properties of pyruvate carboxylase are consistent with its proposed role in the synthesis of PEP from pyruvate and an efficient coupling of purified pyruvate carboxylase and PEP carboxykinase has been demonstrated (Keech and Utter, 1963). Significant levels of pyruvate carboxylase and PEP carboxykinase have been observed in the livers and kidneys of all birds and mammals examined and these enzymes are probably

involved in gluconeogenesis in these tissues.

Veneziale, Gabrielli and Lardy (1970) have recently published data which do not conform to this widely held view of the path of pyruvate carbon in gluconeogenesis. In perfused rat livers, where PEP carboxykinase activity was inhibited by quinolinate, there was significant conversion of pyruvate to glucose without the prior formation of malate, aspartate or PEP. This observation implies that there is an alternative pathway, undefined as yet, which does not require PEP carboxykinase activity. In addition, Bartley and Dean (1969) have observed that the formation of PEP from malate by isolated rat liver mitochondria appears to be independent of PEP carboxykinase activity.

The evidence which implicates the participation of the series of reactions from PEP to triose phosphate in the gluconeogenic pathway is also equivocal and does not exclude the existence of another pathway (Scrutton and Utter, 1968). The phosphoglycerate kinase reaction is approximately as exergonic in the glycolytic direction as the pyruvate kinase reaction and may also be bypassed in gluconeogenesis.

C. Transfer of oxaloacetate from mitochondria to cytosol

Although there is no direct and unequivocal proof that the four-carbon dicarboxylic acids are obligatory intermediates in the conversion of pyruvate to glucose, the scheme shown in Fig. 1.1 is generally accepted as the overall pathway of carbon in gluconeogenesis. However, there is species

variation in the intracellular distribution of PEP carboxykinase which has led to the development of different schemes for the synthesis of PEP in various species. In the avian liver e.g., pigeon (Gevers, 1967) and chicken (Utter, 1959), where PEP carboxykinase is exclusively mitochondrial, PEP must be synthesised in the mitochondrion as shown in Fig. 1.3A. In the rat and mouse where nearly all of the activity is found in the cytosol (Nordlie and Lardy, 1963) the bulk of the PEP for gluconeogenesis would be expected to be synthesised in the cytosol. Oxaloacetate penetrates the mitochondrial membrane very slowly and is transported in the form of malate, aspartate, fumarate or citrate to the cytosol where these compounds are converted back to oxaloacetate (Haynes, 1965; Lardy, Paetkau and Walter, 1965; Walter, Paetkau and Lardy, 1966). Cytosol PEP carboxykinase converts the oxaloacetate to PEP which is ultimately metabolised to glucose (Fig. 1.3.B). The use of malate as a transport derivative fulfils a dual role as this shuttle allows for the transfer of both carbon and reducing equivalents to the cytosol. Reducing equivalents are utilised at the glyceraldehyde-3-phosphate dehydrogenase step in the cytosol in gluconeogenesis and their transfer from the mitochondria is essential in gluconeogenesis from precursors which are more highly oxidised than glucose-6-phosphate, e.g., pyruvate, serine and aspartate. Experiments conducted by Williamson, Anderson and Browning (1970) with butylmalonate in perfused rat liver add support to the concept that malate transport is an integral feature of gluconeogenesis, and show that malate transport can be a rate-limiting step in glucose synthesis.

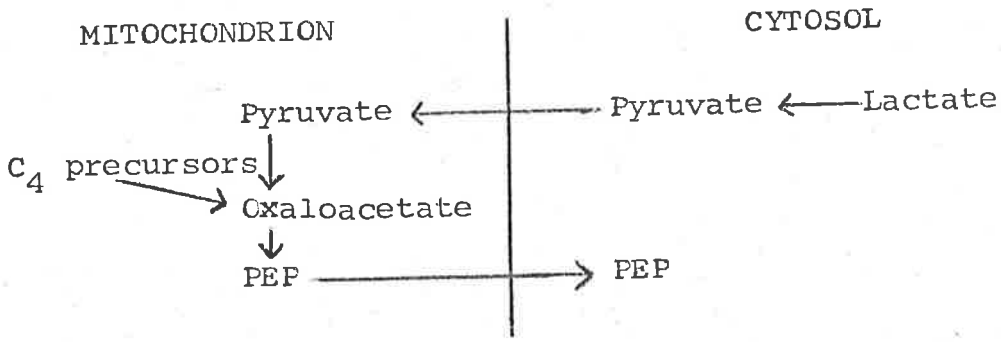


FIG. 1.3.A. Pathway of PEP synthesis in species where PEP carboxykinase is located in the mitochondrion.

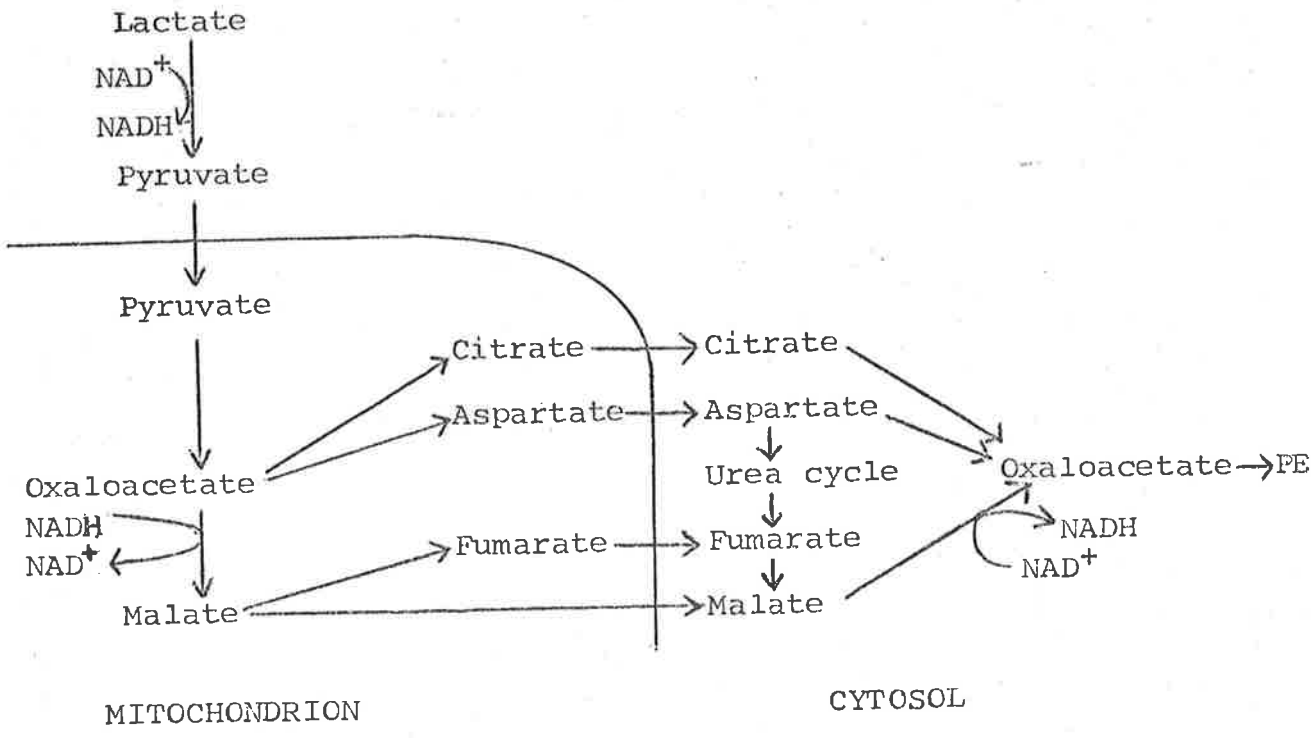


FIG. 1.3.B. Pathways of PEP synthesis in species where PEP carboxykinase is located in the cytosol.

Williamson et al. (1969a) consider that malate is the major form by which carbon leaves the mitochondrion when there is no independent source of reducing equivalents, as would be the situation if pyruvate were the substrate, and that aspartate is the means of transport when the requirements of gluconeogenesis for reducing equivalents are met by dismutation between lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase e.g., when lactate is the substrate. Lardy, Paetkau and Walter (1965), however, suggest that aspartate formed in liver mitochondria may also serve as a source of reducing equivalents in the cytosol - aspartate may be transported to the cytosol where, after donating an amino group for urea synthesis, it gives rise to fumarate. This compound is hydrated to malate, thus providing both reducing equivalents and the four-carbon skeleton for the formation of triose phosphate. Under conditions such as fasting, diabetes or glucocorticoid administration where protein is converted to glucose, aspartate must be used in large amounts for urea synthesis. The main sources of glucose under these conditions are probably malate and aspartate (Ray, Foster and Lardy, 1966), each of which also provides reducing equivalents.

There is evidence that such a scheme for the transport of oxaloacetate may also be operative in species where there is significant PEP carboxykinase activity in the mitochondria. Johnson, Ebert and Ray (1970) have found that the amount of PEP formed in and liberated from rabbit liver mitochondria provided with pyruvate is only a small fraction of the

total carbon leaving the mitochondria as PEP, malate, citrate, and also aspartate if NH_4^+ is added. This suggests that, although 75% of the enzymic activity is mitochondrial, the carbon for gluconeogenesis follows a pathway similar to that proposed for the rat.

1.3. IDENTIFICATION OF REGULATORY ENZYMES IN THE GLUCONEOGENIC PATHWAY

It has been recognised for many years that the basic requirements for gluconeogenesis are a suitable carbon source, reducing equivalents for the reduction of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate, and high energy phosphate for the pyruvate carboxylase, PEP carboxykinase and phosphoglycerate kinase steps.

Attention has been focused more recently on locating the rate-controlling enzymic steps. Studies of the allosteric control properties of isolated enzymes, e.g., pyruvate carboxylase, pyruvate kinase, phosphofructokinase and fructose-1,6-diphosphatase, indicate that they have the potential to act as control sites. However, it is also necessary to carry out studies using intact organs such as perfused liver in order to determine the physiological significance of these control factors, and whether the enzymes are, in fact, regulatory.

Several methods are available for the identification of regulatory enzymes in a metabolic pathway. These include:

(a) comparisons of the maximal catalytic capacities of the enzymes of the pathway

(b) measurements of the rates of product formation from substrates which enter the pathway at different levels

(c) comparison of mass action ratios and equilibrium constants

(d) application of the crossover theorem (Chance et al., 1958) to situations in which the concentrations of the intermediates of the pathway are measured at a series of time intervals during the transition between two steady states.

Descriptions of the use of these methods, together with a discussion of the limitations inherent in each may be found in reviews by Newsholme and Gevers (1967) and Scrutton and Utter (1968). Although none of these methods alone yields completely unequivocal results, a general pattern emerges when the results obtained with the different methods are compared. From these studies it has been concluded that glycolysis and gluconeogenesis in the liver and kidney cortex are controlled by the activities of the enzymes which catalyse the interconversions of fructose-6-phosphate and fructose-1,6-diphosphate, viz., phosphofructokinase and fructose-1,6-diphosphatase, and the enzymes which catalyse the interconversions between pyruvate and PEP, viz., pyruvate carboxylase, PEP carboxykinase, and pyruvate kinase. Although pyruvate kinase and phosphofructokinase are glycolytic enzymes, it is obvious from the complex interrelationships of the two pathways that regulation of the activities of these two enzymes can have a profound influence on the

activity of the gluconeogenic pathway.

In order to understand the control of these pathways it is useful to know the properties and effectors controlling the activities of these enzymes, and also related enzymes.

1.4. PROPERTIES OF THE PROPOSED REGULATORY ENZYMES AND OF RELATED ENZYMES

One would expect a close relationship to exist between the activities of pyruvate carboxylase, PEP carboxykinase, pyruvate kinase, citrate synthase and pyruvate dehydrogenase since these enzymes are all involved in the metabolism of pyruvate and oxaloacetate. Recent studies have indicated that pyruvate carboxylase, pyruvate dehydrogenase and citrate synthase are localised in the mitochondrial matrix, as is PEP carboxykinase in species where there is significant activity of this enzyme in the mitochondria (Schnaitman and Greenawalt, 1968; Erdicska et al., 1968; Marco, Sebastian and Sols, 1969; Chapter 4 of this thesis). Because of the spatial proximity of these enzymes it is imperative that suitable controls be operative to direct the flow of metabolites in the required direction. Pyruvate kinase is found in the cytosol to which oxaloacetate or PEP formed in the mitochondria must be transferred for subsequent conversion to glucose. In the absence of any controls pyruvate kinase could convert all the PEP back to pyruvate, thus resulting in a "futile cycle". A similar situation arises at the level of phospho-fructokinase and fructose-1,6-diphosphatase.

The properties of the above mentioned enzymes which are involved in the metabolism of pyruvate and oxaloacetate

will be discussed. Although it is realised that the properties of fructose-1,6-diphosphatase and phosphofructokinase are also important in the control of glycolysis and gluconeogenesis, these will not be discussed as this thesis is concerned mainly with the gluconeogenic pathway at the level of the three-carbon compounds.

(1) Pyruvate carboxylase

Pyruvate carboxylase is a biotin-containing enzyme catalysing the following reaction:

$$ATP + HCO_3^- + \text{E.biotin} \xrightarrow{Mg^{2+},\text{acetyl-CoA}} \text{E.biotin-}CO_2 + ADP + P_i$$

$$\text{E.biotin-}CO_2 + \text{pyruvate} \rightleftharpoons \text{E.biotin} + \text{oxaloacetate}$$

$$\underline{\text{SUM:}}\ ATP + HCO_3^- + \text{pyruvate} \rightleftharpoons \text{oxaloacetate} + ADP + P_i$$

The control of the activity of pyruvate carboxylase appears to be rather complex. The activity of the enzyme from vertebrate tissues is dependent on the presence of a short chain acyl-CoA (Keech and Utter, 1963; Barritt, Keech and Ling, 1966; Scrutton and Utter, 1967) the most effective of which is acetyl-CoA, although propionyl-CoA also activates the enzyme to only a slightly lesser extent, and butyryl-CoA has been found to activate the enzyme from calf liver. Activation by the latter two derivatives is particularly relevant to the situation in ruminant tissue.

There is a cooperative relationship between the enzyme and acetyl-CoA (Barritt, Keech and Ling, 1966; Scrutton

and Utter, 1967) and the sheep kidney enzyme also exhibits positive homotropic cooperativity with respect to the substrate, $MgATP^{2-}$, with heterotropic cooperativity between $MgATP^{2-}$ and Mg^{2+} (Keech and Barritt, 1967). This would make the enzyme very sensitive to slight changes in the concentrations of these compounds. The kinetics of the reaction with respect to pyruvate are characteristic of negative cooperativity as described by Levitzki and Koshland (1969) (see Chapter 6), the physiological significance of this phenomenon being that the enzyme would tend to be insulated against large fluctuations in activity over a wide range of concentrations of pyruvate.

Malonyl-CoA, methylmalonyl-CoA and succinyl-CoA inhibit the enzyme (Scrutton and Utter, 1967) while acetoacetyl-CoA, in addition to being an inhibitor, destroys the cooperative relationship between chicken liver pyruvate carboxylase and acetyl-CoA (Utter and Fung, 1970). On the other hand, β-hydroxybutyryl-CoA is an activator. Alterations in the redox potential of the cell could be reflected in the β-hydroxybutyryl-CoA/acetoacetyl-CoA, and it is possible that this could affect the activity of pyruvate carboxylase. Calculations of intramitochondrial concentrations of acetyl-CoA in rat liver (Williamson, 1969) yield values which are more than an order of magnitude greater than the apparent K_a of the mammalian enzymes for this compound (Barritt, Keech and Ling, 1966; Utter and Fung, 1970). However, the apparent K_a is determined *in vitro* under optimum conditions and *in vivo* this may be modified by the interactions of other CoA derivatives so that control by the concentration of acetyl-CoA

becomes relevant.

The energy status of the cell, particularly the mitochondria, must play a role in the regulation of the enzymic activity, since ADP is an inhibitor of the purified enzyme from chicken liver (Keech and Utter, 1963), and Walter and Stucki (1970) have reported that the activity of pyruvate carboxylase in isolated rat liver mitochondria is regulated by the intramitochondrial concentration of ADP.

Thus, it is possible that pyruvate carboxylase *in vivo* is subject to direct control by Mg^{2+}, $MgATP^{2-}$, acetyl-CoA and pyruvate and is also influenced by the energy status, and perhaps the redox potential, of the cell. The potential flexibility and sensitivity resulting from such a control system are consistent with the proposed regulatory role of this enzyme.

(2) PEP carboxykinase

PEP carboxykinase catalyses the following reaction:

$$\text{GTP} + \text{oxaloacetate} \xrightleftharpoons{Mn^{2+}} \text{PEP} + CO_2 + \text{GDP}$$

PEP carboxykinase occupies a key position in the gluconeogenic pathway and it has also been demonstrated that it is a regulatory enzyme (Ray, Foster and Lardy, 1966; Veneziale *et al.*, 1967; Ray, Hanson and Lardy, 1970). It would therefore be expected to be subject to some sort of metabolite control. However, no very convincing metabolite controls have been found for the mammalian enzyme except that Barns (1970) has demonstrated inhibition of sheep kidney mitochondrial

PEP carboxykinase by oxaloacetate. Malate inhibition of both mitochondrial and cytosol enzymes has been reported (Ballard, 1970) but at concentrations such that it is unlikely to be of physiological significance.

All the K_m values which have been reported for oxaloacetate are considerably higher than the concentration in the relevant cell compartment. The mitochondrial levels of oxaloacetate, based on $NAD^+/NADH$ ratios, are an order of magnitude less than the 1-10 μM range reported for whole tissues (Williamson, Lund and Krebs, 1967; Williamson, 1969; Loffler and Wieland, 1963; Baird et al., 1968; Ballard, Hanson and Kronfeld, 1968). Williamson (1969) has calculated that in the rat liver the concentration of oxaloacetate is about 5 μM in the cytosol and 0.2 μM in the mitochondria. Most reports indicate that PEP carboxykinase isolated from the cytosol or mitochondria of the liver of various species has a K_m for oxaloacetate of about 100 μM (Holten and Nordlie, 1965; Chang et al., 1966; Foster et al., 1967; Barns, 1970). In contrast to these data, (Ballard, 1970) has recently found that in the presence of Mg^{2+} the cytosol and mitochondrial enzymes from sheep and rat liver exhibit a K_m value for oxaloacetate of 20-30 μM, while in the presence of Mn^{2+} the K_m of the mitochondrial enzyme is reduced to 9 μM although that of the cytosol enzyme is unchanged. This observation also provides another example of differences between the mitochondrial and cytosol enzymes. Holten and Nordlie (1965) have demonstrated that the two enzymes from guinea pig liver show some differences with respect to metal ion activation

and AMP inhibition, and Ballard and Hanson (1969) have found that the cytosol and mitochondrial enzymes of rat liver mitochondria are immunologically distinct.

It is generally assumed that PEP carboxykinase in the cytosol has a gluconeogenic role. The high K_m value for oxaloacetate may be an *in vitro* artifact or it is possible that *in vivo* a positive effector influences the K_m for oxaloacetate. In the mitochondria there is an even greater discrepancy between the concentration of oxaloacetate and the K_m for this compound. However, the intramitochondrial concentration of PEP may be as high as 4 mM (Garber and Ballard, 1969) which contrasts with the range of 0.1 - 0.2 mM reported for whole liver (Ray, Foster and Lardy, 1966; Rolleston and Newsholme, 1967; Weidemann, Hems and Krebs, 1969; Baird and Heitzman, 1970). The K_m of sheep kidney mitochondrial PEP carboxykinase for PEP is approximately 0.2 mM and Barns (1970) has suggested that in view of the relative levels of oxaloacetate and PEP, it may be that the carboxylation reaction is more important *in vivo* than has hitherto been realised. If mitochondrial PEP carboxykinase does, in fact, have an anaplerotic role, the inhibition by oxaloacetate would be significant in the control of its own synthesis.

(3) Pyruvate kinase

Pyruvate kinase is potentially able to divert most of the gluconeogenic flux back towards pyruvate unless it is efficiently controlled. Recent studies have revealed that this enzyme is subject to a complex allosteric control system.

In a comprehensive investigation, Llorente, Marco and Sols (1970) found that the enzyme in fresh extracts of liver and kidney was subject to the following controls, some of which had been demonstrated previously (a) marked positive cooperativity with respect to the substrate, PEP (b) strong allosteric inhibition by alanine (Seubert et al., 1968) and ATP (Tanaka, Sue and Morimura, 1967), each of which raises the apparent K_m value and increases the sigmoidicity of the PEP kinetics; (c) strong activation by fructose 1,6-diphosphate (Taylor and Bailey, 1967) which greatly reduces the apparent K_m and destroys the cooperativity with respect to PEP. Fructose 1,6-diphosphate can fully counteract the inhibitory effects of alanine and ATP.

The concentrations of substrates and effectors for which these phenomena are observed are consistent with the concentrations likely to occur in the cell. The regulatory mechanism is such that a decrease in the fructose-1,6-diphosphate level, as would occur in the switch over from glycolysis to gluconeogenesis, would allow increased inhibition by alanine and ATP, and thus help to prevent diversion of PEP in a "futile cycle". Inhibition of pyruvate kinase by acetyl-CoA, NADH and free fatty acids (Weber, Lea and Stamm, 1968) may also be of physiological significance since these compounds stimulate the rate of gluconeogenesis.

Pyruvate kinase of other non-gluconeogenic tissues, including heart and adipose tissue, does not exhibit any of these allosteric properties (Llorente, Marco and Sols, 1970).

(4) Citrate synthase

The control of citrate synthase activity must be considered in relation to gluconeogenesis because this is one of the factors determining whether oxaloacetate enters the citric acid cycle or whether it is metabolised to glucose.

Inhibition of citrate synthase by fatty acyl-CoA derivatives has been reported (Tubbs, 1963; Wieland and Weiss, 1963), but from data obtained with purified pig heart enzyme (Srere, 1968), perfused rat liver (Williamson et al., 1969a) and arguments presented by other workers (Garland, 1968) it has been concluded that this inhibition is unlikely to be of physiological significance.

Control by the intramitochondrial ATP/ADP ratio (Atkinson, 1965; Shepherd, Yates and Garland, 1965; Garland, 1968; Jangaard, Unkeless and Atkinson, 1968) or more specifically, by the adenine nucleotide energy charge (Atkinson, 1968) may be important, although recent experiments with isolated rat liver mitochondria (Williamson et al., 1967; Williamson et al., 1969d; Wojtczak, 1968) have failed to substantiate this proposal.

Wieland, Weiss and Eger-Neufeldt (1964) and Williamson et al. (1969d) suggest that the oxaloacetate concentration may be more important than adenine nucleotides in the control of activity. The calculated mitochondrial oxaloacetate concentration of 0.1 - 0.4 μM is well below the reported K_m values of 2 to 5 μM (Garland, 1968; Jangaard, Unkeless and Atkinson, 1968). Citrate synthase activity could be controlled indirectly by the redox system since an increase in the

mitochondrial NADH/NAD^+ ratio would tend to restrict citrate synthase activity by decreasing the oxaloacetate concentration through the equilibrium of the malate dehydrogenase reaction. The apparent K_m values for oxaloacetate and acetyl-CoA are increased by ATP (Jangaard, Unkeless and Atkinson, 1968; Garland, 1968) and an increased ATP/ADP ratio would therefore potentiate the inhibition caused by a decreased oxaloacetate concentration.

High NADH/NAD^+ and ATP/ADP ratios tend to favour gluconeogenesis and under these conditions the activity of citrate synthase would be diminished, thus ensuring the availability of oxaloacetate for gluconeogenesis.

(4) Pyruvate dehydrogenase

The properties of the pyruvate dehydrogenase complex are relevant to the control of gluconeogenesis since it can compete with pyruvate carboxylase for pyruvate.

The pig heart enzyme is inhibited by its product, acetyl-CoA, which competes with CoA (Garland and Randle, 1964). Similar effects with acetyl-CoA and NADH (Nicholls and Garland, 1966) have been obtained with the rat liver enzyme.

When highly purified pyruvate dehydrogenase from beef kidney mitochondria is incubated with low concentrations of ATP, it is inactivated and phosphorylated by an ATP-specific kinase (Linn, Pettit and Reed, 1969). Conversely, the reactivation of the enzyme by Mg^{2+} is accompanied by dephosphorylation. The kinase and the phosphatase

responsible for these transformations appear to be regulatory subunits of the pyruvate dehydrogenase complex. The phosphatase will tend to be active when the intramitochondrial ATP/ADP ratio is low, and since the enzyme is inhibited by acetyl-CoA, it is obvious that there is an inverse relationship in the regulation of pyruvate dehydrogenase and pyruvate carboxylase and hence in the regulation of pyruvate oxidation and carboxylation.

1.5. REGULATION OF GLUCONEOGENESIS

The processes of glycolysis and gluconeogenesis both occur in the liver and kidney. The rate and direction of metabolism at a particular time is therefore determined by the relative activities of the regulatory enzymes. The activities of these enzymes will depend on their regulation by modifiers as discussed in Section 1.4., and also on the concentrations of their substrates.

Physiological stimulation of the gluconeogenic rate probably occurs during muscular exercise as a result of the increased production of lactate, and in carbohydrate starvation as a result of the increased availability of substrates such as glucogenic amino acids and glycerol. The initial increase in the gluconeogenic rate may be due to a more complete utilisation of the normal gluconeogenic capacities of the liver and kidney which are not saturated by the normal concentrations of precursors in the plasma (Exton and Park, 1967). Increases in the maximal capacities of these tissues also occur during prolonged exposure to conditions requiring

elevated rates of gluconeogenesis (Krebs and Yoshida, 1963; Krebs et al., 1963; Henning et al., 1966). The increased rates of gluconeogenesis observed in certain hormonal disorders, e.g., diabetes or malfunction of the adrenal cortex, can be reproduced experimentally by administration or withdrawal of the appropriate hormone and have been studied extensively in attempts to identify the enzymic reaction affected. The assayable activities of the key gluconeogenic enzymes, pyruvate carboxylase, PEP carboxykinase, fructose-1,6-diphosphatase and glucose-6-phosphatase in tissue homogenates have generally been found to increase under conditions of increased gluconeogenesis induced by metabolic, dietary or hormonal stimuli. However, the changes in the enzymic activities occur relatively slowly in contrast to the more rapid effects observed on the overall gluconeogenic flux. Changes in the rate of gluconeogenesis in the whole animal are difficult to interpret since both the gluconeogenic and peripheral tissues may be involved in the response observed. Consequently, most of the recent studies on the control of gluconeogenesis have been performed with the isolated gluconeogenic tissue either in the form of tissue slices or as perfused preparations of the whole organ. The effects are thus able to be defined more specifically than is the case in the whole animal.

A. Regulation by variations in the redox potential and energy status of the cell

From intensive studies of the redox state of the pyridine nucleotides and the phosphorylation state of the adenine nucleotides, Krebs and coworkers (Williamson, Lund and Krebs, 1967; Krebs and Veech, 1969) have concluded that these two systems are linked by enzymes which establish near equilibria. This network is likely to be a fundamental component of the energy transforming mechanisms in the cell since it establishes basal levels of the redox state of the two pyridine nucleotide couples in the cytosol and mitochondria and links the redox states to the supply of ATP.

The redox state of the pyridine nucleotides and the phosphorylation state of the adenine nucleotides may also be considered individually with respect to their effects on the rate of gluconeogenesis. The addition of fatty acids (Williamson, Scholz and Browning, 1969), glucagon (Williamson et al., 1969b) and ethanol (Williamson et al., 1969a) to perfused rat liver may enhance the rate of gluconeogenesis by increasing the $NADH/NAD^+$ ratio in the cytosol with a consequent stimulation of the conversion of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate. However, evidence has been presented that it is not the redox state of the pyridine nucleotides which controls the rate of gluconeogenesis. Studies in perfused rat liver (Ross, Hems and Krebs, 1967; Exton and Park, 1969), mouse liver slices (Krebs, Notton and Hems, 1966) and kidney cortex slices (Krebs, Gascoyne and Notton, 1967) show that the rates of gluconeogenesis are, within wide ranges,

independent of the ratio of the concentrations of lactate and pyruvate and hence of the redox state. They suggest instead that the redox state of the pyridine nucleotides may play a role in the regulation of metabolic processes by varying metabolite concentrations. Ethanol inhibits gluconeogenesis from lactate in the liver (Krebs, 1968; Krebs et al., 1969) as a result of a fall in the steady state concentration of pyruvate. Alcohol dehydrogenase activity leads to a more reduced state of the cytoplasmic NADH/NAD^+ couple and this in turn decreases the concentration of pyruvate through the equilibrium of the lactate dehydrogenase system. Ethanol therefore inhibits gluconeogenesis from lactate or other precursors forming pyruvate as an intermediate. Work by Krebs and Veech (1969) indicates that the redox state of the cytoplasmic NADPH/$NADP^+$ couple, together with the concentration of α-ketoglutarate determines the concentration of citrate, which is an allosteric effector of phosphofructokinase (Garland, Randle and Newsholme, 1963; Parmeggiani and Bowman, 1963; Passonneau and Lowry, 1963). It was mentioned in Section 1.3. that although phosphofructokinase is a glycolytic enzyme, the control of its activity is important in the regulation of gluconeogenesis.

In addition to its role in the regulation of the redox potential of the cell compartments, the adenine nucleotide system may have direct effects on the activities of regulatory enzymes. From the properties of the enzymes discussed in Section 1.4. it is apparent that when the ATP/ADP ratio is high, pyruvate kinase and citrate synthase are inhibited, whereas pyruvate carboxylase and PEP carboxykinase (as a result

of nucleoside diphosphate kinase activity) are activated, thus ensuring a high rate of gluconeogenic activity in the cell.

Atkinson (1968) has proposed that the control by the adenine nucleotides may be dependant on the energy charge of the adenylate pool. The energy charge is defined by the following expression which relates the concentrations of the three nucleotides: $([ATP] + 0.5[ADP]) / ([ATP] + [ADP] + [AMP])$.

B. Possible role of fatty acids

It has been shown in many laboratories (Struck, Ashmore and Wieland, 1965; Herrera et al., 1966; Soling et al., 1968; Williamson et al., 1968; Williamson, Browning and Scholz, 1969) that fatty acids increase the rate of glucose production from various substrates in the perfused rat liver. Since plasma free fatty acid concentrations fluctuate over a wide range in vivo during different dietary states, it is conceivable that control of gluconeogenesis by hepatic fatty acid oxidation may represent an important physiological phenomenon.

Studies by Williamson, Browning and Scholz (1969), using rat livers perfused with oleate, have indicated that the location of the control steps in the gluconeogenic pathway is determined both by the nature of the substrate used as a glucose precursor and by the rate of fatty acid oxidation. With lactate and pyruvate as substrates, control sites were observed at pyruvate carboxylase (activation) and phospho-

fructokinase (inhibition) presumably as a result of increased levels of acetyl-CoA and citrate, respectively. When alanine is the substrate there is a relative deficiency in the transport of reducing equivalents to the cytosol. The addition of fatty acids under these conditions caused stimulation of the glyceraldehyde-3-phosphate dehydrogenase step, probably through the elevated $NADH/NAD^+$ ratio maintained in the cytosol during enhanced fatty acid oxidation. Williamson, Browning and Scholz (1969) consider that control of pyruvate carboxylase activity is probably the most relevant interaction between fatty acid oxidation and gluconeogenesis. Other groups have also concluded that fatty acids enhance gluconeogenesis primarily by stimulation of pyruvate carboxylase (Soling et al., 1968) and inhibition of pyruvate oxidation (Teufel et al., 1967; Soling et al., 1968).

Further evidence for a role of fatty acids in the control of gluconeogenesis has been provided by liver perfusion studies with 4-pentenoic acid, the simplest of a series of unsaturated short chain fatty acids causing hypoglycaemia in vivo (Anderson et al., 1958; De Renzo et al., 1958; Corredor, Brendel and Bressler, 1967). Crossover sites have been located at the glyceraldehyde-3-phosphate dehydrogenase and pyruvate carboxylase steps, probably as a result of the decreased $NADH/NAD^+$ ratio in the cytosol and a fall in the level of acetyl-CoA, respectively (Ruderman, Shafrir and Bressler, 1968; Toews, Lowy and Ruderman, 1970; Williamson et al., 1969c; Williamson, Rostand and Peterson, 1970). These sites of inhibition correspond to the sites of

activation discussed for studies with fatty acids.

Exton, Corbin and Park (1969) have also found that high concentrations of free fatty acids stimulate gluconeogenesis in the perfused rat liver. However, when albumin-bound oleate was used, which should represent *in vivo* conditions more closely, little or no change was observed in the rate of gluconeogenesis, although a large increase in ketogenesis occurred. These investigators therefore question the proposal that fatty acids play an important role in the rapid short term regulation of hepatic gluconeogenesis *in vivo*.

Despite this observation, the hypothesis that fatty acids are involved in the regulation of gluconeogenesis remains an attractive one and should not be discarded before more information is available.

C. Hormonal control

(a) Pancreatic hormones

The two pancreatic hormones, insulin and glucagon, constitute a delicately balanced system, and have opposite effects which appear to be mediated by the level of cyclic AMP. Williamson *et al.* (1969b) have concluded that the altered metabolic state of the liver induced by glucagon is associated with a more reduced redox state in both the cytosol and mitochondria, but that the earliest event following glucagon administration is probably an increase in the hepatic level of cyclic AMP.

The increased gluconeogenesis induced by glucagon is suppressed by insulin (Menahan and Wieland, 1969) as a result of a decrease in the tissue level of cyclic AMP

(Jefferson et al., 1968). Administration of anti-insulin serum causes an increase in the liver cyclic AMP concentration, while diabetes induced by alloxan has a similar effect which is rapidly overcome by insulin treatment (Jefferson et al., 1968).

(1) Mode of action of glucagon

The primary site of action of glucagon in the gluconeogenic sequence appears to be the pyruvate carboxylase reaction (Williamson et al., 1969b). Fatty acids have been implicated in the stimulation of gluconeogenesis by glucagon, and it is suggested that the gluconeogenic response may be secondary to increased rates of fatty acid oxidation resulting from activation of hepatic lipase. However, Exton, Corbin and Park, (1969) dispute this hypothesis on the basis of results from liver perfusion experiments where the effect of oleate on gluconeogenesis is additive with that of a maximally effective concentration of glucagon. Mallette, Exton and Park (1969a) propose that glucagon plays a role in the regulation of gluconeogenesis from amino acids by increasing the formation of cyclic AMP, which stimulates the transport of amino acids into the hepatic cell, hence increasing substrate availability and also increasing the conversion of pyruvate to PEP.

A redistribution of Ca^{2+} within the cell has been implicated in the control of gluconeogenesis by glucagon or cyclic AMP (Friedmann and Rasmussen, 1970). This may be related to the observation that pyruvate carboxylase in rat liver mitochondria is inhibited by Ca^{2+}. Kimmich and Rasmussen (1969)

(2) Mode of action of insulin

The decrease in cyclic AMP levels in the liver may account for the rapid effects of insulin on the liver, but it is not known how this relates to the relatively slow changes in enzyme activity and content which occur after administration of the hormone *in vivo* (Weber, Singhal and Srivastava, 1965). Insulin also appears to be important in the regulation of the availability of substrates from peripheral tissues. The decrease in gluconeogenesis after insulin administration may be due partly to diminished release of glycerol from adipose tissue (Fain, Kovacev and Scow, 1965; Kipnis, 1965) and to a reduction in the supply of amino acids as a result of increased protein synthesis (Wool, 1964) and the accumulation of amino acids (Manchester and Young, 1959; Scharff and Wool, 1965; Sanders and Rigge, 1967) in muscle.

(b) Adrenal hormones

(1) Catecholamines

The action of epinephrine on gluconeogenesis appears to be similar to that of glucagon and is also mediated by cyclic AMP (Exton and Park, 1969).

(2) Glucocorticoids

A role for glucocorticoid hormones in the regulation of gluconeogenesis is suggested by observations that adrenalectomy leads to reduced rates of gluconeogenesis which may be restored by glucocorticoids (Subrahmanyam, Joseph and Natarajan, 1967; Henning *et al.*, 1966; Seubert *et al.*, 1968). *In vivo*

administration of glucocorticoids may lead to an increase in the activities of key gluconeogenic enzymes. It is well established that the activity of PEP carboxykinase is increased in rat liver and kidney (Lardy et al., 1964; Seubert et al., 1968) but the behaviour of pyruvate carboxylase is less clear. Although Freedman and Kohn (1964) and Henning, Seiffert and Seubert (1963) have reported an increase in the activity, other workers have been unable to confirm this (Shrago and Lardy, 1966; Struck, Ashmore and Wieland, 1966). These examples of chronic adaptation appear to be secondary to an increase in gluconeogenic flux (Ray, Foster and Lardy, 1964) which may occur partly in response to an increased availability of amino acids from peripheral tissues (Smith and Long, 1967; Sutherland and Haynes, 1967; Kostyo, 1965).

It appears that glucocorticoids also have a permissive action on the stimulation of hepatic gluconeogenesis. Adrenalectomy impairs the gluconeogenic response to low concentrations of glucagon and epinephrine in livers from fed or fasted rats, and a normal response may be restored by administration of dexamethasone (Friedmann, Exton and Park, 1967). The impairment of the response is not due to reduced accumulation of cyclic AMP, but is the result of decreased sensitivity of gluconeogenesis to activation by the nucleotide.

(c) Other hormones

There are some indications that thyroid hormones and growth hormone may be involved in the control of gluconeogenesis.

(1) Thyroid hormones

Freedland and Krebs (1967) have reported that thyroxine treatment *in vivo* increases the rates of gluconeogenesis from lactate and glycerol, but not from fructose in the perfused rat liver. Consistent with this report is the observation that the activities of pyruvate carboxylase, PEP carboxykinase and mitochondrial glyceraldehyde-3-phosphate dehydrogenase are decreased in the livers of thyroidectomised rats (Menahan and Wieland, 1969; Bottger, Kriegel and Wieland, 1970). Administration of triiodothyronine restores pyruvate carboxylase to the normal level, while PEP carboxykinase and glyceraldehyde-3-phosphate dehydrogenase exceed the normal level.

(2) Growth hormone

Growth hormone increases the hepatic glucose output in intact dogs (Altszuler *et al.*, 1968) and stimulates amino acid gluconeogenesis and ureogenesis by 40-50% in the perfused rat liver (Jefferson (1968) in Exton *et al.*, 1970).

(d) Summary

At the present time it appears that glucose output by the liver is probably determined by the activities of glucagon and catecholamines on the one hand, and insulin on the other, acting on systems synthesising and degrading cyclic AMP (see Robison, Butcher and Sutherland, 1968). Glucagon and catecholamines elicit a maximum response in the liver only in the presence of normal levels of glucocorticoid

hormones. The relative importance of the roles of other hormones, such as thyroid hormones and growth hormone, has yet to be assessed.

1.6. RENAL GLUCONEOGENESIS

Renal gluconeogenesis appears to be similar in many respects to hepatic gluconeogenesis. On a weight basis the gluconeogenic capacity of the kidney cortex probably equals or exceeds that of the liver (Krebs, 1964; Bowman, 1970). However, there are some rather striking differences in the regulation of this process in the two tissues.

(1) Effect of acid-base status on the rate of gluconeogenesis

Metabolic acidosis causes an increase in the rate of gluconeogenesis in rat kidney cortex slices (Goodman, Fuisz and Cahill, 1966; Goorno, Rector and Seldin, 1967; Kamm et al., 1967) and the enhanced rate of glucose formation by slices from diabetic and starved rats is returned to normal by alkali feeding (Kamm and Cahill, 1969). Hepatic gluconeogenesis remains elevated despite alkali loading indicating that hepatic gluconeogenesis is independent of acid-base status (Herrera et al., 1966).

In the isolated perfused rat kidney the rate of gluconeogenesis is sensitive to the pH of the perfusate (Bowman, 1970). Goodman et al. (1966) showed that metabolic acidosis led to an enhancement of renal gluconeogenesis with glutamine, glutamate, α-ketoglutarate and oxaloacetate, but not with fructose or glycerol as substrates. Attempts

to determine more specifically the rate limiting step which is affected by pH have produced data suggesting that the conversion of oxaloacetate to PEP may be the site of interaction (Alleyne, 1968).

(2) Effects of hormones and cyclic AMP

In contrast to results obtained with liver preparations, glucagon (Nishiitsutsuji-Uwo, Ross and Krebs, 1967; Bowman, 1970) and epinephrine (Bowman, 1970) have no effect in the perfused kidney. Cyclic AMP does, however, stimulate glucose production in rat kidney cortex slices (Pagliara and Goodman, 1969) and in the perfused kidney (Bowman, 1970) and it has been suggested that the appropriate receptors (e.g., glucagon- and epinephrine-sensitive adenyl cyclases) are absent from renal tissue. Another possibility is that exogenous and endogenous cyclic AMP may not be available to the same reactive sites. In support of this is the fact that neither parathyroid extract nor vasopressin altered the gluconeogenic rate although these hormones activate renal adenyl cyclases (Chase and Aurbach, 1967; Pagliara and Goodman, 1969).

1.7. GLUCONEOGENESIS IN RUMINANT SPECIES

Most studies on gluconeogenesis have been carried out in rats. These are monogastric animals whose normal diet provides an adequate amount of glucose which is readily absorbed from the alimentary tract.

In adult ruminants, particularly those on hay or low grain diets, very little glucose is absorbed from the

rumen because the dietary carbohydrates are fermented by rumen microorganisms to volatile fatty acids of which acetic, propionic and butyric acids predominate. These volatile fatty acids account for 50 - 80% of the adult ruminant's energy requirements (Carroll and Hungate, 1954; Hungate, Mah and Simesen, 1961; Bergman et al., 1965; Leng and Leonard, 1965; Gray et al., 1967). Although only propionic acid is directly glucogenic (Annison, Leng, Lindsay and White, 1963), the metabolism of acetic and butyric acids could spare the utilisation of glucose for oxidation and lipogenesis. It has been estimated that approximately 50% of the propionate absorbed is converted to glucose, and depending on the diet, this may account for 20 - 40% of the animal's total glucose requirement (Bergman, Roe and Kon, 1966; Leng, Steel and Luick, 1967). About one sixth of the propionic acid produced in the rumen is converted to lactic acid during its absorption and this is metabolised in the liver via pyruvate, oxaloacetate and PEP. Propionate is converted to oxaloacetate through propionyl-CoA, methylmalonyl-CoA, succinyl-CoA and the citric acid cycle. The glucogenic amino acids obtained from degradation of microbial protein provide another source of glucose precursors. Lactate from glycolysis in muscle, the central nervous system and erythrocytes is also a substrate for glucose synthesis.

The importance of gluconeogenesis in ruminants is emphasised when it is realised that the glucose turnover rates in ruminant and non-ruminant animals are similar (see Ballard, Hanson and Kronfeld, 1969). It has long been recognised that in the sheep glucose is utilised chiefly by the nervous

system, foetus and mammary gland during lactation. However, recent studies by Bergman *et al.* (1970) have revealed that the portal drained viscera (gastrointestinal tract, pancreas and spleen) utilise considerable amounts of glucose and are more important in this regard than has previously been realised.

1.8. AIMS OF THIS PROJECT

The activities of the key gluconeogenic enzymes have been discussed with respect to the relationship between their acute control and the factors which regulate the flux through the gluconeogenic pathway. It has been found that under prolonged conditions of physiological perturbation there are marked increases in the activities of some of these enzymes. The most extensive studies of this nature have been carried out in rat liver, but there is relatively little information available about the enzymatic aspects of renal gluconeogenesis.

Gluconeogenesis is an essential process in the metabolism of ruminants, and as the sheep is an animal of economic significance to Australia, it may prove valuable to have a complete understanding of this important metabolic pathway. Since a considerable amount of information was already available in the literature about hepatic gluconeogenesis in other species, the original aim of the project was to compare the enzymatic aspects of hepatic and renal gluconeogenesis at the three-carbon level in the sheep. Therefore, a study of the behaviour of pyruvate carboxylase and PEP carboxykinase in the liver and kidney cortex of the sheep under various conditions of physiological

perturbation, e.g., fasting, diabetes and administration of glucocorticoid hormones, was undertaken to add to our knowledge of the process of gluconeogenesis in these conditions and to enable comparisons to be made between the two tissues. When it became evident that there were some striking differences in hepatic gluconeogenesis between the sheep and other species, particularly the rat, it was decided that attention should be focussed on hepatic pyruvate carboxylase and PEP carboxykinase in the sheep. The main aim of the project then was to determine the intracellular localisation of pyruvate carboxylase and PEP carboxykinase and the nature of the increase in the hepatic activity of pyruvate carboxylase which was found to occur in fasted and diabetic sheep. Such information was necessary for further studies on the factors responsible for the induction of the enzymic activity.

In order to understand the acute control of pyruvate carboxylase *in vivo*, it is essential that the enzyme be characterised as fully as possible, albeit that this must at present be done *in vitro*, with all the limitations in interpretation which this implies. Acetyl-CoA and $MgATP^{2-}$ had previously been demonstrated to play important roles in the short term control of the enzymic activity, and when it became evident that pyruvate carboxylase purified from sheep liver exhibited negative cooperativity in the binding of the substrate, pyruvate, this phenomenon was studied in detail.

CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

Sodium pyruvate (dimer free), ADP, ATP, IDP, CoA, GSH, NADH, pyridoxal phosphate, sodium glutamate, α-ketoglutarate, 5,5'-dithiobis(2-dinitrobenzoic acid), Tris (Trizma Grade), digitonin, dithioerythritol and β-phenylpyruvate were products of Sigma Chemical Co., St. Louis, Mo., U.S.A.

Oxaloacetate was obtained from the California Corporation for Biochemical Research.

Dithiothreitol and hydroxypyruvate were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Malate dehydrogenase and aspartate transaminase were obtained from the Boehringer Corporation (London) Ltd.

Avidin (10.3 units per mg.) was the product of Worthington Biochemical Corporation, Freehold, New Jersey.

Ammonium sulphate (Enzyme Grade) was obtained from Mann Research Laboratories, Inc., New York, U.S.A.

N-ethylmorpholine (Eastman Organic Chemicals, U.S.A.) and pyruvic acid (B.D.H. technical grade) were purified by distillation under reduced pressure.

PEP (monocyclohexylammonium salt) was synthesised by the method of Clark and Kirby (1963).

Sephadex G200 and DEAE-Sephadex (A50) (Pharmacia, Sweden) and DEAE cellulose (Whatman, DE23) were used in the purification of pyruvate carboxylase.

Polyethyleneglycol (Carbowax) was obtained from Union Carbide.

Sucrose (A.R.) was the product of C.S.R. Co., Sydney, Australia.

Alloxan was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England; Cortisyl (cortisone acetate) from Roussel Labs., Wembley Park, England; Solu-Cortef (hydrocortisone sodium succinate) from Upjohn Co., Michigan, U.S.A.; and Decadron phosphate (dexamethasone 21-phosphate) from Merck, Sharp and Dohme, Australia.

Freund's complete adjuvant was purchased from the Commonwealth Serum Laboratories, Melbourne, Australia.

$Ba^{14}CO_3$ and uniformly labelled [U-^{14}C] glucose were obtained from the Radiochemical Centre, Amersham, Great Britain.

Scintillation fluid. PPO (2,5-diphenyl oxazole) and dimethyl POPOP (1,4-bis-2(4-methyl-5-oxazolyl)-benzene were obtained from the Packard Instrument Co., U.S.A. These compounds were dissolved in sulphur-free toluene according to the method of Bousquet and Christian (1960).

2.2. ANIMALS

Dr. I.G. Jarrett of the Commonwealth Scientific and Industrial Research Organization, Division of Nutritional Biochemistry provided merino ewes (age 2-3 years) and supervised the various treatments which will be described in subsequent chapters.

Female albino Wistar rats were obtained from the Waite Agricultural Research Institute of the University of Adelaide.

2.3. PREPARATION AND PURIFICATION OF ACETYL-CoA

Acetyl-CoA was prepared from CoA by a modification of the method of Simon and Shemin (1953).

CoA (35 mg) was dissolved in a small volume of water and a drop of bromothymol blue was added to the solution at 0°. After the solution had been neutralised with 1 M tris base, sodium borohydride (1-2 mg) was added to ensure that the CoA was fully reduced. Five minutes later, the solution was acidified with a drop of constant boiling HCl to destroy excess borohydride, and after the frothing had subsided, the pH was readjusted to 7.0 with tris. The reduced CoA was acetylated with redistilled acetic anhydride (0.05 ml) and allowed to stand in ice for 5 min. The pH was adjusted to 6.8 and the volume was adjusted to 5.0 ml.

For use in kinetic studies, acetyl-CoA was purified by ascending paper chromatography. After the CoA had been acetylated the final neutralisation step was omitted and the solution was applied to Whatman 3 MM chromatography paper, with reduced CoA as a standard. The solvent system used was isobutyric acid/water/conc. NH_4OH (66/33/1, v/v/v) (Zetterstrom and Ljunggren, 1951). After the bands of acetyl-CoA and CoA had been located by their absorption of ultraviolet light, the acetyl-CoA was eluted with 10^{-5} M EDTA, pH 7.0. The eluate was concentrated by freeze-drying and the concentration of acetyl-CoA was determined by measuring the extinction at 259 mμ.

2.4. PREPARATION OF $NaH^{14}CO_3$

$^{14}CO_2$ was distilled under vacuum at room temperature from $Ba^{14}CO_3$ and 7% (v/v) $HClO_4$ and was absorbed by an equivalent amount of NaOH. The distillation flask was warmed gently to ensure the complete liberation of the $^{14}CO_2$. The solution of $NaH^{14}CO_3$ was diluted to 50 µC per ml with 0.2 M $NaHCO_3$ freshly prepared with CO_2-free glass distilled water. The specific activity of the $NaH^{14}CO_3$ solution was determined by dilution in 0.1 M tris. Aliquots of this solution were dried on 1 inch squares of Whatman 3 MM paper which had previously been moistened with a solution of $BaCl_2$ (0.1%, w/v), and were counted in a Packard Tricarb Scintillation Spectrometer. A solution of 50 µC per ml corresponded to approximately 3×10^5 counts per min per µmole.

2.5. PEP CARBOXYKINASE ASSAY

PEP carboxykinase was assayed by a modification of the $^{14}CO_2$ fixation method originally described by Utter and Kurahashi (1954) and was similar to that used by Barns and Keech (1968) except that the transaminase system was not included and oxaloacetate was stabilised by the formation of the oxaloacetate-dinitrophenylhydrazone.

The reaction mixture contained in µmoles (total volume 0.5 ml) tris-citrate, (pH 6.5), 10; PEP, 1.2; IDP, 0.5; $MnCl_2$, 1.5; GSH, 0.8; $NaH^{14}CO_3$, 5.0 and enzyme.

After incubation at 30° for 5 or 10 min. the reaction was terminated by the addition of 0.05 or 0.10 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6 M HCl. The reaction mixtures were centrifuged and then aliquots of the supernatant were applied in triplicate onto 1 inch squares of Whatman 3 MM filter paper, dried for 5 min. at 100° and the radioactivity was determined in a Packard Tricarb Scintillation Spectrometer. The efficiency of the counting was reduced by the yellow colour and so the results were corrected for quenching by the channels ratio method (Baillie, 1960).

2.6. PYRUVATE CARBOXYLASE ASSAYS

(a) Radiochemical assay

The activity of pyruvate carboxylase was assayed by a $^{14}CO_2$ fixation method similar to that described by Barritt, Keech and Ling (1966).

The reaction mixture contained in μmoles (total volume 0.5 ml) tris-Cl or N-ethylmorpholine-Cl (pH 8.4), 100; ATP, 1.25; $MgCl_2$, 4.0; sodium pyruvate, 5.0; acetyl-CoA, 0.18; $NaH^{14}CO_3$, 5.0 and enzyme. After incubation at 30° for 5 or 10 min. the reaction was terminated by the addition of 0.05 or 0.10 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6 M HCl. The samples were treated and radioactivity was determined as described in Section 2.5.

(b) Spectrophotometric assay

During the purification of sheep liver pyruvate carboxylase the enzymic activity was assayed by the spectro-

photometric method described by Utter and Keech (1963). The pyruvate carboxylase reaction was coupled to the malate dehydrogenase system in which NADH was oxidised to NAD^+ during the conversion of oxaloacetate to malate. The rate of oxidation of NADH was followed in a Unicam SP 800 Recording Spectrophotometer at 340 mμ and 30°. The assay mixture contained in μmoles (total volume 1.0 ml) Tris-Cl (pH 8.4), 100; ATP, 2.5; $MgCl_2$, 7.0; $NaHCO_3$, 20; pyruvate, 10; acetyl-CoA, 0.35; malate dehydrogenase, approximately 2 units; NADH, 0.12; and enzyme. Blank rates were determined, where necessary, in the absence of added acetyl-CoA.

2.7. COMPARISON OF DIFFERENT ASSAY METHODS FOR PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE

The activities of PEP carboxykinase and pyruvate carboxylase may be assayed by the fixation of labelled bicarbonate to form labelled oxaloacetate, as described in Sections 2.5. and 2.6 (a), respectively. In the literature there are numerous examples where these enzymes are assayed in coupled systems so that the oxaloacetate is immediately converted to malate, aspartate or citrate. Although an uncoupled system is probably more specific than a coupled one, it is possible that when the activity is assayed in a crude homogenate oxaloacetate could be diverted through other pathways with loss of label so that incorrect estimates of the activity would be obtained.

The activities of the two enzymes were therefore assayed in a crude homogenate of liver from a normal sheep

by three different systems:

(a) uncoupled, oxaloacetate stabilised as the 2,4-dinitrophenylhydrazone

(b) coupled, oxaloacetate converted to malate by malate dehydrogenase

(c) coupled, oxaloacetate converted to aspartate by glutamate-oxaloacetate transaminase.

The data presented in Table 2.1 show that the estimate of PEP carboxykinase activity was identical in all three systems. However, the assay for pyruvate carboxylase activity increased approximately 2.5-fold when a coupled system was used. Similar results were obtained when an incomplete coupling system, containing only the enzyme was included. This suggested that the effect was due either to bovine serum albumin, in which the coupling enzymes had been diluted before addition to the assay system, or to the presence of ammonium sulphate in which the coupling enzymes were suspended. Equivalent concentrations of bovine serum albumin or ammonium sulphate were added separately to the assay system. It can be seen that the addition of ammonium sulphate gave almost the same results as a complete coupling system and it was concluded that the increased activity was probably due not to a stabilisation of oxaloacetate, but instead to activation of pyruvate carboxylase. Activation of purified sheep kidney pyruvate carboxylase by NH_4^+ has been observed (Nielsen, 1970).

The uncoupled assay system is used routinely in this laboratory for studies with purified pyruvate carboxylase

TABLE 2.1: COMPARISON OF DIFFERENT ASSAY METHODS FOR PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE

A sheep liver homogenate (25%, w/v) was prepared at 2° in 0.25 M sucrose containing 20 mM tris-Cl (pH 7.2), 0.1 mM EDTA and 0.5 mM GSH. The homogenate was sonicated for four periods of 10 sec. and diluted ten-fold before assay.

The normal assay system for pyruvate carboxylase contained in μmoles (total volume0.5 ml) tris-Cl (pH 8.4), 100; ATP, 1.25; $MgCl_2$, 4.0; sodium pyruvate, 5.0; acetyl-CoA, 0.18; $NaH^{14}CO_3$, 5.0; and 0.10 ml of enzyme.

The normal assay system for PEP carboxykinase contained in μmoles (total volume 0.5 ml) tris-citrate (pH 6.5), 10; PEP, 1.2; IDP, 0.5; $MnCl_2$, 1.5; GSH, 0.8; $NaH^{14}CO_3$, 5.0; and 0.10 ml of enzyme.

The following additions were made where indicated: sodium glutamate, 10; pyridoxal phosphate, 0.02; NADH, 0.12; ammonium sulphate, 5; bovine serum albumin, 0.5 mg; malate dehydrogenase, 3 units; glutamate-oxaloacetate transaminase, 4.5 units.

After incubation for 5 min. at 30° the reaction was stopped by the addition of 0.05 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6M HCl. The results are expressed as μmoles of CO_2 fixed/mg of protein/min.

TABLE 2.3 COMPARISON OF DIFFERENT ASSAY METHODS FOR PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE

Additions	Pyruvate Carboxylase ($x10^3$)	PEP Carboxykinase ($x10^3$)
None	3.81	3.16
Malate dehydrogenase, NADH	10.2	3.24
Glutamate-oxaloacetate transaminase, pyridoxalphosphate, glutamate	10.6	3.20
Malate dehydrogenase	9.49	
Glutamate-oxaloacetate transaminase	9.37	
Ammonium sulphate	9.71	
Bovine serum albumin	3.27	

and it was concluded that this same assay system was satisfactory for comparisons of activities in crude homogenates or cell fractions, as the same quantitative changes in enzyme levels following physiological perturbation of the animal were observed, irrespective of the type of assay used.

2.8. METHODS OF DISRUPTING TISSUE FOR ASSAY OF ENZYMIC ACTIVITIES

It was found that if tissue homogenates were assayed for pyruvate carboxylase and PEP carboxykinase activities, the activity of pyruvate carboxylase was negligible although substantial PEP carboxykinase activity was evident. It is well known that the activities of many mitochondrial enzymes are latent because of permeability barriers presented to substrates by the inner mitochondrial membrane, and that the mitochondria must be disrupted for the total activity to be assayed.

Liver homogenates (25%, w/v) were prepared with a Potter-Elvehjem homogeniser and were diluted ten-fold for assay of the enzymic activities.

The following treatments were used to disrupt the tissue before assay:

(1) The homogenate was frozen and thawed rapidly three times in an ethanol-dry ice bath.

(2) The homogenate was sonicated for four periods each of ten sec. with a "Soniprobe" (Dawe Instruments, Ltd., England) at setting 8, 3 amperes.

(3) The homogenate was freeze-dried and then reconstituted to the original volume with water.

(4) The tissue was frozen in liquid nitrogen immediately after removal from the animal, ground to a fine powder and homogenised in buffer.

Further details of the treatments are indicated in Table 2.2.

(a) Pyruvate carboxylase

From the results presented in Table 2.2 it can be seen that sonication of the homogenate increased the assayable activity approximately 10 fold. Similar results were obtained with an homogenate prepared from tissue which had been frozen in liquid nitrogen. The activity was not increased further by subsequent freezing and thawing of the homogenate.

The results of the second experiment demonstrate that the activity is the same in either a sonicated homogenate or reconstituted freeze dried homogenate. Freeze drying or sonicating also solubilises the enzyme completely.

The enzyme from chicken liver is cold labile (Scrutton and Utter, 1965) but the results presented for Experiment 3 show that the activity of sheep liver pyruvate carboxylase is the same in homogenates prepared at 0° or 23°. Contrary to an earlier report (Ling and Keech, 1966) it has recently been observed in this laboratory that the purified sheep kidney enzyme is not cold labile (R. Bais, J.C. Wallace and D.B. Keech, unpublished observations).

TABLE 2.2: DISRUPTION OF TISSUE FOR ASSAY OF ENZYMIC ACTIVITIES

Sheep liver homogenates were prepared as follows:

Experiment 1. The liver was homogenised in 0.04 M tris-acetate, pH 7.4, at 23°, for assay of pyruvate carboxylase activity. PEP carboxykinase activity was assayed in an homogenate prepared at 2° in 0.1 M tris-citrate (pH 6.5) containing 0.5 mM GSH.

Experiment 2.A. The liver homogenates were prepared as for Experiment 1.

Experiment 2.B. The liver was homogenised at 2° in 0.25 M sucrose containing 0.02 M tris-Cl (pH 7.2), 0.5 mM GSH and 0.1 mM EDTA.

Experiment 3.A. Sucrose homogenates were prepared at 2° and 23°.

Experiment 3.B. Tris-acetate homogenates were prepared at 2° and 23°.

The homogenates were treated as indicated in the table and described in the text. The activities of pyruvate carboxylase and PEP carboxykinase were assayed by the methods described in Sections 2.5. and 2.6.(a), respectively.

TABLE 2.2: DISRUPTION OF TISSUE FOR ASSAY OF ENZYMIC ACTIVITIES

Experiment	Treatment	Pyruvate Carboxylase (units/g wet wt)	PEP Carboxykinase (units/g wet wt)
1.	(1) Homogenate	0.08	0.64
	(2) Homogenate sonicated	0.85	
	(3) Homogenate frozen and thawed		0.86
	(4) Homogenate of nitrogen powder	0.80	0.82
	(5) Homogenate from (4) frozen and thawed	0.82	0.87
2A	(1) Homogenate sonicated	0.96	0.83
	(2) Freeze-dried homogenate, reconstituted	0.92	0.73
	(3) 38,000 x g supernatant from (2)	0.98	0.81
2B	(1) Homogenate sonicated	0.97	
	(2) 100,000 x g supernatant from (1)	0.94	
	(3) Freeze-dried homogenate, reconstituted	0.97	
	(4) 38,000 x g supernatant from (3)	0.96	
3A	(1) Sucrose homogenate (2°) sonicated	1.01	
	(2) Sucrose homogenate (23°) sonicated	0.95	
3B	(1) Tris-acetate homogenate (2°) sonicated	0.98	
	(2) Tris-acetate homogenate, 23° (sonicated)	0.97	

(b) PEP carboxykinase

The results for PEP carboxykinase in Table 2.2 were similar to those for pyruvate carboxylase, in that sonicating, freeze drying or freezing in liquid nitrogen were all equally effective in disrupting the tissue, although the activity was not greatly increased from that observed in an untreated homogenate. This suggests that a large proportion of the activity is present in the cytosol, as will be confirmed in Chapter 4.

2.9. PREPARATION OF FREEZE-DRIED MITOCHONDRIA

Freeze-dried mitochondria were prepared from livers obtained from the Metropolitan and Export Abattoirs Board. The livers were stored in ice as soon as possible after slaughter of the animals.

The temperature at all stages of the preparation was maintained at 4°. After removal of connective tissue the livers were chopped into small pieces and homogenised for 1 min. in a Waring Blendor with 3.5 vol. (w/v) of 0.25 M sucrose containing 0.1 mM EDTA. The homogenate was centrifuged at 600 x g for 20 min. and the resulting supernatant was further centrifuged at 23,000 x g for 15 min. The mitochondrial precipitate was suspended in 0.1 mM EDTA to a volume half that of the original homogenate and then recentrifuged at 23,000 x g for 20 min. The final residue was suspended in a minimum volume of 0.1 mM EDTA, frozen quickly in a dry ice/ethanol mixture and freeze-dried overnight. The freeze-dried mitochondria were powdered in a Waring Blendor and stored

under vacuum at room temperature.

2.10. PURIFICATION OF PYRUVATE CARBOXYLASE FROM FREEZE-DRIED MITOCHONDRIA

Throughout the purification procedure protein was determined spectrophotometrically by the method of Warburg and Christian (1941) as described by Layne (1957). The enzymic activity was measured by the spectrophotometric method described in 2.6.(b).

Two different methods were used for the purification of pyruvate carboxylase. The first method which involves chromatography on DEAE-Sephadex (M.C. Scrutton, personal communication to J.C. Wallace) is the simpler and yields enzyme of high specific activity. The second method is a modification of the procedure described by Nielsen (1970) for the purification of sheep kidney pyruvate carboxylase, and was used for purification of the enzyme used in some of the kinetic studies which were carried out early in the course of this investigation.

(a) Purification of pyruvate carboxylase by DEAE-Sephadex chromatography

(1) Buffers

(i) Extraction buffer: 42 ml of 0.5 M tris-acetate (pH 6.7) 30 ml of 0.05 M ATP, 3.0 ml of 1.0 M $MgCl_2$ and 798 ml of H_2O.

(ii) Buffer A contained 0.025 M potassium phosphate (pH 7.2), 0.03 M ammonium sulphate, 1 mM EDTA and 0.1 mM dithioerythritol.

(iii) Buffer B contained 0.825 M potassium phosphate (pH 7.2), 0.3 M ammonium sulphate, 1 mM EDTA and 0.1 mM dithioerythritol.

(iv) Storage buffer for purified pyruvate carboxylase contained 0.1 M potassium phosphate (pH 7.2), 1.6 M sucrose and 0.06 M ammonium sulphate.

The following procedures were all carried out at room temperature (23°).

(2) Extraction of pyruvate carboxylase from mitochondrial powder

Mitochondrial powder (90 g) was extracted with 1164 ml of extraction buffer, the pH being maintained at 6.5 by the addition of 1 M tris base. Stirring was continued for 10 min. after the powder had been thoroughly dispersed. The suspension was centrifuged at 23,000 x g for 20 min. and the pH of the supernatant was immediately adjusted to 6.9 - 7.2 with 1 M tris base.

(3) Ammonium sulphate precipitation of protein

Solid ammonium sulphate (19.6 g per 100 ml of supernatant) was added slowly with stirring, the pH being maintained at 6.9 - 7.0 with tris base. Stirring was continued for a further 20 min. after all the ammonium sulphate had dissolved and the precipitated protein was then recovered by centrifuging at 23,000 x g for 15 min.

(4) Precipitation with polyethylene glycol

This step provided a slight purification as well as desalting the protein solution prior to DEAE-Sephadex chromatography.

The ammonium sulphate precipitate was dissolved in Buffer A to give a solution of volume 400 ml. Solid polyethylene glycol (14.5 g per 100 ml of enzyme solution) was added with constant stirring. Stirring was continued for 5 min. after all the polyethylene glycol had dissolved and the precipitated protein was recovered by centrifuging at 23,000 x g for 15 min. The precipitate was dissolved in about 50 ml of Buffer A. This suspension was centrifuged at 23,000 x g for 15 min. to remove insoluble material and the supernatant was retained.

(5) DEAE-Sephadex chromatography

The supernatant from (4) was applied to a DEAE-Sephadex column (19 cm x 4.5 cm) equilibrated with Buffer A. The protein was eluted with a buffered linear salt gradient from 0.03 M to 0.3 M ammonium sulphate (i.e., 750 ml of Buffer A and 750 ml of Buffer B). The flow rate was 85 - 90 ml per hr and 15 - 20 ml fractions were collected.

The enzymic activity was detected using the spectrophotometric assay and the protein concentration was monitored at 280 and 260 mμ. The fractions of highest specific activity were pooled and the protein was precipitated with solid ammonium sulphate (27.7 g per 100 ml of solution), the pH being maintained at 7.0 by the addition of 1 M KOH. The

precipitate recovered after centrifuging at 37,000 x g for 15 min. was dissolved in the storage buffer to give a solution of concentration approximately 20 mg per ml. The solution was rapidly frozen in an ethanol/dry ice mixture and stored at -15°.

The results from a typical purification procedure are shown in Table 2.3.A.

(b) Purification of pyruvate carboxylase by DEAE-cellulose chromatography

All procedures were carried out at room temperature.

(1) Extraction of pyruvate carboxylase from mitochondrial powder

Mitochondrial powder (100 g) was extracted with 1,500 ml of 0.04 M Tris-Cl (pH 7.4) containing 0.1 mM EDTA, the pH of the suspension being maintained at 7.2 by the addition of tris base. The suspension was stirred and then centrifuged at 23,000 x g for 20 min.

(2) Ammonium sulphate fractionation

Solid ammonium sulphate (13 g/100 ml of extract) was added with stirring to the supernatant from (1). The precipitate obtained after centrifuging was discarded and solid ammonium sulphate (6.6 g/100 ml) was added to the supernatant. The precipitate was recovered by centrifuging and was dissolved to a final volume of 500 ml in 0.04 M Tris-Cl (pH 7.4) containing 0.1 mM EDTA.

TABLE 2.3: PURIFICATION OF SHEEP LIVER PYRUVATE CARBOXYLASE

Method	Treatment	Protein (mg)	Activity (units)	Specific Activity (units/mg of protein)
A.	Initial extract	14,000	-	-
	Ammonium sulphate precipitate	3,040	5,460	1.80
	Polyethylene glycol precipitate	1,450	3,730	2.57
	DEAE-Sephadex chromatography, pH 7.2	56	1,600	29.6
B.	Initial extract	33,900	-	-
	Ammonium sulphate precipitate	5,430	1,260	0.23
	DEAE-cellulose chromatography, pH 6.5	181	640	3.83
	DEAE-cellulose chromatography, pH 7.5	50	328	6.56
	Sephadex G200 chromatography, pH 7.0	27	230	8.52

(3) Polyethylene glycol fractionation

Solid polyethylene glycol (3.5 g/100 ml) was added to the redissolved precipitate from (2). The suspension was centrifuged and additional polyethylene glycol (12 g/100 ml) was added to the supernatant. The precipitated protein was recovered by centrifuging and was dissolved in about 70 ml of 0.01 M potassium phosphate (pH 6.5) containing 0.1 mM EDTA, 0.1 mM GSH and 0.1 mM dithiothreitol. The solution was centrifuged to remove insoluble material and sucrose was dissolved in the supernatant to a concentration of 0.4 M.

(4) DEAE-cellulose chromatography, pH 6.5

The enzyme from (3) was applied to a DEAE-cellulose column (5.0 x 18.0 cm) equilibrated with 0.1 M potassium phosphate (pH 6.5) containing 0.04 M sucrose, 0.1 mM GSH and 0.1 mM dithiothreitol. The protein was eluted with a linear salt gradient (1,500 ml) of 0 to 0.33 M KCl. The flow rate was 100 ml per hr and 25 ml fractions were collected.

The fractions containing enzyme of highest specific activity were pooled and the protein was precipitated by the addition of polyethylene glycol (20 g/100 ml). The suspension was centrifuged and the precipitate was dissolved in 0.01 M potassium phosphate (pH 7.5), containing 0.4 M sucrose, 0.1 mM EDTA, 0.1 mM dithiothreitol and 0.1 mM GSH.

(5) DEAE-cellulose chromatography, pH 7.5

The enzyme from (4) was applied to a DEAE-cellulose column (1.6 x 27 cm) equilibrated with the sucrose-phosphate buffer, pH 7.5. The protein was eluted with a linear salt gradient (300 ml) of 0. to 0.33 M KCl. The flow rate was 16 ml per hr and 4 ml fractions were collected.

The fractions of highest specific activity were pooled and the protein was precipitated by the addition of solid ammonium sulphate (24.3 g/100 ml). The precipitate was dissolved in 0.01 M potassium phosphate (pH 7.0) containing 0.4 M sucrose, 0.1 mM EDTA, 0.1 mM dithiothreitol.

(6) G200 Sephadex chromatography

The enzyme solution from (5) was applied to a G200 Sephadex column (25 x 1.8 cm) equilibrated with 0.1 M potassium phosphate (pH 7.0), containing 0.2 M KCl and 0.1 mM EDTA. The protein was eluted with approximately 40 ml of the same buffer at a flow rate of 40 - 60 ml per hr. Fractions of 0.5 ml were collected. The fractions of highest specific activity were pooled and the protein precipitated and freeze-dried. The dried material was dissolved in 2 ml of H_2O and dialysed against 0.01 M potassium phosphate (pH 7.0) containing 0.4 M sucrose and 0.1 mM EDTA. The solution was frozen in an ethanol/dry ice bath and stored at -15°.

The results from a typical purification procedure are shown in Table 2.3.B.

CHAPTER 3

EFFECTS OF FASTING, DIABETES AND GLUCOCORTICOIDS ON GLUCONEOGENIC ENZYMES AND GLUCOSE TURNOVER RATES IN SHEEP

3.1. INTRODUCTION

Changes in the activities of the key gluconeogenic enzymes have been observed in the liver, and to a lesser extent, in the kidney, of the rat after various physiological perturbations. The levels of activity of glucose-6-phosphatase (Weber and Cantero, 1954; Langdon and Weakley, 1955) and PEP carboxykinase (Shrago et al., 1963; Lardy et al., 1964; Henning et al., 1966) increase in rat liver during fasting, diabetes or glucocorticoid administration. However, complete agreement has not been reached concerning the behaviour of pyruvate carboxylase. Henning et al. (1963) have presented evidence that the activity is increased in the liver after cortisol treatment and other investigators (Freedman and Kohn, 1964; Prinz and Seubert, 1964; Wagle, 1964) have shown that the activity is increased in diabetic rat liver. In contrast to these reports, Shrago and Lardy (1966) and Struck, Ashmore and Wieland (1966) were unable to demonstrate any increase in the activity of pyruvate carboxylase after administration of glucocorticoids and Krebs (1966) found no change in the activity of this enzyme in the livers of either fasted or alloxan diabetic rats.

In view of the importance of gluconeogenesis in the metabolism of ruminants, it would be useful to have information about the enzymatic aspects of this process in the two gluconeogenic tissues, liver and kidney cortex. Although pyruvate carboxylase and PEP carboxykinase have been studied in the livers of cows in relation to bovine ketosis (Baird et al., 1968; Ballard et al., 1968; Baird and Heitzman, 1970)

no investigations appear to have been made on the effects of perturbations on key gluconeogenic enzymes in the sheep.

In the study reported in this chapter, the assayable activities of key gluconeogenic enzymes, in particular pyruvate carboxylase and PEP carboxykinase, were determined in the livers and kidneys of sheep under various conditions including fasting of normal, pregnant or phlorhizinised animals, diabetes induced either by pancreatectomy or by an injection of alloxan, and administration of glucocorticoid hormones.

Glucose turnover rates were also measured under most of these conditions so that the changes observed in the activities of the enzymes could be compared with the rates of glucose turnover.

3.2. MATERIALS AND METHODS

(a) Animals

Female albino rats (300 - 400 g body wt.) were fed on a standard laboratory pelleted diet. Food was withheld from starved animals for periods varying from 16 to 72 hr. before slaughter.

Merino ewes (age 2 - 3 years) were fed daily a mixture of 600 g of chaffed wheaten hay and 400 g of chaffed lucerne hay for several weeks before starting the experiments. Fasted animals were deprived of food for the periods indicated in the text.

All fasted animals had access to water *ad libitum*.

Cortisone acetate (dispersed in sodium CM-cellulose) and dexamethasone were administered as intramuscular injections.

Phlorhizin was administered as a subcutaneous injection (1 g suspended in 7 ml olive oil) at the rate of 1.0 ml on the first day and 0.5 ml on each of the subsequent 5 days.

Sheep were made diabetic either by pancreatectomy or by an intravenous injection of alloxan (60 mg/kg body wt).

Pregnant ewes (110 - 130 days gestation) were fasted for the periods indicated in the text.

(b) Sampling procedure

The animals were killed by severing the neck and cervical cord and the tissues were removed within 30 sec. Thin slices (about 3 mm) of the parietal lobe of the liver of the sheep and of the cortex of the right kidney were immediately immersed in liquid nitrogen. The whole liver and both kidneys of the rat were removed and similarly immersed. About 20 min. later for the sheep tissues and 5 min. later for the tissues of the rat, the frozen material was ground to a fine powder and thoroughly mixed in a stainless steel mortar cooled in liquid nitrogen. Weighed portions of the frozen powder were taken for the determination of glucose-6-phosphatase, fructose-1,6-diphosphatase, PEP carboxykinase, pyruvate carboxylase and glycogen.

(c) Enzyme assays

(i) PEP carboxykinase

Frozen tissue powder (1.0 g) was homogenised in 3.0 ml of 0.1M Tris-citrate (pH 6.5) containing 10^{-3}M GSH at 0°. Each tissue was assayed in duplicate. The reaction

mixture contained in μmoles (total volume 0.6 ml): Tris-citrate (pH 6.5), 10; PEP, 1.2; IDP, 0.5; $MnCl_2$, 1.5; GSH, 0.8; $NaH^{14}CO_3$, 5.0 (counts per min. per μmole: 6×10^5 for rat liver, 3×10^5 for all other tissues) and 0.01 ml of tissue homogenate (approximately 0.5 mg protein). PEP was omitted from the controls. The reaction mixture was incubated at 30° for 10 min. and the enzymic activity was stopped by the addition of 0.1 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6M HCl. Radioactivity was determined as described in Section 2.5.

(ii) Pyruvate carboxylase

Frozen tissue powder (1.0 g) was homogenised in 3.0 ml of 0.04M Tris-acetate (pH 7.4) at room temperature. Each tissue was assayed in duplicate. The assay mixture (total volume 0.5 ml) contained (in μmoles) N-ethylmorpholine-Cl (pH 8.4), 100; ATP, 1.25; $MgCl_2$, 4.0; sodium pyruvate, 5.0; acetyl CoA, 0.18; $NaH^{14}CO_3$, 5.0; and 0.01 ml of tissue homogenate (approximately 0.5 mg protein). Two control assays, lacking pyruvate and acetyl CoA, were included in each experiment. The reaction mixture was incubated at 30° for 10 min. and the enzymic activity was stopped by the addition of 0.1 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6M HCl. Radioactivity was determined as described in Section 2.5.

(iii) Protein was estimated by the biuret method (Gornall, Bardawill and David, 1949) as described by Cleland and Slater (1953).

(d) The following assays were carried out by collaborators in the Commonwealth Scientific and Industrial Research Organization by the methods indicated:

Glucose-6-phosphatase was measured as the amount of orthophosphate released on incubation of the tissue homogenate with glucose-6-phosphate (Harper, 1963). For the determination of fructose-1,6-diphosphatase activity, the 100,000 x g supernatant from the tissue homogenate was incubated with fructose-1,6-diphosphate (Weber and Cantero, 1959) and the amount of orthophosphate released was estimated.

Glycogen was extracted from the frozen tissue powder in 30% (w/v) KOH heated in a boiling water bath and estimated by the method of Carroll, Longley and Roe (1956).

The concentrations of glucose and total ketone bodies in blood were determined by the methods of Huggett and Nixon (1957) and Reid (1960), respectively.

The concentration of glucose in the urine was determined by a glucose oxidase method (Huggett and Nixon, 1957) and nitrogen was determined by Kjeldahl digestion followed by microtitration in the Pregl apparatus.

(e) Glucose turnover rates

(i) Theory

The overall rate of glucose metabolism in the body may be estimated by the rate at which the radioactivity of injected glucose is diluted in the glucose pool. The glucose pool is defined as the body glucose that dilutes the injected

^{14}C-glucose. It consists mainly of free glucose dissolved in the body fluids, although it can include other compounds in rapid equilibration with glucose (Searle, Strisower and Chaikoff, 1956; Steele *et al.*, 1956; Baxter, Kleiber and Black, 1955). Very little glycogen is included in the pool since its turnover is relatively slow (Stetten and Stetten, 1954), and Steele *et al.* (1956) have shown that in the calculation of the glucose pool size the error attributable to liver glycogen is negligible.

The dilution of the radioactive glucose represents primarily the flow of glucose into the blood from a variety of sources: hepatic glucose production by glycogenolysis and gluconeogenesis, renal gluconeogenesis and to a limited extent, glucose absorption from the alimentary tract. Glucose absorption is negligible in monogastric animals in a post-absorptive state and in ruminants fed predominantly hay diets. Thus in ruminants the glucose entry rate mainly represents the rate of gluconeogenesis. In the dynamic steady state the rate of glucose utilisation is equal to the glucose entry rate, which may then be regarded as a glucose turnover rate.

Glucose entry rates in sheep have been estimated by using a single injection of [U-^{14}C]glucose (Annison and White, 1961; Kronfeld and Simesen, 1961) or by using a single injection followed by a continuous infusion of [U-^{14}C]glucose (a primed infusion) (Annison and White, 1961; Kronfeld and Simesen, 1961; Ford, 1963; Bergman, 1963).

The method used here was similar to that described by Bassett, Mills and Reid (1966) in estimations of glucose

entry rates in normal and cortisol treated sheep, and was based on the approach adopted by Kronfeld and Simesen (1961). These workers used the single injection technique and progressively "peeled" the radioisotope-disappearance curve until there remained a single exponential function which was coincident with the rectilinear regression line relating the specific radioactivities and time from 40 min. to 160 min. They assumed that first order kinetics were applicable and used this function to calculate the various parameters of glucose metabolism.

Since the experiments reported in this chapter were carried out, White et al. (1969) have published an evaluation of the methods available for studying glucose metabolism in sheep. They have shown that the procedure described by Kronfeld and Simesen (1961) is an oversimplification, and that in order to calculate these parameters accurately the whole of the isotope dilution curve up to 8 hr should be analysed by methods based on compartmental analysis. White et al. (1969) have found that with normal sheep the simplified analysis for the single injection technique gives results similar to those obtained by other methods, but point out that this analysis may give rise to large errors in some physiological situations, e.g., in pregnant sheep.

Insufficient data were available to subject the results in this chapter to compartmental analysis in the manner recommended. Nevertheless, they should provide a qualitative, if not an accurate quantitative, indication of the rates

of glucose turnover in the sheep.

(ii) Experimental

Cannulae were inserted into the left and right jugular veins. [U-^{14}C]glucose was injected into the right jugular (1.0 μC/kg body wt.) and at 15 min. intervals from 60 to 180 min. blood samples were withdrawn from the left jugular into tubes containing oxalate and fluoride. The plasma was removed and used for determination of glucose concentration and radioactivity. The glucose was isolated as the pentaacetate derivative (Jones, 1965) and a sample of the glucose used for injection was treated in the same way to form standard labelled pentaacetate. 50 mg of the pentaacetate was dissolved in 2.5 ml of scintillation fluid and the radioactivity was determined in a Packard TriCarb Scintillation Spectrometer.

(iii) Calculations

The logarithm of the specific activity of the plasma glucose was plotted against time. The resulting straight line was extrapolated to give the specific activity of the glucose at zero time. The initial dilution of the specific activity could thus be determined and this enabled the glucose pool size to be calculated.

The turnover rate was calculated from the first order reaction rate

$$U = 2.3\ (b/t)\ \log\ a_o/a_t$$

where U is the turnover rate (mg/min/100 ml of plasma); b is the plasma glucose concentration (mg/100 ml) which remains constant; a_o and a_t are the specific activities of the plasma glucose at the initial and final times, respectively; t is the time in min. (Dunn et al., 1957). When the glucose space is taken into account, the turnover rate may be expressed as mg/min/kg body wt. The glucose space is the percentage of the animal's volume occupied by the glucose if its concentration is assumed to be equal to that in the accessible pool (i.e., the plasma).

The glucose turnover rate is a metabolic rate which is more closely proportional to the surface area of the body than to the body weight. The power exponent used is usually 0.75, which is the same as that relating basal metabolic rate to body size. Thus the glucose turnover rate is better expressed in terms of mg of glucose/min./kg body $wt^{0.75}$ when the rates for animals of different weights are to be compared.

3.3. RESULTS

(a) Effect of fasting on specific activity of gluconeogenic enzymes in the liver and kidney

The activities of the four key gluconeogenic enzymes, pyruvate carboxylase, PEP carboxykinase, fructose-1,6-diphosphatase and glucose-6-phosphatase, were measured in the livers and kidneys of fed and fasted sheep. The results obtained are presented in Tables 3.1 and 3.2, and it can be seen that there were significant increases in the levels of glucose-6-phosphatase, fructose-1,6-diphosphatase and pyruvate carboxylase but no change in PEP carboxykinase. The changes in the activities of the kidney enzymes were similar to those in the liver but less marked.

Since the changes in the activities of the enzymes in sheep differed from those reported for the rat (see Section 3.1), the experiments were repeated with rats to ensure that the results reported in the literature could be duplicated. Although the rats were starved for varying periods of time, from 16 - 72 hr, no further increment in the activities of the enzymes was observed after the first 16 hr and so the results were grouped together for statistical analysis. These results are presented in Tables 3.1 and 3.2.

When the results for the two species are compared, one of the most striking features is the behaviour of hepatic pyruvate carboxylase which increases four-fold in the starved sheep while no significant change is observed in the starved rat. A further major difference is that liver PEP carboxykinase shows a four-fold increase in activity in the rat

TABLE 3.1: EFFECT OF FASTING ON THE SPECIFIC ACTIVITY OF GLUCOSE-6-PHOSPHATASE AND FRUCTOSE-1,6-DIPHOSPHATASE IN THE LIVER AND KIDNEY OF SHEEP AND RATS

		Glucose-6-Phosphatase (x10)		Fructose-1,6-Diphosphatase (x10)	
Sheep Liver	Control	0.64 ± 0.04	(6)	0.97 ± 0.07	(6)
	Fasted (6 days)	1.10 ± 0.08	(6)	1.50 ± 0.10	(6)
		$P < 0.001$		$P < 0.01$	
Sheep Kidney	Control	1.17 ± 0.08	(6)	1.18 ± 0.08	(6)
	Fasted (6 days)	1.72 ± 0.12	(6)	1.63 ± 0.12	(6)
		$P < 0.01$		$P < 0.05$	
Rat Liver	Control	0.51 ± 0.04	(5)	0.85 ± 0.07	(5)
	Fasted (1-3 days)	1.29 ± 0.10	(5)	0.96 ± 0.08	(5)
		$P < 0.001$		n.s.	
Rat kidney	Control	0.77 ± 0.06	(5)	1.07 ± 0.09	(5)
	Fasted (1-3 days)	1.01 ± 0.08	(5)	1.19 ± 0.10	(5)
		$P < 0.05$		n.s.	

The activities of glucose-6-phosphatase and fructose-1,6-diphosphatase were assayed by the methods described in Section 3.2(c) in liver and kidney preparations, from sheep which had been fasted for 6 days and rats which had been fasted for 1-3 days. The specific activities are expressed as μmoles of phosphorus produced per mg of protein per min. The mean ± S.E.M. is given with the number of animals in parentheses. The statistical significance for the comparison between the fed and fasted animals is indicated.

n.s. = not significant.

TABLE 3.2: EFFECT OF FASTING ON THE SPECIFIC ACTIVITY OF PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN THE LIVER AND KIDNEY OF SHEEP AND RATS

		Pyruvate Carboxylase ($x10^3$)		PEP Carboxykinase ($x10^3$)	
Sheep Liver	Control	3.86 ± 0.40	(12)	3.35 ± 0.23	(14)
	Fasted (6 days)	14.8 ± 2.0	(7)	2.96 ± 0.29	(7)
		$P < 0.001$		n.s.	
Sheep Kidney	Control	7.10 ± 0.55	(9)	5.24 ± 0.33	(9)
	Fasted (6 days)	10.8 ± 3.2	(5)	5.01 ± 0.26	(5)
		$P < 0.01$		n.s.	
Rat Liver	Control	3.51 ± 0.24	(5)	0.62 ± 0.08	(5)
	Fasted (1-3 days)	4.19 ± 0.32	(5)	2.53 ± 0.30	(5)
		n.s.		$P < 0.001$	
Rat Kidney	Control	4.95 ± 0.27	(5)	5.11 ± 0.35	(5)
	Fasted (1-3 days)	6.28 ± 0.39	(5)	7.85 ± 0.41	(5)
		$P < 0.05$		$P < 0.001$	

The activities of pyruvate carboxylase and PEP carboxykinase were assayed by the methods described in Section 3.2(c) in homogenates of liver and kidney from sheep which had been fasted for 6 days and rats which had been fasted for 1-3 days. The specific activities are expressed as μmoles of CO_2 fixed per min. per mg of protein. The mean ± S.E.M. is given with the number of animals in parentheses. The statistical significance of the comparison between the fed and fasted animals is indicated.

n.s. = not significant.

whereas in the liver of the sheep the activity of this enzyme remains unchanged after 6 days' starvation. An increase in glucose-6-phosphatase was found in both rats and sheep on starvation, but although no significant change in the activity of fructose-1,6-diphosphatase was observed in the rat tissues, a small but significant rise was apparent in sheep liver.

Determination of the concentration of glycogen in the livers of sheep and rats indicated that whereas in the rat the glycogen was almost depleted after fasting overnight, in the sheep the normal level of 36.3 ± 5.0 mg/g wet wt (mean ± S.E.M. for 6 sheep) was only reduced to 17.0 ± 3.5 mg/g wet wt after 6 days' starvation. At the same time an increase in the concentration of ketone bodies from a normal level of 1.7 ± 0.3 mg/100 ml of blood to 9.7 ± 3.1 mg/100 ml of blood (6 observations in each group) was observed.

The most striking feature in these results was the behaviour of pyruvate carboxylase and PEP carboxykinase. Therefore, in subsequent studies attention was focused on the activities of these two enzymes.

(b) Effect of glucocorticoids and diabetes on pyruvate carboxylase and PEP carboxykinase in rat liver

As was discussed in Section 3.1, it is generally agreed that diabetes and administration of glucocorticoids cause a marked increase in the activity of PEP carboxykinase in rat liver, but there are conflicting reports about the behaviour of pyruvate carboxylase. Prior to an

investigation of the effects of these perturbations on the enzymes in sheep, the activities were assayed in the livers of rats injected with dexamethasone or made diabetic by an injection of alloxan. The results are presented in Table 3.3. The rats were severely diabetic, as indicated by the increased level of activity of PEP carboxykinase and the elevated blood glucose levels which exceeded 250 mg/100 ml, but there was only a 50% increase in the activity of pyruvate carboxylase. Dexamethasone had a similar effect on the level of pyruvate carboxylase activity.

(c) Effect of glucocorticoids on PEP carboxykinase and pyruvate carboxylase in the sheep

The results shown in Table 3.4 indicate that, in contrast to data reported for rats, there is no change in the activity of PEP carboxykinase in the livers of sheep in which the plasma glucose levels have been maintained at approximately twice the normal level during several days of cortisone administration. Furthermore, a significant decrease in the activity of pyruvate carboxylase occurred in both the liver and kidney. When the synthetic glucocorticoid, dexamethasone, was administered the results were similar to those obtained with cortisone. The results shown in Table 3.4 are the means of values obtained for varying periods of hormone administration. Since the effects were similar over the varying time period, the results were pooled for statistical analysis.

When the time course for the effect of glucocorticoid administration isexamined it can be seen that the liver and

TABLE 3.3: EFFECTS OF DEXAMETHASONE AND ALLOXAN DIABETES ON PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN RAT LIVER

	Pyruvate Carboxylase ($x10^3$)	PEP Carboxykinase ($x10^3$)
Control	3.51 ± 0.24 (5)	0.62 ± 0.08 (5)
Dexamethasone	5.29 ± 0.15 (3) $P < 0.005$	-
Diabetic (Alloxan)	5.24 ± 0.48 (4) $P < 0.02$	3.07 ± 0.69 (4) $P < 0.01$

The activities of pyruvate carboxylase and PEP carboxykinase were assayed by the methods described in Section 3.2(c) in homogenates of liver from normal rats, rats injected with 1 mg of dexamethasone per day for 3 days, and rats which had been given an injection of alloxan (60 mg/kg body wt) 4 days prior to killing. The specific activities are expressed as μmoles of CO_2 fixed per min per mg of protein. The mean ± S.E.M. is given with the number of animals in parentheses. The statistical significance of the comparison with the normal animals is indicated.

TABLE 3.4: EFFECTS OF GLUCOCORTICOID ADMINISTRATION ON THE SPECIFIC ACTIVITY OF PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN THE LIVER AND KIDNEY OF SHEEP

	Treatment	Liver ($x10^3$)	Kidney ($x10^3$)
A. Pyruvate Carboxylase	Control	3.86 ± 0.40 (12)	7.10 ± 0.55 (9)
	Cortisone	1.88 ± 0.28 (5)	3.98 ± 0.16 (4)
		P < 0.01	P < 0.01
	Dexamethasone	1.25 ± 0.29 (4)	3.38 ± 0.10 (2)
		P < 0.01	P < 0.02
B. PEP Carboxy-kinase	Control	3.35 ± 0.23 (14)	5.24 ± 0.33 (9)
	Cortisone	3.47 ± 0.26 (5)	6.27 ± 0.25 (4)
		n.s.	n.s.
	Dexamethasone	3.88 ± 0.40 (4)	5.92 ± 0.10 (2)
		n.s.	n.s.

The activities of pyruvate carboxylase and PEP carboxykinase were assayed by the methods described in Section 3.2(c) in homogenates of liver and kidney from normal sheep, and sheep which had been injected with cortisone or dexamethasone. The details of the hormone administration are given in Table 3.5. The specific activities are expressed as μmoles of CO_2 fixed per min. per mg of protein. The mean ± S.E.M. is given with the number of animals in parentheses. The statistical significance of the comparison with the normal animals is indicated.

n.s. = not significant.

TABLE 3.5: TIME COURSE OF THE EFFECT OF GLUCOCORTICOID ADMINISTRATION ON THE SPECIFIC ACTIVITY OF PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN THE LIVER AND KIDNEY OF THE SHEEP

Treatment	Dose/day (mg/kg)	No.of days	Pyruvate Carboxylase		PEP Carboxykinase	
			Liver ($x10^3$)	Kidney ($x10^3$)	Liver ($x10^3$)	Kidney ($x10^3$)
Control	0		3.86	7.10	3.35	5.24
Cortisone	2.5	3	2.65		3.45	
	5.0	3	1.25	3.90	2.70	6.32
	5.0	5	2.10	4.12	3.14	6.80
	5.0	5	2.17	3.92	4.23	5.60
	5.0	7	1.18	3.99	3.82	6.36
Cortisone	See text	1	2.93	4.09	3.48	10.2
Cortisone (fasted)	2.5	6	5.47	4.47	3.73	9.31
Dexamethasone	0.13	2	1.36	3.47	3.27	6.02
	0.13	3	1.99		4.96	
	0.25	3	1.04		3.97	
	0.25) 0.63)	5) 2)	0.60	3.88	3.30	5.82

The activities of pyruvate carboxylase and PEP carboxykinase were assayed by the methods described in Section 3.2(c) in homogenates of liver and kidney from sheep which had been given daily intramuscular injections of the hormones as indicated. One sheep was fasted throughout the cortisone treatment, and another sheep was given intensive treatment over 24 hr. The values for the controls are taken from Table 3.4. The specific activities are expressed as μmoles of CO_2 fixed per min. per mg of protein.

kidney differ in their response. The results for cortisone are presented in Table 3.5. There is a tendency for the decrease in the activity of liver pyruvate carboxylase to continue as the treatment is prolonged, whereas the kidney enzyme shows no further decrease after 3 days' treatment. The liver enzyme also decreases to a greater extent than that in the kidney. This is similar to the situation in fasted sheep where the response of the liver enzyme was the more pronounced.

The time course for the effect of dexamethasone (Table 3.5) was similar to that for cortisone, although, consistent with the reported properties of synthetic glucocorticoids, dexamethasone was more potent in its action. It can also be seen that a larger dose of hormone caused a greater reduction in enzyme activity for the same time period. The activity of PEP carboxykinase in the liver and kidney remained constant during the administration of either hormone.

Normal levels of PEP carboxykinase were observed in sheep liver after 2 or 3 days' treatment, but the possibility existed that there was an initial increase in the level of PEP carboxykinase which subsequently returned to normal. Therefore one sheep was given intensive treatment over a period of only 24 hr. This involved an initial intramuscular injection of cortisone acetate (2.5 mg/kg) followed 8 hr later by a second injection and 21 hr later by an intravenous injection of hydrocortisone succinate (2.5 mg/kg). At the time of slaughter (24 hr after the initial injection) the plasma glucose was elevated from a normal level of about 50 mg/100 ml

to 90 mg/100 ml. The enzyme pattern (Table 3.5) is similar to that in animals treated for long periods of time. Liver PEP carboxykinase is unchanged in activity although there does appear to be an increase in kidney PEPcarboxykinase activity.

Since fasting causes an increase in the level of pyruvate carboxylase while glucocorticoids cause a decrease it was of interest to measure the enzymic activities in an animal which had been injected daily with cortisone during a 6 day period of starvation. The cortisone administration almost completely prevented the marked increase in pyruvate carboxylase usually observed after fasting. PEP carboxykinase remained unchanged in the liver, although there was a slight increase in activity in the kidney.

(d) Effect of diabetes on pyruvate carboxylase and PEP carboxykinase in sheep

It can be seen from Table 3.6 that diabetes induced in sheep either by pancreatectomy or by an injection of alloxan leads to an increase in the levels of both pyruvate carboxylase and PEP carboxykinase in the liver. The data presented in Table 3.7 indicate that the alloxan treated animals were more severely diabetic than those which had been pancreatectomised. The conditions of hyperglycaemia and ketosis were more severe in the alloxan diabetics, and despite approximately the same food intake, a much greater excretion of glucose and nitrogen occurred in the alloxan diabetic sheep. This could be indicative of a greater degree of gluconeogenesis from protein in these

TABLE 3.6: EFFECT OF DIABETES ON SPECIFIC ACTIVITY OF PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN LIVER AND KIDNEY OF SHEEP

	Treatment	Liver ($x10^3$)	Kidney ($x10^3$)
A. Pyruvate Carboxylase	Control	3.86 ± 0.40 (12)	7.10 ± 0.55 (9)
	Diabetic (Alloxan)	18.4 ± 1.7 (4)	5.76 ± 0.43 (4)
		$P < 0.001$	n.s.
	Diabetic (Depancreatised)	14.1 ± 3.4 (3)	5.35 ± 0.24 (3)
		$P < 0.001$	n.s.
B. PEP Carboxykinase	Control	3.35 ± 0.23 (14)	5.24 ± 0.33 (9)
	Diabetic (Alloxan)	8.07 ± 0.73 (4)	6.83 ± 2.00 (4)
		$P < 0.001$	n.s.
	Diabetic (Depancreatised)	5.76 ± 0.41 (3)	5.78 ± 1.43 (3)
		$P < 0.01$	n.s.

The activities of pyruvate carboxylase and PEP carboxykinase were assayed as described in Section 3.2(c) in homogenates of liver and kidney from normal sheep and sheep which had been made diabetic either by pancreatectomy or by an injection of alloxan (60 mg/kg body wt). The specific activities are expressed as μmoles of CO_2 fixed per min per mg of protein. The mean ± S.E.M. is given with the number of animals in parentheses. The statistical significance of the comparison with the normal animals is indicated.

n.s. = not significant.

TABLE 3.7: METABOLITE LEVELS IN NORMAL AND DIABETIC SHEEP

	Control	Alloxan Diabetic	Depancreatised
Food intake (g/day)	1000	625 ± 38	914 ± 117
Plasma glucose (mg/100 ml)	51.3 ± 1.3	184 ± 26	159 ± 21
Ketones in blood (mg/100 ml)	1.4 ± 0.2	36 ± 4	11 ± 2
Glucose in urine (g/day)	0	53 ± 6	30 ± 3
Nitrogen in urine (g/day)	6.7 ± 0.7	12.6 ± 0.5	5.6 ± 0.5

The concentrations of glucose, ketone bodies and nitrogen were determined as described in Section 3.2(d) over a 4 day period in normal, depancreatised and alloxan diabetic sheep. The results are given as mean ± S.E.M.

animals and would be consistent with the higher specific activities of both enzymes in the liver.

It is somewhat surprising that there is no increase in the activity of either enzyme in the kidneys of diabetic sheep.

(e) Effect of fasting on pyruvate carboxylase and PEP carboxykinase during phlorhizin administration in sheep

Treatment with phlorhizin imposes a glucose demand on the animal due to a lowered renal threshold for glucose.

The results presented in Table 3.8 show no change in the activity of either enzyme in liver or kidney following the daily administration of phlorhizin for 6 days to fed sheep. The adequacy of the phlorhizin treatment was indicated by the daily excretion in the urine of 15-20 g of glucose. However, when two animals were fasted during the phlorhizin treatment a substantial increase in the activity of pyruvate carboxylase in the liver was observed. There was no change in PEP carboxykinase in the liver but an increase in the activity of this enzyme occurred in the kidney.

The concentration of total ketone bodies was increased to 7-8 mg/100 ml of blood in the phlorhizinised animals when fasted, compared with a value of 2-3 mg/100 ml when fed.

(f) Effect of fasting on pyruvate carboxylase and PEP carboxykinase in pregnant sheep

It has long been recognised that pregnant sheep, particularly those with twin foetuses, are susceptible to severe hypoglycaemia and ketosis during the last few weeks of pregnancy. In an experimental situation starvation can

TABLE 3.8: EFFECT OF PHLORHIZIN ON THE SPECIFIC ACTIVITY OF PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN THE LIVER AND KIDNEY OF SHEEP

	Treatment	Liver ($x10^3$)	Kidney ($x10^3$)
A. Pyruvate Carboxylase	Control	3.86 ± 0.40 (12)	7.10 ± 0.55 (9)
	Phlorhizin (Fed)	3.68 ± 0.19 (2)	6.78 ± 0.37 (2)
		n.s.	n.s.
	Phlorhizin (Fasted)	13.3 ± 1.7 (2)	8.98 ± 0.73 (2)
		$P < 0.001$	n.s.
B. PEP Carboxykinase	Control	3.35 ± 0.23 (14)	5.24 ± 0.33 (9)
	Phlorhizin (Fed)	3.46 ± 0.68 (4)	8.14 ± 0.29 (2)
		n.s.	$P < 0.005$
	Phlorhizin (Fasted)	3.78 ± 0.27 (2)	7.92 ± 0.70 (2)
		n.s.	$P < 0.01$

The activities of pyruvate carboxylase and PEP carboxykinase were assayed by the methods described in Section 3.2(c) in homogenates of liver and kidney from normal sheep and sheep which had been injected with phlorhizin. Phlorhizin was administered daily for 6 days as indicated in Section 3.2(a) and two of the animals were fasted throughout the period of phlorhizin administration. The specific activities are expressed as μmoles of CO_2 fixed per min per mg of protein. The mean ± S.E.M. is given with the number of animals in parentheses. The statistical significance of the comparison with the normal animals is indicated.

n.s. = not significant.

reproduce most features of this ovine pregnancy toxaemia. The results in Table 3.9 show the activities of the enzymes and the plasma glucose levels in ewes which were starved for increasing periods of time during the later stages of pregnancy. As the time of starvation was increased hypoglycaemic ketosis (30 - 60 mg of total ketone bodies/100 ml of blood) developed and pyruvate carboxylase increased markedly in both the liver and kidney. In the more severely hypoglycaemic sheep there was a tendency for PEP carboxykinase to increase in activity in the liver, while a very pronounced increase in activity was observed in the kidney.

(g) Glucose turnover rates

The values obtained for various parameters of glucose metabolism under conditions of physiological perturbation are presented in Table 3.10.

Fasting caused a decrease in the glucose pool size and in the glucose turnover rate, in agreement with observations by Kronfeld and Simesen (1961) and Bassett, Mills and Reid (1966). Cortisone treatment appeared to cause a slight increase in the turnover rate while the value obtained for the animal which was fasted during the period of cortisone administration was slightly less than the controls. These observations are similar to those reported by Bassett, Mills and Reid (1966). Alloxan diabetes caused a three-fold increase in the pool size, while the turnover rate increased by about 50%. The most pronounced changes in glucose turnover rates occurred during phlorhizin administration. Both the pool size and the glucose

TABLE 3.9: EFFECT OF FASTING ON ENZYMIC ACTIVITIES AND PLASMA GLUCOSE CONCENTRATION IN PREGNANT SHEEP

Time fasted (days)	Plasma Glucose (mg/100 ml)	Foetus Wt. (kg)	Pyruvate Carboxylase		PEP Carboxykinase	
			Liver ($x10^3$)	Kidney ($x10^3$)	Liver ($x10^3$)	Kidney ($x10^3$)
0	50.4	3.2	4.15	8.04	2.91	5.86
2	65.5	4.9	5.26	6.86	2.95	5.22
3	24.9	4.0	7.43		3.35	
4	12.5	0.8	9.22		3.09	
5	21.9	1.9	13.3	8.96	4.68	12.3
6	9.0	3.7	16.5	12.1	5.83	15.9
11	9.5	3.0	18.0	9.06	5.72	14.5

The activities of pyruvate carboxylase and PEP carboxykinase were assayed by the methods described in Section 3.2(c) in homogenates of liver and kidney from pregnant sheep fasted during the latter stages of gestation. The specific activites are expressed as μmoles of CO_2 fixed per min per mg of protein. Plasma glucose concentrations were estimated as indicated in Section 3.2(d).

TABLE 3.10: PARAMETERS OF GLUCOSE METABOLISM IN NORMAL SHEEP AND IN SHEEP UNDER CONDITIONS OF PHYSIOLOGICAL PERTURBATION

	Body Wt. (kg)	Plasma Glucose (mg/100 ml)	Glucose Pool (g)	Glucose Space (% of body wt.)	Turnover Rate mg/min/kg$^{0.75}$
Control (6)	35.5 ± 1.8	57.8 ± 3.9	5.57 ± 0.36	27.6 ± 1.8	3.07 ± 0.15
Diabetic (4)	38.7 ± 2.7	228 ± 32	18.4 ± 2.3	21.7 ± 2.4	4.33 ± 0.55
Fasted	25.5	44.7	2.92	25.7	2.20
	27.1	54.7	3.37	22.8	2.35
Phlorhizin	42.7	52.0	6.52	29.4	5.73
	50.7	53.1	8.93	33.2	7.15
Phlorhizin (fasted)	37.6	41.6	7.07	45.2	5.80
	47.7	43.5	8.14	39.3	5.18
Cortisone	20.5	55.3	2.73	24.0	3.51
	24.4	54.2	3.41	25.8	4.07
Cortisone (fasted)	21.0	67.5	4.12	29.1	2.29

The various parameters of glucose metabolism were calculated from the rate of dilution of a single injection of [U-^{14}C]glucose as described in Section 3.2(e).

The sheep were treated as indicated in Section 3.2(a). Diabetes wasinduced by an injection of alloxan. Cortisone (2.5 mg/kg body wt) was administered daily for 5 days. One sheep was fasted throughout the period of cortisone administration. The animals were not fed on the day of the experiment. The results are expressed as mean ± S.E.M. for the control and diabetic animals, with the number of animals in parentheses. The individual values are shown for treatments in which only two animals were examined.

space were increased while the glucose turnover rates were approximately twice those obtained in controls. Fasting had very little effect on one of the phlorhizinised animals, except that the glucose space was increased still further, but in the other animal there was a decrease of about 30% in the turnover rate.

3.4. DISCUSSION

(a) Fasting

In the fed rat the hepatic activity of PEP carboxykinase is very low, and the four-fold increase in the activity in the fasted rat is consistent with increased glucose production via oxaloacetate. However, in the fed sheep the activity of this enzyme is relatively high and when the animal is fasted the gluconeogenic demands for metabolic flux through the oxaloacetate-PEP pathway may be no greater than in the fed state, since during fasting the glucose turnover rate is reduced. In the fed sheep most of the glucose used is synthesised through the gluconeogenic pathway and so a decrease in glucose turnover rate in the fasted animal may be indicative of decreased flux through this pathway. The decrease is not very large and emphasises the important contribution to glucose synthesis of sources other than propionate as the concentrations of volatile fatty acids in the rumen of starved sheep are low and the entry ofpropionate from other sources is probably negligible. The entry rate of non-esterified fatty acids is increased (Leat and Ford, 1966) on starvation and it has been

estimated that glycerol, available from increased lipolysis (Bergman, 1968) could replace approximately half of the propionate which would normally be available.

Tissue protein is another source of gluconeogenic substrates during starvation. Work in Cahill's laboratory (Owen et al., 1969) on humans has shown that alanine is quantitatively the most important single gluconeogenic precursor among the amino acids tested. The amounts of alanine extracted by the liver are far in excess of the amount which could be derived from protein breakdown (Felig et al., 1969), and from data obtained using rats it has been suggested that alanine may serve a carrier function in the transport of nitrogen from peripheral tissues to the liver (Brosnan, Krebs and Williamson, 1967; Mallette, Exton and Park, 1969). It has also been speculated that alanine is the carrier of carbon atoms of gluconeogenic amino acids. In muscle and other tissues partial degradation of glutamate and aspartate to pyruvate would then allow the three carbon atoms, together with the nitrogen of these amino acids to be transported to the liver in the form of alanine. Purines and pyrimidines would be other sources of nitrogen. If alanine has a similar role in sheep, this may account for the high activity of pyruvate carboxylase in the liver of the fasted sheep.

(b) Glucocorticoids

Neither fasting nor glucocorticoid administration led to an increase in the activity of hepatic PEP carboxykinase

in sheep. The sheep is not the only species in which the behaviour of PEP carboxykinase on fasting differs from that in the rat, since no increase occurs in the livers of pigeons (Gevers, 1967) or lactating cows (Ballard et al., 1968) when fasted. Furthermore, the effect of glucocorticoids in causing an increase in PEP carboxykinase in the liver of the rat does not apply to all species. Lardy et al. (1964) showed that the activity of this enzyme was not increased in guinea pigs and results presented here indicate that there is no increase in hepatic PEP carboxykinase in sheep in which the plasma glucose concentration has been maintained at a level higher than normal during a period of glucocorticoid administration. Bassett, Mills and Reid (1966) have reported that the hyperglycaemia in sheep during cortisol administration cannot be accounted for by increased gluconeogenesis. They suggest that an inhibition of glucose utilisation occurs, leading to an accumulation of glucose in the plasma which in turn leads to a normal rate of glucose utilisation at the higher plasma concentration. Under these conditions there would be no need for an increase in the activity of PEP carboxykinase.

The only other report of the effects of glucocorticoids on PEP carboxykinase in ruminants is that of Baird and Heitzman (1970) who found that this enzyme was increased in the livers of lactating cows when compared with non-lactating cows, and that this increased activity was depressed to a normal level 48 hr after administration of Voren, an ester of dexamethasone. However, in agreement with the results obtained in sheep,

the hormone had no significant effect on the activity of PEP carboxykinase in the livers of non-lactating cows.

The fall in the activity of pyruvate carboxylase following glucocorticoid administration to a fed sheep may be associated with the mobilisation of protein and increased availability of amino acids which could augment the supply of oxaloacetate already coming from propionate, and thus lead to a lowered activity of pyruvate carboxylase.

(c) Diabetes and phlorhizin

Of the treatments used, only diabetes and prolonged starvation of pregnant ewes led to an increase in the activity of hepatic PEP carboxykinase.

The change in the activity of PEP carboxykinase in the diabetic animal is consistent with increased glucose production by the liver as indicated by the increased glucose turnover rate. It is interesting that there was a very marked increase in glucose entry rate in the phlorhizinised animals although their urinary glucose excretion was less than that in the diabetic animals. Lack of insulin in diabetes causes inhibition of glucose uptake and utilisation by peripheral tissues. Therefore, in spite of the amount of glucose excreted, it may be that the total requirement for glucose is not very much greater than normal. On the other hand, phlorhizinised animals excrete glucose, but probably continue to utilise it at a normal rate. In dogs infused with phlorhizin for short time periods the hepatic glucose output is increased and compensates for the glucose excreted, while the uptake of glucose by peripheral

tissues is unaffected (Kolodny, Kline and Altszuler, 1962).

In the diabetic animals the increases in pyruvate carboxylase and PEP carboxykinase are probably associated with the increased glucose production by the liver. However, although there is a large increase in glucose entry rate in the phlorhizinised animals, both enzymes remain unchanged, except that pyruvate carboxylase increases when the animals are also fasted. This suggests that the increased glucose production may be from precursors which are different from those utilised in diabetes.

(d) Fasting during pregnancy

In pregnant sheep the rate of endogenous glucose production and utilisation is higher than in the non-pregnant animal (Bergman, 1963, 1964). When a pregnant sheep is fasted the glucose entry rate decreases, but is still higher than that observed in a comparably fasted non-pregnant sheep.

The turnover of glycerol is greatly elevated during hypoglycaemic ketosis (Bergman, 1968) but is obviously insufficient to meet the animal's requirement for glucose.

As pregnant fasted sheep become progressively hypoglycaemic there is an increase in ketone body production and plasma free fatty acid levels (Reid and Hogan, 1959; Reid and Hinks, 1962). When the blood glucose level falls below a critical level of 25-30 mg/100 ml there is a disproportionate increase in blood ketones, associated with little further increase in free fatty acids. Reid and Hinks (1962) have suggested that in severely hypoglycaemic pregnant ewes, fatty acid synthesis, mobilisation and oxidation are affected by

endocrine changes, including increased plasma cortisol levels, probably associated with reduction of insulin secretion (Reid, 1960). It is interesting to note that it was under conditions such as these that the hepatic activity of PEP carboxykinase tended to increase in fasted pregnant ewes.

(e) Comparison of the changes in the activities of pyruvate carboxylase and PEP carboxykinase in the liver and kidney

In the ruminant liver, gluconeogenesis is an essential metabolic process under all conditions and glycolysis probably occurs only to a very limited extent. Very little is known about glucose metabolism in the kidney of ruminants. The results obtained during this investigation suggest that under some conditions there may be important differences in the control of metabolic processes in the liver and kidney.

Under most conditions of perturbation, except diabetes, pyruvate carboxylase in liver and kidney responded similarly, although the response in the kidney was usually less pronounced. On the other hand, kidney PEP carboxykinase increased more readily than the liver enzyme. Significant increases in activity were observed in the kidney of fasted pregnant ewes and phlorhizinised animals, whereas the activity of the liver enzyme tended to increase only during prolonged starvation of pregnant ewes and remained unchanged with phlorhizin treatment. It is possible that the increased activity in the kidney may be associated with metabolic acidosis, which has been shown in rats to increase the activity of PEP carboxykinase in the

kidney (Allayne, 1968).

In the diabetic sheep both pyruvate carboxylase and PEP carboxykinase increased in activity in the liver, and although the high concentration of ketone bodies in the blood may lead to acidosis, neither enzyme increased in activity in the kidney. The reason for this apparently anomalous behaviour is not known. It suggests either that the enzymes in the liver and kidney are controlled by different factors or else that in diabetes the environments in the liver and kidney differ, such that conditions necessary for the induction of the enzymic activity do not arise in the kidney.

CHAPTER 4

INTRAMITOCHONDRIAL AND INTRACELLULAR DISTRIBUTIONS OF PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN SHEEP LIVER

4.1. INTRODUCTION

It was shown in the previous chapter that the activity of pyruvate carboxylase increased markedly in the livers of fasted and diabetic sheep, whereas hepatic PEP carboxykinase increased only in the diabetic animal. Before a study of the nature and cause of the changes in the activity of these enzymes could be undertaken it was necessary to determine the intracellular distribution of each of them in the livers of normal sheep and to establish whether this distribution was altered when the sheep were subjected to physiological perturbations.

There is species variation in the subcellular distribution of PEP carboxykinase in the liver. In the rat and mouse (Nordlie and Lardy, 1963) most of the activity is found in the cytosol, while in other mammalian species examined, viz., guinea pigs, rabbits (Nordlie and Lardy, 1963), pigs (Swiatek et al., 1970), cows (Ballard, Hanson and Kronfeld, 1968), sheep (Ballard and Hanson, 1969) and humans (Ballard and Hanson, 1969) there is significant activity in both the cytosol and mitochondria. With the exception of the pig (Swiatek et al., 1970), it has been found that in those species in which the effects of diet and hormonal treatments have been tested only the cytosol enzyme is inducible (Lardy et al., 1964; Lardy, 1965; Nordlie, Varrichio and Holten, 1965). In pigeons (Gevers, 1967) and chickens (Utter, 1959), PEP carboxykinase is confined to the mitochondria and its activity is not increased by starvation.

The intracellular distribution of pyruvate carboxylase is less clearly understood. The enzyme in avian liver is located primarily in the mitochondria (Keech and Utter, 1963). Some studies have indicated that the enzyme is exclusively mitochondrial in rat liver (Freedman and Kohn, 1964; Brech, Shrago and Wilken, 1970; Bottger et al., 1969; Ballard, Hanson and Reshef, 1970), while other authors claim that there is significant activity in the cytosol (Henning et al., 1966). Furthermore, agreement has not been reached concerning the behaviour of pyruvate carboxylase following physiological perturbation of the animal. Although some investigators have reported increased levels of activity in the livers of fasted, diabetic and glucocorticoid treated rats (Freedman and Kohn, 1964; Prinz and Seubert, 1964; Wagle, 1964; Henning et al. 1966), others have found no change in the activity under these conditions (Krebs, 1966; Shrago and Lardy, 1966; Struck, Ashmore and Wieland, 1966; Brech, Shrago and Wilken, 1970).

In this investigation the intracellular distributions of pyruvate carboxylase and PEP carboxykinase have been determined in normal, starved and diabetic sheep. In all three conditions approximately one-third of the total PEP carboxykinase activity was found in the mitochondria, the remainder being in the cytosol. In the liver of the diabetic sheep the activity increased to a similar extent in both fractions. On the other hand, the data indicate that under all of the conditions investigated, pyruvate carboxylase is exclusively mitochondrial *in vivo*.

4.2. METHODS

(a) Cell fractionation procedure: The cell fractionation procedure was similar to that described by Keech and Utter (1963). The tissue was homogenised with five strokes in a Potter-Elvehjem homogeniser at 2° in 0.25M sucrose containing 0.1mM EDTA, 0.5mM GSH and 20mM tris-Cl, pH 7.2. Particulate fractions were suspended in this medium and sonicated for four periods of 10 sec. before assay.

(b) Mitochondrial fractionation procedure: Mitochondria were fractionated by the method of Schnaitman and Greenawalt (1968) as modified by Erdiczka, Gerbitz and Fette (1969). The fractionation scheme is shown in Fig. 4.1. The sucrose medium used throughout the procedure contained 0.25M sucrose, 0.1mM EDTA, 0.5mM GSH and 20mM tris-Cl, pH 7.2. The initial mitochondrial suspension contained approximately 100 mg of protein per ml. The inner and outer membrane fractions were suspended in the above sucrose medium.

(c) Enzyme assays: All assays were carried out at 30°. Product formation was proportional to enzyme concentration and time under the conditions used.

(i) PEP carboxykinase was assayed by the method described in Section 2.5.

(ii) Pyruvate carboxylase was assayed by the method described in Section 2.6.(a).

(iii) Monoamine oxidase was assayed by the method of Tabor, Tabor and Rosenthal (1954) as modified by Schnaitman, Erwin and Greenawalt (1967). The rate was measured against

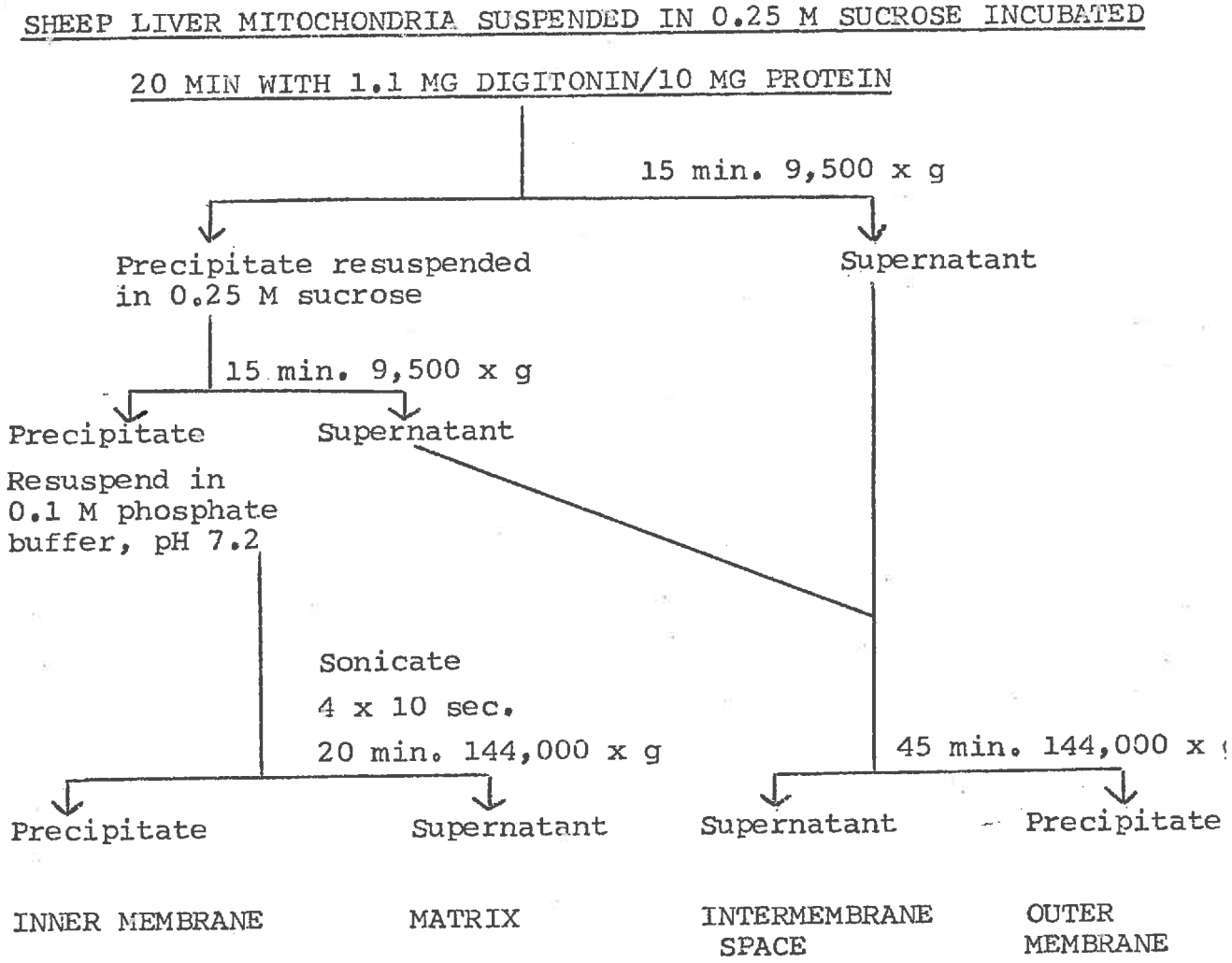


FIG. 4.1. Flow-sheet for digitonin fractionation of isolated mitochondria.

a control cuvette from which benzylamine hydrochloride had been omitted.

(iv) Succinate dehydrogenase was assayed by the method of Bonner (1955), except that the reaction mixture contained 1mM KCN and the pH was 7.6. Succinate was omitted from the control.

(v) Citrate synthase was assayed by a modification of the method of Srere (1969) in which the rate of reduction of 5,5'-dithiobis- (2-nitrobenzoate) (DTNB) was followed. The system contained 0.13mM acetyl-CoA. The control rate was that observed in the absence of added oxaloacetate. The rate in both the test and control followed a biphasic pattern with a rapid initial rate (probably due to the reaction of DTNB with GSH as well as citrate synthase activity) and then a slower rate being the citrate synthase activity alone. However, the same result for net citrate synthase activity was obtained by taking the difference between test and control rates for either the fast or slow phases ofthe reaction.

(vi) Glutamate dehydrogenase was assayed by a method based on that of Colman and Frieden (1966). The reaction mixture contained 100 mM tris-Cl (pH 7.4), 5mM α-ketoglutarate, 5mM ADP, 50mM NH_4Cl, 0.3mM NADH and enzyme. α-Ketoglutarate was omitted from the control.

(d) Partial purification of pyruvate carboxylase from sheep liver cytosol

A 20% (w/v) homogenate of liver from diabetic sheep was prepared at 4° in 0.25M sucrose containing 0.1mM EDTA and 20mM tris-Cl, pH 7.2. The homogenate was centrifuged for 60 min. at 35,000 x g and the resulting supernatant was centrifuged for 30 min. at 105,000 x g. The supernatant was taken to 35% saturation by addition of a cold saturated solution of neutralised ammonium sulphate. The precipitate obtained after centrifuging was dissolved and stored at -15° in a solution containing 1.6M sucrose, 0.06M $(NH_4)_2SO_4$, and 0.1M potassium phosphate, pH 7.2. This enzyme was used for all immunochemical experiments.

(e) Purification of mitochondrial pyruvate carboxylase

Pyruvate carboxylase was purified from the mitochondria of normal sheep liver to a specific activity of 15 units per mg of protein by the method described in Section 2.10(a).

(f) Preparation of antibody to purified sheep liver mitochondrial pyruvate carboxylase

A rabbit was immunised with sheep liver mitochondrial pyruvate carboxylase (15 units per mg of protein). The enzyme was diluted with Freund's adjuvant and approximately 1 mg of protein was injected subcutaneously into each of four sites. This procedure was repeated two weeks later and the animal was bled by heart puncture one week after the second immunisation.

γ-Globulin was isolated and purified from the serum by a modification of the method of Goldstein, Slizys and Chase (1961). Serum obtained from the rabbit before immunisation was treated in an identical manner.

Cold saturated $(NH_4)_2SO_4$ (40 ml) was added to 40 ml of serum. The solution was stirred for one hr at 4° and then centrifuged at 35,000 x g for 20 min. The precipitate was dissolved in glass distilled water to give a volume of 35 ml. The solution was dialysed against 0.0175M potassium phosphate, pH 7.2, and applied to a column containing 100 ml of packed DEAE cellulose equilibrated with the same buffer. The protein was eluted with the equilibrating buffer, fractions of 4 ml were collected and those containing protein were pooled. The purified γ-globulin was concentrated by membrane ultrafiltration and dialysed against 0.15M KCl in 0.02M potassium phosphate, pH 7.2. The protein concentration of the solution was adjusted to 60 mg per ml.

This procedure resulted in a four-fold purification with almost no loss of antibody activity.

(g) Antibody titration experiments

The incubation mixture (total volume, 0.20 ml) contained 10 µl enzyme; 90 µl of a mixture containing 200mM tris-Cl (pH 8.4), 16mM $MgCl_2$, 5mM ATP, 0.7mM acetyl-CoA and 20mM $NaHCO_3$; and 100 µl of antibody in 0.15M KCl in 0.02M potassium phosphate (pH 7.2). The mixture was incubated for 60 min. at room temperature and then centrifuged for 15 min. at 700 x g. Aliquots (0.10 ml) of supernatant were

taken for assay, using the system described in Section 2.6(a). Since the antigen-antibody precipitate was enzymatically active, care was taken to avoid including any of the precipitate in the sample assayed.

In preliminary experiments, the time course (up to 24 hr) of the precipitation process was followed by estimation of the amount of protein precipitated and it was found that complete precipitation had occurred after 60 min.

(h) Ouchterlony double diffusion analysis

Ouchterlony double diffusion analysis was carried out on a medium of 1% (w/v) agar in 0.2M KCl and 0.02M potassium phosphate buffer, pH 7.2. A centre well 5 mm in diameter was filled with approximately 50 µl of antibody to purified mitochondrial pyruvate carboxylase. Alternating outer wells spaced at 13 mm from the centre contained either 30 m units of purified mitochondria pyruvate carboxylase or 30 m units of enzyme from cytosol of diabetic liver. The plates were developed for 48 hr at 25° in a humidified chamber.

(i) Protein estimation

Protein was determined by the biuret method (Gornall, Bardawill and David, 1949).

(j) Animals

Normal, fasted and diabetic merino ewes were treated as described in Section 3.2(a).

(k) Electron microscopy

(i) Liver sections

Liver sections from normal, fasted and diabetic sheep were fixed for 30 min with 1% (w/v) osmium tetroxide, dehydrated in acetone and embedded in araldite. Sections were cut with glass knives, stained with uranyl acetate and lead acetate and viewed in a Siemens Elmiskop I with an 80 Kv and 50 μ objective aperture.

(ii) Isolated mitochondria

The mitochondria were isolated from the livers of normal and diabetic sheep as described in Section 4.2(a). The mitochondrial pellet was treated in the same manner as the liver sections, except that the sample was fixed for 1 hr with 2% (w/v) glutaraldehyde followed by 1% (w/v) osmium tetroxide for 30 min.

4.3. RESULTS

(a) Intramitochondrial localisation of pyruvate carboxylase and PEP carboxykinase in livers of normal sheep

In preliminary cell fractionation experiments only a small proportion of pyruvate carboxylase activity was found in the cytosol fraction of livers from normal sheep. However, in the livers of fasted and diabetic sheep where a several-fold increase occurred in the total cellular level of pyruvate carboxylase activity, a substantial proportion of the activity was found in the cytosol. The most plausible explanation

for this observation was either that considerable mitochondrial breakage was occurring during the fractionation procedure or that the increase in total activity was due partly to the appearance of a cytosol form of the enzyme.

In an attempt to resolve these alternatives, mitochondrial marker enzymes were used to indicate the extent of contamination of the cytosol fraction with mitochondrial protein. The intramitochondrial localisation of pyruvate carboxylase and PEP carboxykinase was first determined to ensure that appropriate enzymes were used as mitochondrial markers in subsequent cell fractionation experiments. Folloving digitonin fractionation of isolated mitochondria, monoamine oxidase was used as a marker for the outer membrane fraction (Okamoto et al., 1967; Beattie, 1968; Schnaitman and Greenawalt, 1968), succinate dehydrogenase for the inner membrane (Beattie, 1968; Schnaitman and Greenawalt, 1968) and glutamate dehydrogenase (Norum, Farstad and Bremer, 1966; Sottocasa et al., 1967; Brdiczka et al., 1968) and citrate synthase (Brdiczka et al., 1968; Marco, Sebastian and Sols, 1969) were used as markers for the mitochondrial matrix. The distribution of total protein and of the enzymes in the four submitochondrial fractions is shown in Fig. 4.2. A possible explanation for the large proportion of the monoamine oxidase activity in the intermembrane space fraction is that the digitonin treatment disrupted a considerable amount of the outer membrane into fragments too small to be sedimented under the conditions employed.

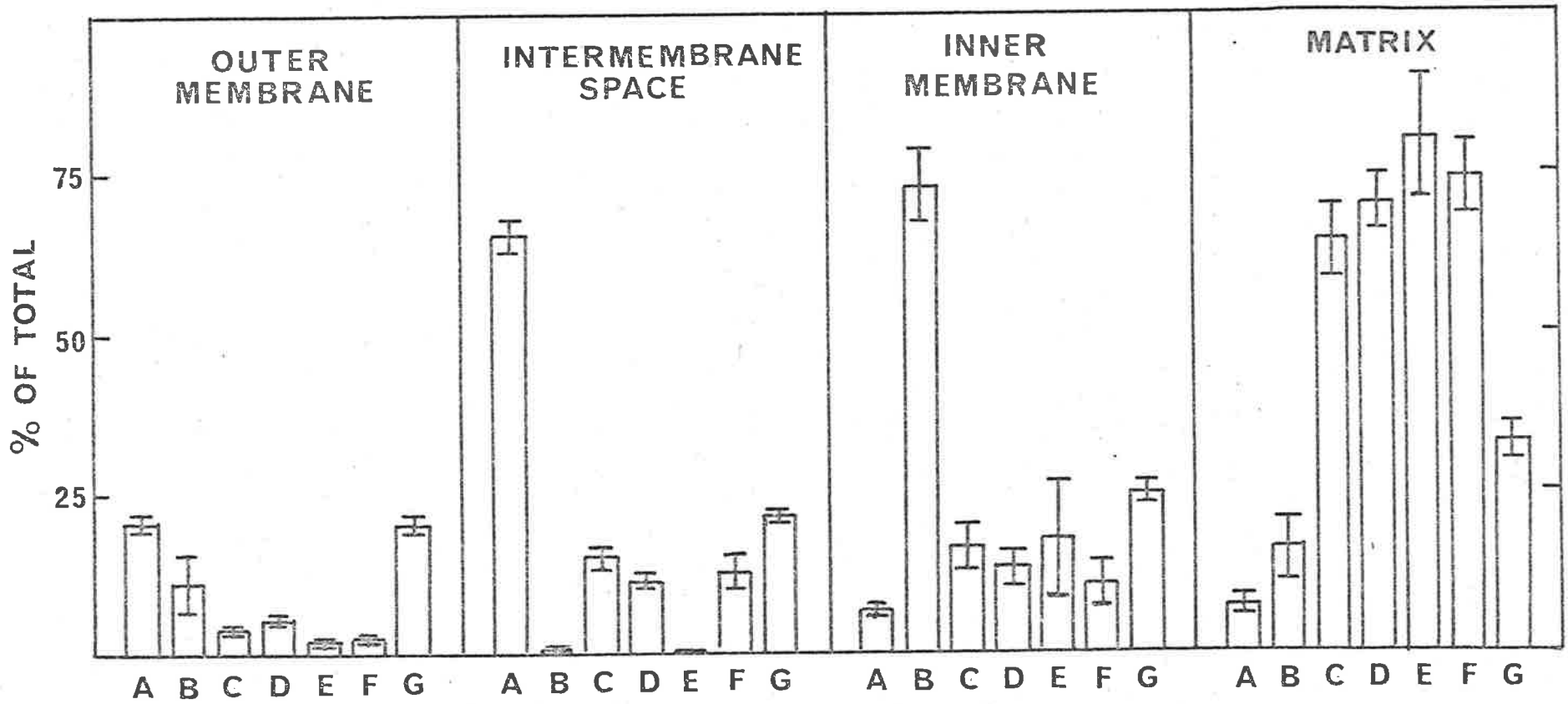


FIG. 4.2. Distribution of protein and enzymic activities in submitochondrial fractions. Mitochondria isolated from sheep liver were fractionated with digitonin (Section 4.2.(b)) and enzymic activities and protein were assayed as described in Section 4.2.(c). Each value is expressed as a percentage of the total and is the mean ± S.E.M. for four separate experiments. A, monoamine oxidase; B, succinate dehydrogenase; C, citrate synthase; D, glutamate dehydrogenase; E, pyruvate carboxylase; F, PEP carboxykinase; G, protein.

From Fig. 4.2 it can be seen that glutamate dehydrogenase, citrate synthase, pyruvate carboxylase and PEP carboxykinase have similar distributions and are located within the mitochondrial matrix. The activities of these enzymes found in the inner membrane fraction could arise from contamination by matrix protein and the presence of a few intact inner membrane complexes. There is also some activity of these enzymes in the intermembrane space fraction. After the removal of the outer membrane the inner membrane complexes are more fragile and any damage to the inner membrane could result in the loss of some of the matrix protein into the intermembrane space fraction. Nevertheless, the distribution of enzymes presented in Fig. 4.2 is in essential agreement with similar studies reported for other species (Sottocasa *et al.*, 1967; Bottger *et al.*, 1969). The recoveries of enzymic activity and total protein were almost quantitative throughout the fractionation procedure. It may be concluded therefore that in the sheep the mitochondrial activities of pyruvate carboxylase and PEP carboxykinase are localised within the matrix. Rat liver pyruvate carboxylase is also found within the mitochondrial matrix (Bottger *et al.*, 1969; Marco, Sebastian and Sols, 1969), and a similar site has been reported for both pyruvate carboxylase and PEP carboxykinase in mitochondria from pigeon liver (Landriscina *et al.*, 1970).

The mitochondrial activities of sheep liver pyruvate carboxylase and PEP carboxykinase are latent unless the mitochondria have been disrupted, *e.g.*, by sonicating or

freeze-drying. This suggests that the enzymes are separated from exogenous substrates by a permeability barrier, which is compatible with an inner membrane or matrix localisation as the outer membrane provides no significant permeability barrier to the substrates of these enzymes (Pfaff et al., 1968; Lardy and Ferguson, 1969). However, with the techniques available at present it is not possible to differentiate between enzymes loosely bound to the matrix surface of the inner membrane, and those in a soluble form in the matrix or in a protein reticular network in the matrix (Hackenbrock, 1968; Wrigglesworth, Packer and Branton, 1970).

(b) Intracellular distribution of pyruvate carboxylase and PEP carboxykinase in livers of normal, starved and diabetic sheep

Since glutamate dehydrogenase (Christie and Judah, 1953; Hogeboom and Schneider, 1953; Beaufay et al., 1959; Delbruck et al., 1959; Boyd, 1961) and citrate synthase (Fette, 1966) are exclusively mitochondrial in o ther species and have the same intramitochondrial localisation as pyruvate carboxylase and PEP carboxykinase (see Fig. 4.2) they were used as marker enzymes for mitochondrial breakage in subsequent cell fractionation experiments.

From Table 4.1 it can be seen that in normal sheep 90% of the pyruvate carboxylase activity in the hepatic cell is found in the mitochondrial fraction. As well as an increase in total activity in the liver of fasted animals there is also an increase in the proportion of the activity found in the

TABLE 4.1: INTRACELLULAR DISTRIBUTION OF ENZYMES IN LIVERS FROM NORMAL, FASTED AND DIABETIC SHEEP

Liver samples from normal, fasted and diabetic sheep were fractionated (Section 4.2.(a)) and the activities of pyruvate carboxylase, PEP carboxykinase, citrate synthase and glutamate dehydrogenase were assayed as described in Section 4.2.(c). The results are expressed as units per 10 g of liver (wet wt). The values given are the mean ± standard error of the mean for the number of observations indicated in parentheses. The results are shown for the mitochondria and cytosol, since, with the exception of glutamate dehydrogenase, the activities in the microsomal fraction were negligible. The extramitochondrial glutamate dehydrogenase activity was distributed between the cytosol and microsomal fractions, and it was assumed that this was due to the sedimentation of aggregated species of the enzyme with the microsomes. The activity was therefore included in the figure shown for the cytosol.

TABLE 4.1: INTRACELLULAR DISTRIBUTION OF ENZYMES IN LIVERS FROM NORMAL, FASTED AND DIABETIC SHEEP

		Mitochondria	Cytosol	% in Cytosol
PEP-carboxykinase				
Normal	(4)	1.50 ± 0.13	3.24 ± 0.65	66
Starved	(4)	0.92 ± 0.25	2.95 ± 0.53	76
Diabetic	(4)	2.24 ± 0.25	7.06 ± 1.00	76
Pyruvate carboxylase				
Normal	(4)	2.27 ± 0.47	0.25 ± 0.08	10
Starved	(5)	5.18 ± 1.44	2.46 ± 0.77	32
Diabetic	(4)	15.3 ± 3.0	9.29 ± 1.53	38
Glutamate dehydrogenase				
Normal	(4)	795 ± 70	170 ± 33	18
Starved	(5)	381 ± 48	203 ± 27	35
Diabetic	(4)	374 ± 46	306 ± 98	45
Citrate synthase				
Normal	(4)	22.8 ± 1.7	3.54 ± 0.28	13
Starved	(5)	10.1 ± 0.8	5.47 ± 0.52	35
Diabetic	(4)	10.9 ± 2.8	4.84 ± 0.87	31

TABLE 4.2: ANALYSIS OF VARIANCE FOR THE INTRACELLULAR DISTRIBUTION OF PYRUVATE CARBOXYLASE, GLUTAMATE DEHYDROGENASE AND CITRATE SYNTHASE IN SHEEP LIVER

Source	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Probability
NORMAL					
Between enzymes	2	104.4	52.2	3.09	* 0.05
Within enzymes	9	151.6	16.9		
Total	11	256.0			
FASTED					
Between enzymes	2	226.9	113.4	1.44	* 0.2
Within enzymes	12	946.3	78.9		
Total	14	1173.2			
DIABETIC					
Between enzymes	2	105.0	52.5	0.38	* 0.2
Within enzymes	9	1250.6	139.0		
Total	11	1355.6			

* indicates greater than.

cytosol from approximately 10% in the normal to 30% in the fasted liver. In diabetic animals there is an even greater increase in total activity, with approximately 40% of this activity in the cytosol fraction. However, analyses of variance (Table 4.2) indicate that the relative distributions between mitochondria and cytosol of pyruvate carboxylase and the marker enzymes, glutamate dehydrogenase and citrate synthase, are not significantly different within any of the three conditions. Although there are changes in the activities of glutamate dehydrogenase and citrate synthase in starvation and diabetes, these changes are not correlated with the changes in the activity of pyruvate carboxylase, so that it is unlikely that *in vivo* a similar increase is occurring in the proportion of each of these enzymes in the cytosol. In a sheep injected with dexamethasone (0.50 mg/kg body wt.) daily for five days, the total cellular activity of pyruvate carboxylase had decreased to about 20% of the activity found in normal sheep. Only 12% of the total pyruvate carboxylase, glutamate dehydrogenase and citrate synthase activities were isolated in the cytosol fraction. This indicated that there was no redistribution of pyruvate carboxylase when the activity was decreased during glucocorticoid treatment. It therefore appears that, *in vivo*, pyruvate carboxylase is exclusively a mitochondrial enzyme.

In Chapter 3 it was shown that there was a two-fold increase in the activity of PEP carboxykinase in liver homogenates of diabetic sheep. From Table 4.1 it can be seen that approximately

one-third of the activity is located in the mitochondria in the normal animal and that the activity increases in both fractions in diabetes. There is also a slight decrease in activity in the mitochondria and cytosol in the fasted animal. If mitochondrial contamination of the cytosol is taken into account for the data obtained for PEP carboxykinase, it is evident that approximately one-third of the total activity is mitochondrial under all three conditions.

From the data obtained for the mitochondrial marker enzymes during cell fractionation it is concluded that liver mitochondria from starved or diabetic sheep are significantly more susceptible to breakage during the cellular fractionation procedure. Matsubara and Tochino (1969) have reported that the mitochondria of alloxan diabetic rats show a slightly abnormal conformation and swelling of cristae. It was observed by Hall, Sordahl and Stefko (1960) that mitochondria from livers of diabetic rats and cats were larger and more fragile than normal mitochondria. Brech, Shrago and Wilken (1970) found, however, that the distribution and activity of pyruvate carboxylase was the same in the mitochondria and cytosol of normal and diabetic rats. The lack of any increase in the proportion of activity in the cytosol is in contrast to the results reported here for diabetic sheep where the mitochondria appear to be more fragile than those from diabetic rat liver.

(c) Electronmicroscopy

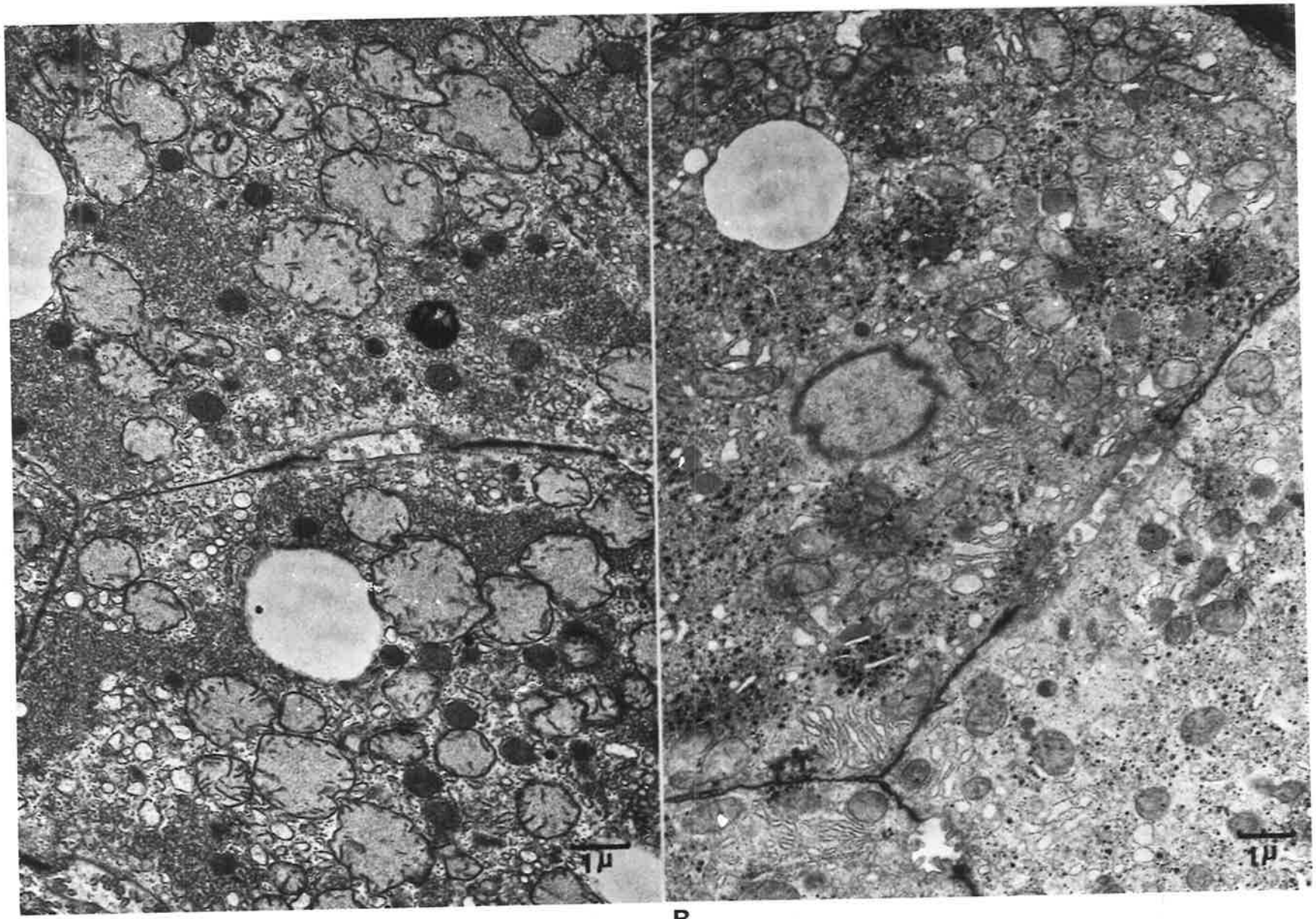
Sections from livers of normal, fasted and diabetic sheep were examined under the electronmicroscope to determine

whether there were any gross morphological differences in the mitochondria which could perhaps account for their increased fragility.

Fig. 4.3 shows a comparison of sections taken from the livers of normal and diabetic sheep. In the section from the diabetic sheep there are more fat droplets and fewer glycogen granules, but the most striking difference is that many of the mitochondria in sections taken from livers of diabetic sheep are several times their normal size. It seems likely that these mitochondria would be more susceptible to damage during homogenisation than those of normal size. This is substantiated in Fig. 4.4 which shows mitochondria isolated from normal and diabetic livers. The intact mitochondria are of comparable size while the diabetic preparation contains none of the larger mitochondria seen in the sections of diabetic liver. It does, however, contain a large amount of membrane fragments and membrane vesicles, presumably from disrupted mitochondria. It would be expected that matrix enzymes from these mitochondria would be isolated in the cytosol fraction. Sections of livers from fasted sheep contain mitochondria which, although not as large as thosein sections from diabetic liver, are considerably larger than normal. These changes are demonstrated more clearly by comparing the frequency distributions for the axial dimensions of the mitochondria in each of the three conditions (Fig. 4.5). The increase in size appears to occur more in the long axis than in the short axis, but for both these parameters the results are similar in that the mitochondria have increased more in size in diabetes

FIG. 4.3. Electronmicrographs of liver sections from (A) diabetic sheep and (B) normal sheep. The samples were prepared for electronmicroscopy as described in Section 4.2.(k)(i).

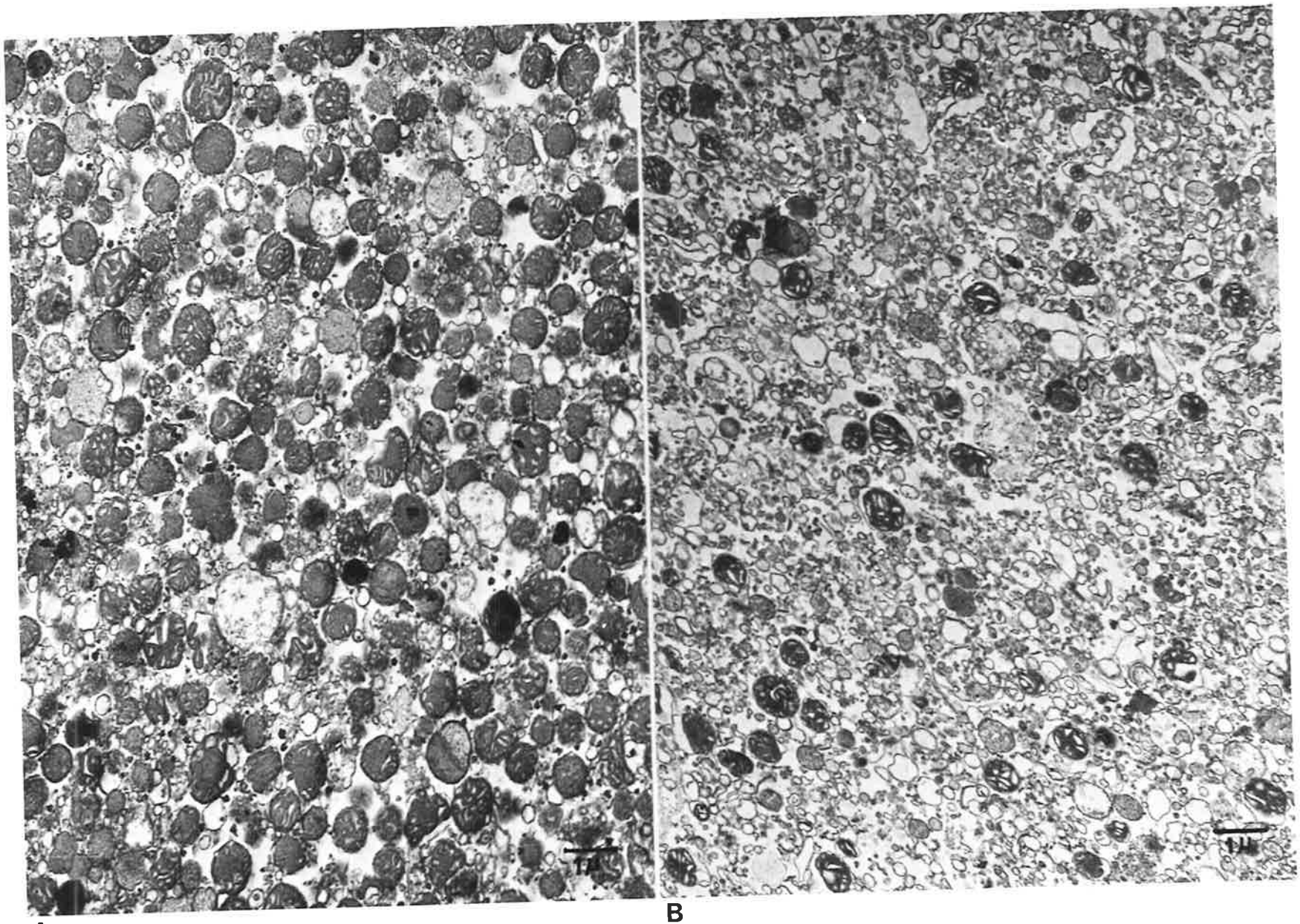
Magnification: 10,000 x.



A

B

FIG. 4.4. Electronmicrographs of mitochondria isolated from livers of (A) normal and (B) diabetic sheep. The mitochondria were isolated by the method described in Section 4.2.(a) and the mitochondrial pellet was treated as indicated in Section 4.2.(k)(ii). Magnification: 10,000 x.



A B

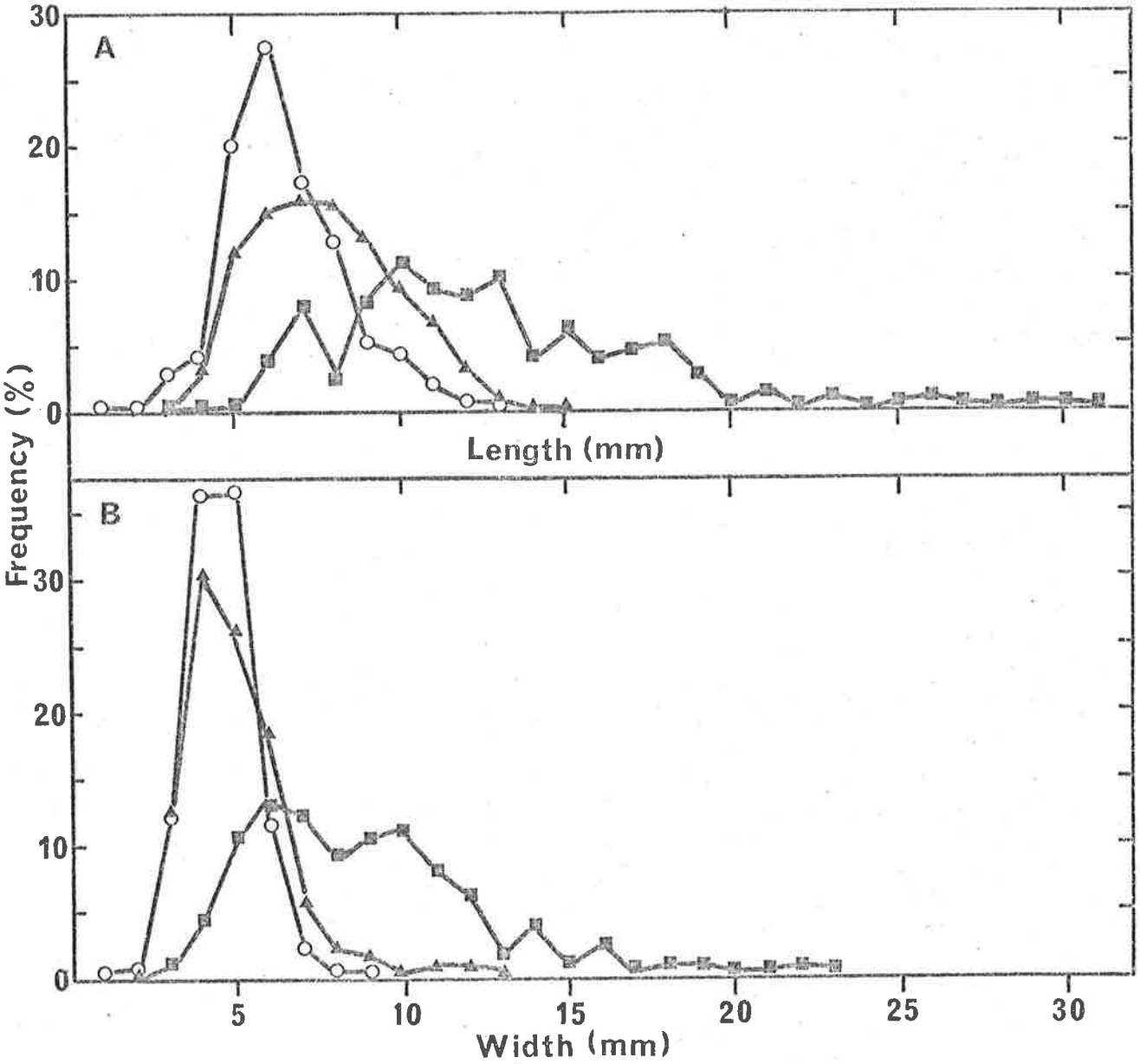


FIG. 4.5. Frequency distributions of the axial dimensions of the mitochondria in liver sections from normal, fasted and diabetic sheep. Tissue sections were prepared for electron microscopy as described in Section 4.2.(k). In photographic prints (magnification: 8,000 x) all the mitochondria were assessed with respect to the lengths (in mm) of the long axis (A) and of the short axis (B) (perpendicular to the long axis). Each curve represents the pooled results for several hundred mitochondria from at least 3 animals.
(o _____ o), normal; (▲ _____ ▲), fasted;
(■ _____ ■), diabetic.

than in fasting.

In biopsy sections from diabetic rat liver, Harano, DePalma and Miller (1969) observed branched and atypical mitochondria which showed an average increase in size of 60%. Although the size of the mitochondria in diabetic sheep liver sections is variable (see frequency distributions in Fig. 4.5) the axial dimensions of many of these mitochondria are two or three times greater than the corresponding dimensions of the mitochondrial in normal liver sections. This again suggests that the morphology of sheep liver mitochondria is more severely affected in diabetes than that of rat liver mitochondria.

(d) Immunochemical comparison of purified mitochondrial pyruvate carboxylase and pyruvate carboxylase from the cytosol fraction of sheep liver

In order to confirm the conclusion that the pyruvate carboxylase activity in the cytosol is due to increased fragility and subsequent disruption of enlarged mitochondria, the immunochemical properties of pyruvate carboxylases isolated from both sources were investigated.

It can be seen from the data presented in Fig. 4.6 that where a constant amount of each enzyme was incubated with increasing amounts of antibody prepared against the purifid mitochondrial enzyme, the antibody reacted equally well with the immunogen and with the enzyme isolated from the cytosol of diabetic liver. γ-Globulin obtained from the rabbit before immunisation had no effect on the enzymic activity.

When a constant amount of antibody was incubated with increasing amounts of mitochondrial and cytosol enzyme, the same amount of each enzyme was precipitated by the antibody and after the equivalence point, the enzymic activity was recovered quantitatively in the supernatant (Fig. 4.7). These data, supported by the Ouchterlony double diffusion analysis shown in Fig. 4.8, provide strong evidence that the mitochondrial and cytosol enzymes are immunologically identical.

Thus, these findings support the previous conclusion that the increased proportion of pyruvate carboxylase in the cytosol fraction from diabetic and starved animals is mitochondrial in origin.

4.4. DISCUSSION

(a) Pyruvate carboxylase

The results presented here indicate that the liver pyruvate carboxylase in normal sheep is exclusively mitochondrial. Throughout both the cellular and mitochondrial fractionations, the distribution of pyruvate carboxylase was similar to that of the mitochondrial enzymes, citrate synthase and glutamate dehydrogenase. Thus, it may be concluded that the total cell activity of the enzyme is confined to the mitochondrial matrix. These findings are in agreement with the observations of Bottger *et al.* (1969) and at variance with those of Henning *et al.*, (1966). Henning and coworkers report that up to 40% of the total pyruvate carboxylase in rat liver can be isolated in the cytosol fraction if acetyl-

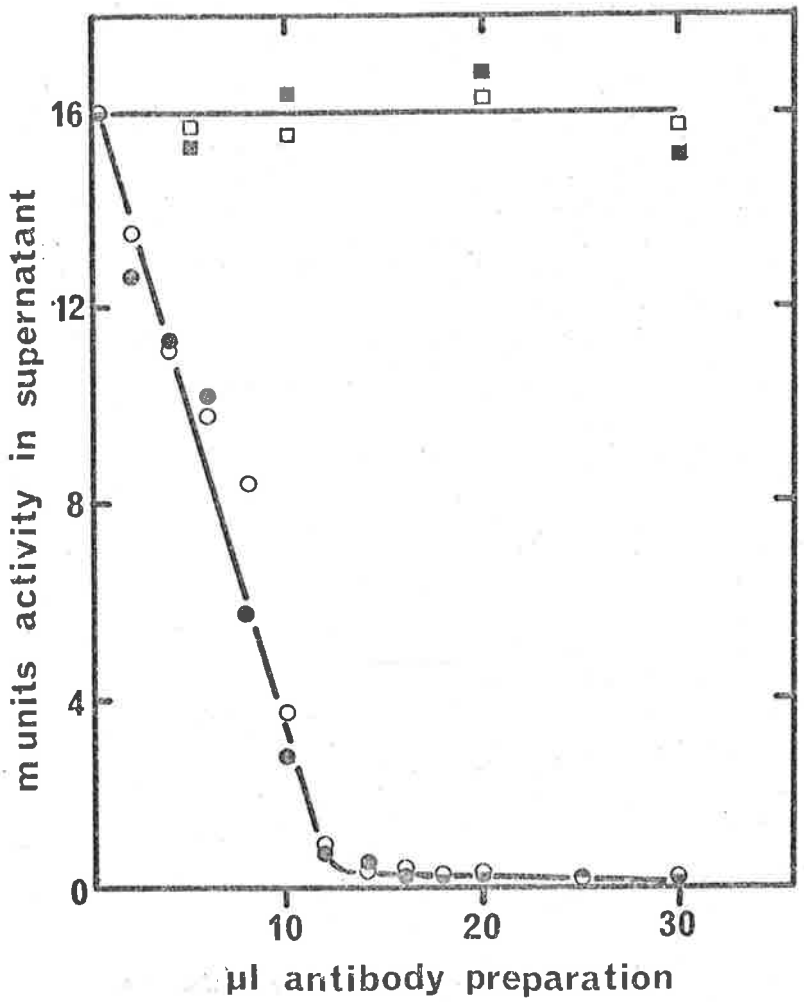


FIG. 4.6. Titration of purified mitochondrial pyruvate carboxylase and pyruvate carboxylase from cytosol of diabetic liver against antibody prepared with mitochondrial enzyme as the antigen. Enzyme (16 munits) was incubated with increasing amounts of antibody as described in Section 4.2.(g), and the supernatant obtained after centrifuging the incubation mixture was assayed for residual enzymic activity. (● ——— ●), purified mitochondrial pyruvate carboxylase plus immune γ-globulin; (■ ——— ■); purified mitochondrial pyruvate carboxylase plus preimmune γ-globulin; (o ——— o), partially purified cytosol pyruvate carboxylase plus immune γ-globulin; (□ ——— □), partially purified cytosol pyruvate carboxylase plus preimmune γ-globulin.

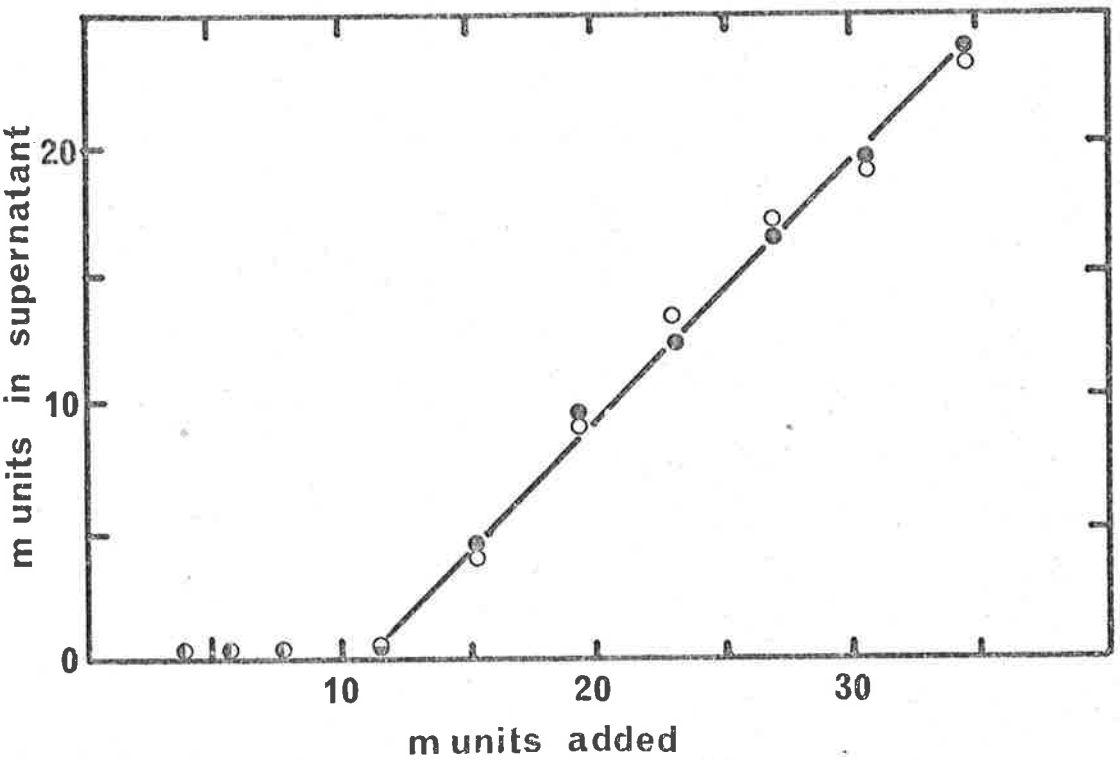
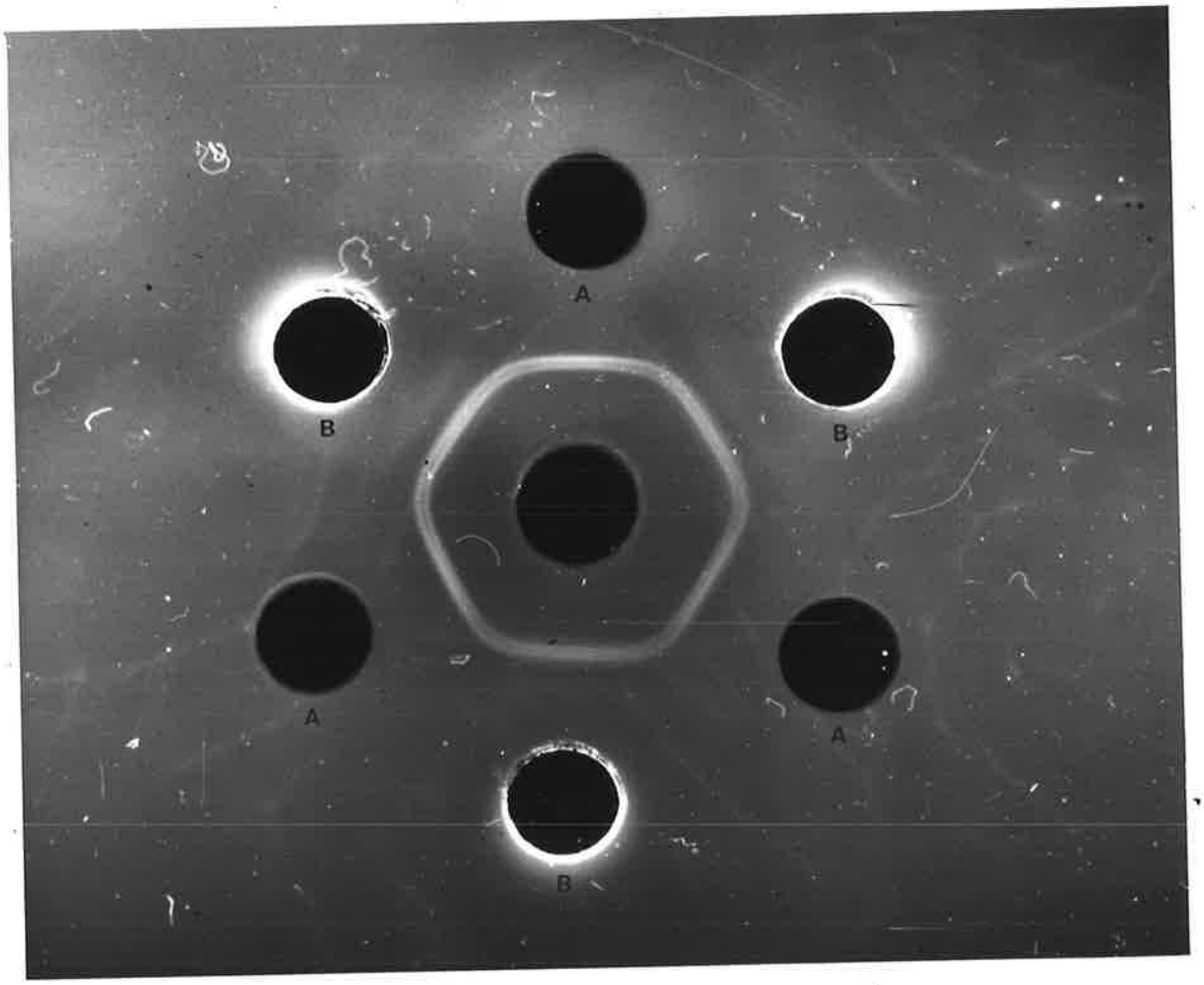


FIG. 4.7. Titration of an antibody preparation against pyruvate carboxylase from mitochondria of normal liver (● ——— ●) and cytosol of diabetic liver (o ——— o). Antibody preparation (8µl) was included in each incubation mixture with increasing amounts of enzyme up to a total of 35 munits (Section 4.2.(g)).

FIG. 4.8. Ouchterlony double diffusion analysis of mitochondrial pyruvate carboxylase antibody, mitochondrial pyruvate carboxylase from normal liver, and cytosol pyruvate carboxylase from diabetic liver.

The centre well contained 50 µl of antibody and the outer wells contained 30 m units of (A) mitochondrial pyruvate carboxylase and (B) cytosol pyruvate carboxylase. The plates were developed for 48 hr in a humidified chamber at room temperature prior to photography.


A
B
B
A
A
B

CoA or monovalent cations are included in the homogenising medium. They suggest that this enzyme is loosely bound to the outside surface of the mitochondria and hence functions in the cytosol. If such a situation existed in sheep liver, significant pyruvate carboxylase activity should have been observed in the outer membrane or intermembrane space fractions during mitochondrial fractionation. Additional evidence for an exclusively mitochondrial localisation of pyruvate carboxylase in rats has been presented by Ballard, Hanson and Reshef (1970). In immunochemical titration experiments similar to those reported in this chapter, they found that there were no differences between the soluble and mitochondrial activities of pyruvate carboxylase from various rat tissues. They concluded that the soluble activities of pyruvate carboxylase detected in many rat tissues must be the result of mitochondrial damage during tissue homogenisation.

Although the level of pyruvate carboxylase activity in sheep liver increases several fold during diabetes and starvation, the proportions of the enzyme in the mitochondrial and cytosol fractions are still the same as for the two marker enzymes. Furthermore, from the immunochemical studies it is evident that in the diabetic and starved states, the total cell content of pyruvate carboxylase is immunologically homogeneous. Therefore, the observed increases in the level of pyruvate carboxylase following these perturbations must be confined to the mitochondria.

(b) PEP carboxykinase

In sheep liver, PEP carboxykinase occurs in both the cytosol and the mitochondria but it is not yet known whether these activities are identical. In other species, these two isoenzymes have been shown to be distinct immunologically (Ballard and Hanson, 1969) and kinetically (Holten and Nordlie, 1965) and differ in their responses to gluconeogenic stimuli (Lardy et al., 1964; Lardy, 1965; Nordlie, Varrichio and Holten, 1965). However, sheep liver PEP carboxykinase is unusual in two respects: (a) although diabetes causes a two to three fold increase in enzymic activity, there is a slight decrease in the fasted animal, and (b) in each condition the cytosol and mitochondrial enzymes behave in an identical manner. In the livers of sheep, pigs (Sviatek et al., 1970) and guinea pigs (Nordlie and Lardy, 1963) where there is a high level of PEP carboxykinase in both the cytosol and mitochondria, it is not clear what purpose is served by the dual occurrence. In other species where this enzyme is found predominantly in one cell compartment, it would seem that PEP synthesis for gluconeogenesis occurs in that particular compartment. It has been speculated (Smith and Osborne-White, 1969) that in the sheep, where propionate is normally a major substrate for gluconeogenesis, the malate resulting from the metabolism of propionate in the mitochondria may be converted to PEP by malate dehydrogenase and PEP carboxykinase in the cytosol. Reducing requivalents would therefore be generated in the cytosol where they are required for subsequent steps

in the synthesis of glucose. On the other hand, when lactate is the precursor for gluconeogenesis, the mitochondrial PEP carboxykinase may be responsible for PEP synthesis, since $NADH_2$ would be generated in the cytosol during the lactate dehydrogenase step. Although Barns (1970) has suggested that the oxaloacetate inhibition of sheep kidney mitochondrial PEP carboxykinase may be indicative of an anaplerotic role for this enzyme, the similar responses of the hepatic mitochondrial and cytosol activities to starvation and diabetes would tend to suggest that both enzymes participate in gluconeogenesis. Despite this apparently coordinate control of synthesis of both PEP carboxykinase activities by gluconeogenic stimuli, their responses to these are independent of the factors controlling the level of pyruvate carboxylase (see Section 3.3).

(c) Mitochondria

A rather interesting feature emerging from this study is the behaviour of the liver mitochondria in sheep under conditions of physiological perturbation. Other tissues, viz., kidney cortex, skeletal muscle and cardiac muscle have been examined under similar conditions. The mitochondria tended to be more variable in size even in sections from normal animals and hence any changesin size resulting from the treatments were less readily detected. There was some indication of an increase in the size of kidney mitochondria in diabetic sheep but the changes were much less obvious than in the liver. Changes in mitochondrial size comparable in magnitude with those

found in sheep liver do not seem to have been reported for diabetic animals of other species.

In a sheep treated with dexamethasone the mitochondria were enlarged, but the low proportion of the activities of mitochondrial enzymes isolated in the cytosol fraction suggests that these mitochondria were similar to those from normal liver with respect to fragility. It has been demonstrated by Wiener et al. (1968) that although the liver mitochondria in cortisone treated rats are larger than normal, a comparison of various characteristics of the mitochondria in liver sections from normal and glucocorticoid treated rats reveals that on a relative basis the mitochondria are identical. This may also be true for the mitochondria in sheep treated with glucocorticoids, but the mitochondria in livers of fasted and diabetic sheep appear to be swollen and are unduly fragile during their isolation. In fasted and diabetic sheep the liver is infiltrated with vast quantities of lipid which may affect the mitochondria. Conversely, in glucocorticoid treated sheep there appears to be no mobilisation of lipid and the mitochondria are not abnormally fragile. Matsubara and Tochino (1969) have observed that liver mitochondria from alloxan diabetic rats exhibit decreased respiratory control and P/O ratios and contain 2 - 3 times the normal level of free fatty acids. They suggest that the fatty acids produce mitochondrial conformational changes which cause the uncoupled respiration. In adrenalectomised, thyroidectomised or hypop hysectomised rats, neither hyperlipaemia nor fatty liver

occurred after the administration of alloxan and the mitochondria appeared to be normal (Matsubara and Tochino, 1970).

It should therefore be interesting to determine whether an infusion of fatty acids in sheep has any effect on the ultrastructure of the hepatic mitochondria. At the present time the enlarged mitochondria cannot be isolated satisfactorily from the livers of diabetic sheep and hence the properties of these mitochondria have not yet been examined *in vitro*.

CHAPTER 5

STUDIES ON THE CHANGES IN THE ACTIVITIES OF PYRUVATE CARBOXYLASE AND PEP CARBOXYKINASE IN FASTED AND DIABETIC SHEEP

5.1. INTRODUCTION

In Chapter 4 it was shown by the use of appropriate marker enzymes during cell fractionation experiments and by immunochemical techniques that the marked increase in the activity of pyruvate carboxylase in the livers of fasted and diabetic sheep is confined to the mitochondria *in vivo*. This chapter presents information obtained from an investigation of the means whereby the activity of pyruvate carboxylase is increased.

An increase in the activity of an enzyme could be effected by

(1) activation of preexisting enzyme protein so that the catalytic efficiency per enzyme molecule is increased.

Possible mechanisms by which this could occur are:

(a) activation of the enzyme by a positive effector. An activation of this nature is unlikely to be detected in an *in vitro* assay system under optimum conditions.

(b) formation of active enzyme from inactive enzyme by enzymically catalysed chemical modification of the enzyme protein e.g., phosphorylation of liver and muscle phosphorylase (Sutherland and Wosilait, 1955; Helmreich and Cori, 1964), dephosphorylation of pyruvate dehydrogenase (Linn, Pettit and Reed, 1969), adenylation of *E. coli* glutamine synthetase (Mecke and Holzer, 1966; Shapiro and Stadtman, 1968).

(c) conversion of apoenzyme to holoenzyme by incorporation of a prosthetic group e.g., incorporation of haem into yeast

apocytochrome oxidase (Tuppy and Birkmayer, 1969) and of biotin into rat liver pyruvate apocarboxylase (Deodhar and Mistry, 1969a,b) and rat adipose tissue acetyl-CoA carboxylase (Jacobs, Kilburn and Majerus, 1970).

(2) An increase in the amount of active enzyme protein present as a result of a change in the rate of degradation or synthesis de novo. In some instances the rates of both processes may be altered.

The methods available for studying the turnover of proteins have been reviewed recently by Schimke (1970) and will only be described briefly here.

There are many examples in the literature where the effects of inhibitors of RNA or protein synthesis on the levels of enzymes have been tested. Abolition of the increase in the level of the enzyme could tentatively be interpreted as evidence for the participation of de novo protein synthesis but it is well known that such drugs may have multiple and indirect actions (Revel, Hiatt and Revel, 1964; Henig and Rabinovitz, 1965; Laszlo et al., 1966; Soeiro and Amos, 1966; Pastan and Friedman, 1968; Stewart and Farber, 1968). It is therefore difficult to be certain whether the drug has affected RNA or protein synthesis specifically, or whether it has affected some other metabolic function which in turn has a secondary effect on the observed enzymic activity.

A more direct method of distinguishing between alternatives (1) and (2) is the use of immunochemical techniques

to measure the relative content of enzyme protein under varying conditions. An increase in the amount of immunochemically reactive enzyme protein may be regarded as evidence for a decrease in the rate of degradation or an increase in the rate of synthesis de novo of the enzyme protein. However, similar results would be obtained if there existed inactive preformed enzyme or apoenzyme which reacted less efficiently with the antibody than the active enzyme used as an antigen. It is unlikely that the differences between active and inactive forms of an enzyme would be sufficient to cause differences in their immunochemical reactivity but this possibility cannot be ignored. More convincing results may be obtained by combining immunochemical and radioisotope techniques to demonstrate both an increased content of immunochemically reactive protein and a net uptake of radioactive isotope in specific enzyme protein. The relative rate of synthesis of the enzyme protein may also be measured by this approach. The rate of degradation may be estimated from the decay of radioactivity after the single administration of a labelled precursor, but the results may be confounded by the extensive reutilisation of isotopic amino acids.

Methods involving the use of inhibitors orradioactive amino acids need careful consideration before their use in an animal the size of the sheep. Furthermore, a preliminary study of the time course of the increase in the activity of pyruvate carboxylase in diabetes suggests that this increase occurs relatively slowly. Large quantities of the compounds would be required for their repeated administration over a period

of several days. In addition, the slow increase in activity would enhance the possibility that during this time drugs could affect other processes which in turn could affect the level of pyruvate carboxylase activity, so that a secondary effect on pyruvate carboxylase activity may be observed. Therefore, it was decided to adopt initially the approach of determining whether the increased activity of pyruvate carboxylase was, in fact, associated with an increase in the amount of immunochemically reactive enzyme protein. The fact that positive results were obtained indicates that the use of a confirmatory and possibly a more definitive approach is warranted in future experiments to study the mechanism by which the change in the level of pyruvate carboxylase occurs.

5.2. METHODS

(a) Animals

Sheep were subjected to the following treatments:

(i) Normal sheep - fed 600 g of chaffed wheaten hay and 400 g of chaffed lucerne hay daily

(ii) Fasted sheep - fasted for 5 days before slaughter

(iii) Alloxan diabetic sheep - 2 sheep were injected with 60 mg of alloxan/kg body wt. In one of the animals the plasma glucose concentration was 250 mg/100 ml, but the ketone body concentration was normal. In the other animal the concentrations of both glucose and ketones were elevated to levels of 550 mg/100 ml of plasma and 14 mg/100 ml, respectively.

(iv) "Stabilised" diabetic sheep: A sheep was made diabetic by an injection of alloxan (60 mg/kg body wt.). Within 2 days the plasma glucose concentration had increased from 36 mg/100 ml to 225 mg/100 ml. After the diabetic condition had been allowed to develop for about 5 days the glucose level was 225 mg/100 ml of plasma and the total ketone body concentration had risen from 3 mg/100 ml of plasma to 24 mg/100 ml. A continuous intravenous insulin infusion of 5 units/24 hr reduced the plasma glucose concentration to 150 mg/100 ml of plasma and the ketone body concentration to the normal level. This treatment was continued for 6 days. Ten units of insulin per 24 hr for the following 14 days maintained the plasma glucose level at 25-30 mg/100 ml of plasma and the ketones at 3 mg/100 ml. Within 24 hr after the termination of the insulin infusion the glucose concentration had increased to 230 mg/100 ml of plasma and the ketones to 6 mg/100 ml, while 72 hr after the withdrawal of insulin the glucose level was 264 mg/100 ml and the ketones had increased to 9 mg/100 ml.

(b) Surgical procedure

An intravenous injection of pentothal was used for the initial induction of anaesthesia. The trachea was intubated with an endotracheal tube and anaesthesia was maintained with a fluothane-oxygen mixture.

An incision parallel to the costal margin of the ribs was made, the lateral lobe of the liver was exposed and a small

triangular wedge was excised from the periphery. The incised area was repaired with a plug of haemostatic sponge and sutured.

(c) Enzyme assays

Liver homogenates (25%, w/v) were prepared at 2° in 0.25 M sucrose containing 0.1 mM EDTA, 0.5 mM GSH and 0.02 M tris-Cl (pH 7.2). The homogenate was sonicated for four periods each of 10 sec., diluted ten-fold in the sucrose medium and then assayed for PEP carboxykinase and pyruvate carboxylase activities as described in Sections 2.5 and 2.6(a), respectively.

(d) Antibody titration experiments

(i) Preparation of tissue extract

Sonicated liver homogenates were prepared as described in (c) above. This treatment completely solubilised the pyruvate carboxylase activity (see Section 2.8) and the supernatant obtained after centrifuging at 105,000 x g for 30 min. was used for immunochemical studies.

(ii) Preparation of antibody

Purified sheep liver pyruvate carboxylase of specific activity 29 units/mg of protein was used for the preparation of antibody which was treated as described in Section 4.2.(f).

(iii) Incubation system

In the experiments where the amount of antibody was varied against a constant amount of tissue extract the incubation mixture (total volume 0.30 ml) contained 150 µl of a mixture containing 200 mM tris-Cl (pH 8.4), 16 mM $MgCl_2$, 5 mM ATP, 0.7 mM acetyl-CoA and 20 mM $NAHCO_3$; antibody in 100 µl of 0.15 M KCl containing 0.02 M potassium phosphate (pH 7.2); and 50 µl of liver extract.

In the experiments where the amount of tissue extract was varied against a constant amount of antibody, the incubation mixture was as described above, except that the antibody was added in 50 µl of 0.15 M KCl containing 0.02 M potassium phosphate (pH 7.2), and the enzyme was added in 100 µl of the sucrose medium.

The mixture was incubated at 23° for 60 min. and then centrifuged at 700 x g for 10 min. Aliquots of the supernatant (50 µl) were taken for assay in the system described in Section 2.6(a).

5.3. RESULTS

(a) Time course of the increase in the activity of pyruvate carboxylase and PEP carboxykinase with the onset of diabetes

In an alloxan diabetic sheep treated with a continuous intravenous infusion of insulin the plasma glucose concentration can be maintained within normal limits (50 - 60 mg/100 ml of plasma) (O.H. Filsell and I.G. Jarrett, unpublished observations). After the withdrawl of insulin the plasma glucose concentration increases rapidly and reaches a diabetic level of 120 - 180 mg/100 ml of plasma within 2 to 3 hr. A system such as this would be useful for an investigation of the time course of the changes in the hepatic enzyme content if biopsy samples of the liver could be obtained without undue difficulty or physiological complications.

In a preliminary experiment it was found that when

liver biopsy samples were removed under anaesthesia from a normal sheep and from a sheep in which the diabetic state was uncontrolled, the values obtained for the activities of pyruvate carboxylase and PEP carboxykinase were in agreement with those reported in Section 3.3. This method of sampling the liver is therefore a valid approach at least with respect to the effects of anaesthesia on the activities of the enzymes.

In an investigation of the time course of induction of pyruvate carboxylase and PEP carboxykinase with the onset of diabetes, a liver sample was taken from a stabilised diabetic sheep (t = 0), the insulin infusion was terminated 24 hr later (t = 24), and subsequent liver samples were removed 24 hr (t = 48) and 72 hr (t = 96) after the withdrawal of insulin. Three liver samples were taken from a normal sheep (t = 0, 96 and 144 hr).

The results for the time course experiment are shown in Fig. 5.1. At the beginning of the experiment (t = 0) the activities of pyruvate carboxylase and PEP carboxykinase in the normal sheep agreed well with those generally found in the normal sheep (see Section 3.3(a)). The slight increase in the activities of both enzymes by the end of the experiment may be attributed to the stress of the repeated surgical procedures and to partial starvation, since by the end of the experiment the animal was not eating all of the normal ration provided.

In the alloxan diabetic sheep stabilised with the insulin infusion (t = 0) the plasma concentrations of glucose (36 mg/100 ml of plasma) and ketones (3 mg/100 ml)

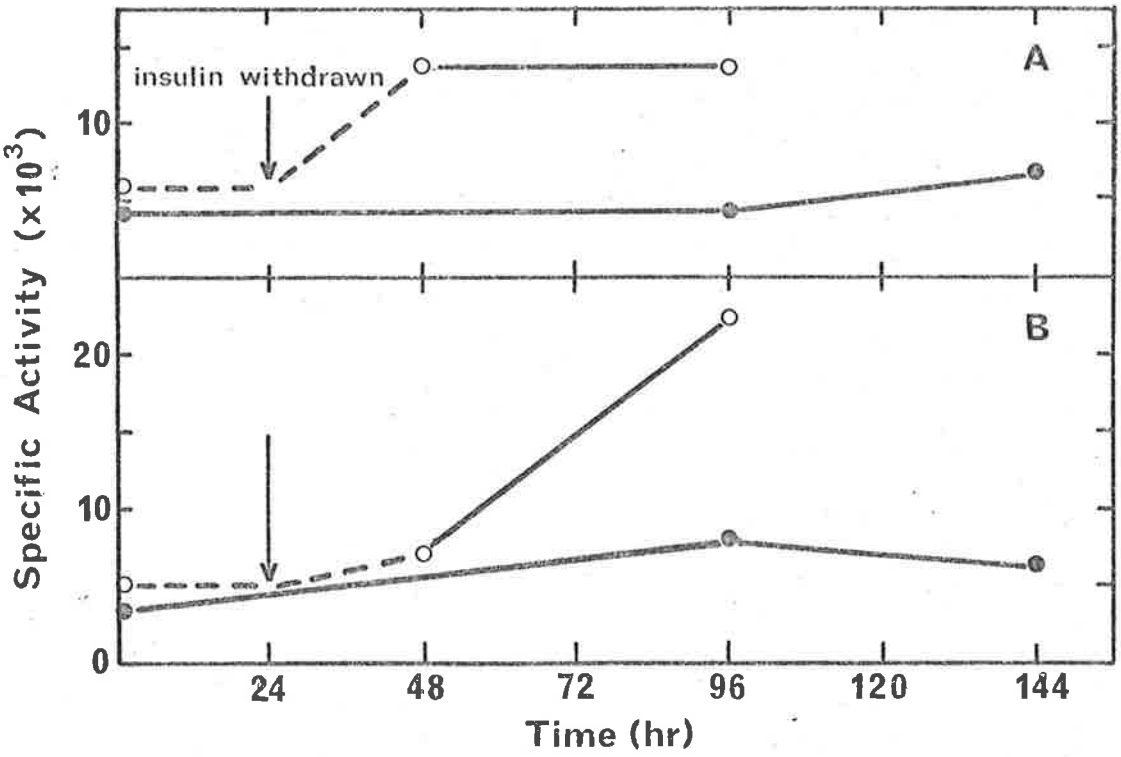


FIG. 5.1. Time course of the increase in the hepatic activity of pyruvate carboxylase and PEP carboxykinase with the onset of diabetes. Liver samples were removed under anaesthesia from a stabilised diabetic sheep before and after the withdrawal of the insulin infusion, at the times indicated (Section 5.2.(a) and (b)). Liver samples were also taken from a normal sheep as indicated. The activities of pyruvate carboxylase and PEP carboxykinase in the liver samples were determined as described in Section 5.2.(c). The results are expressed as specific activity (μmoles of CO_2 fixed per min. per mg of protein).

were within the normal range. However, the diabetic state was not completely controlled as the activities of both enzymes were slightly higher than in the normal animal, in spite of the fact that the stabilised diabetic animal was eating normally. It was also observed in electronmicrographs of liver sections from the stabilised diabetic sheep that the mitochondria were slightly enlarged. Nevertheless, the diabetic state was controlled as assessed by the return to normal levels of such physiological parameters as plasma levels of glucose, ketone bodies and non-esterified fatty acids. Within 24 hr (t = 48) after the withdrawal of insulin, the activity of PEP carboxykinase had increased to the level usually seen in chronically diabetic sheep. The plasma glucose level had increased to 230 mg/100 ml, which is comparable with the level before the commencement of the insulin infusion, and the ketone concentration had increased to 6 mg/100 ml of plasma. The rapid increase in the glucose concentration could be due either to inhibition of glucose utilisation by peripheral tissues or to an increase in the rate of gluconeogenesis. The increase in PEP carboxykinase activity which was evident 24 hr after the withdrawal of insulin suggests that the rate of gluconeogenesis had increased by this time. However, very little change in the activity of pyruvate carboxylase was apparent. Although 72 hr (t = 96) after the withdrawal of insulin the plasma glucose concentration had increased still further to 264 mg/100 ml, the ketone body concentration of 9 mg/100 ml was still considerably less than the level of 24 mg/100 ml observed before the insulin infusion. PEP carboxy-

kinase showed no further increase at this time and pyruvate carboxylase had attained the usual diabetic level.

(b) Antibody titration experiments

Fig. 5.2 shows the results of experiments in which a constant amount of tissue extract from sheep under different conditions of physiological perturbation was titrated against an increasing amount of antibody prepared against purified mitochondrial pyruvate carboxylase from the livers of normal sheep. The amount of antibody required to precipitate completely the enzymic activity in a given amount of liver extract, as estimated by the extrapolation of the linear portion of the titration curves to zero activity, is proportional to the amount of catalytic activity present in the absence of antibody (Fig. 5.2 inset).

When the experiments were carried out with a constant amount of antibody in the presence of increasing amounts of tissue extract, it can be seen that although the level of pyruvate carboxylase activity per g of liver varied over a six-fold range, the equivalence point was the same for all types of liver extract when based on the amount of enzymic activity added (Fig. 5.3).

Thus, under all of the conditions tested, the increase in the activity of pyruvate carboxylase is associated with a proportionate increase in the hepatic content of immunochemically reactive enzyme protein.

It is possible that the liver from normal sheep may contain apoenzyme or proenzyme which does not react with the

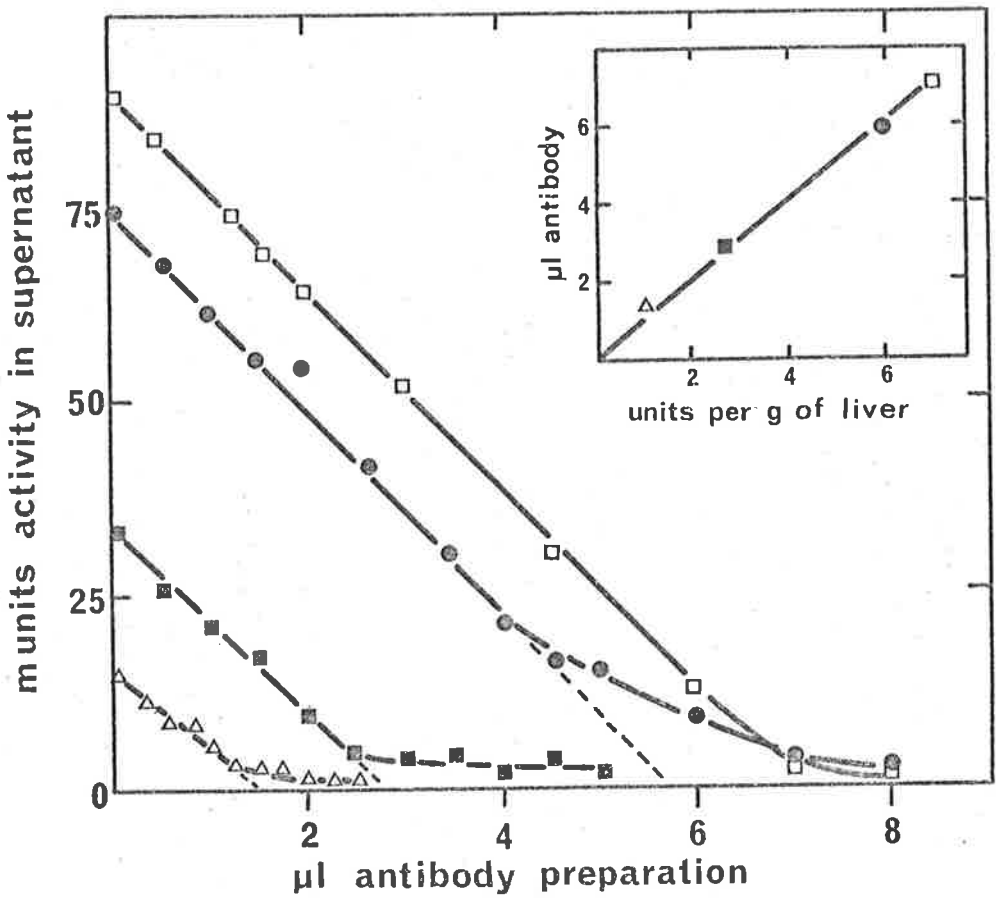


FIG. 5.2. Titration of liver extract from normal, fasted and diabetic sheep with antibody prepared against pyruvate carboxylase purified from the liver of normal sheep. Inset: µl of antibody required to precipitate completely (as estimated by extrapolation) the activity in each extract, expressed in terms of units/g wet wt (50 munits of activity/50 µl of extract is equivalent to 4 units/g wet wt of liver). Liver extract (50 µl) was incubated with antibody as described in Section 5.2.(d) (iii), and the supernatant obtained after centrifuging was assayed for residual enzymic activity. (△ ——— △), normal; (▪ ——— ▪), diabetic, not ketotic; (● ——— ●), fasted; (□ ——— □), diabetic.

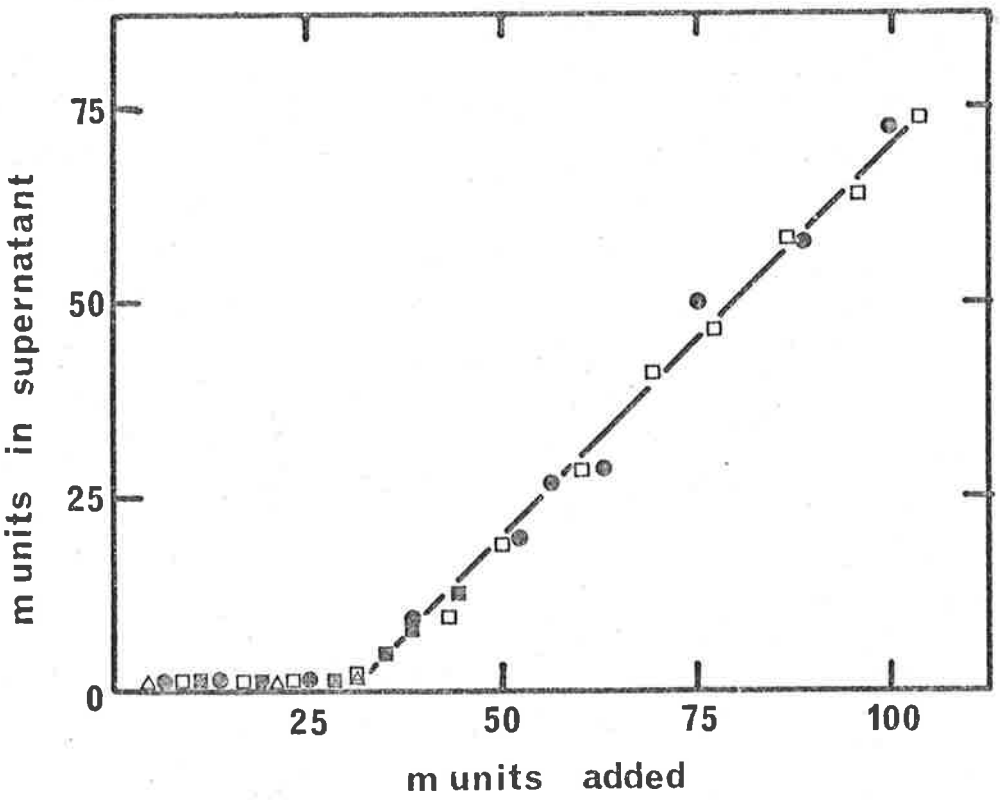


FIG. 5.3. Titration of a constant amount of antibody against increasing amounts of liver extract from normal, fasted and diabetic sheep. Liver extract was incubated with antibody as described in Section 5.2.(d) (iii) and the supernatant obtained after centrifuging was assayed for residual enzymic activity.
(△ ——— △), normal; (■ ——— ■), diabetic, not ketotic; (● ——— ●), fasted; (□ ——— □), diabetic.

antibody. In liver from biotin deficient rats (Deodhar and Mistry, 1969a,b) and chickens (Madappally and Mistry, 1970a) there is apoenzyme such that normal levels of holoenzyme may be synthesised upon the addition of biotin. It is therefore feasible that the increased amount of active enzyme found in fasted and diabetic sheep could represent the synthesis of holoenzyme by incorporation of biotin into preformed apoenzyme.

Attempts to detect the presence of apoenzyme in sheep liver have been unsuccessful with experiments conducted in vivo and in vitro.

(1) There is no difference between the levels of pyruvate carboxylase in the livers of normal sheep and of sheep which have received an intramuscular injection of 400 µg of biotin once every two days to a total of five injections. While this finding suggests that it is unlikely that the full expression of pyruvate carboxylase activity in the normal sheep is limited by a deficiency of biotin, it does not eliminate the possibility that under normal conditions the holoenzyme synthetase is controlled in some way to prevent incorporation of biotin into apoenzyme.

(2) The incubation of a normal sheep liver homogenate with biotin in the system described by Kosow and Lane (1961) failed to reveal the presence of any apoenzyme (L.A. Penberthy, J.C. Wallace and D.B. Keech, unpublished observations).

It therefore appears unlikely that apoenzyme, lacking biotin, is present although pyruvate carboxylase may exist as a proenzyme in some other form.

5.4. DISCUSSION

The results obtained for the time course experiment suggest that this system has potential as a tool for investigating metabolic changes in the sheep with the onset of diabetes. In the present experiment the results were influenced by complications arising from the repeated surgery and it may be preferable to use a series of different animals for the time curves. An investigation of the time course of the changes in mitochondrial size was attempted in conjunction with the assays for enzymic activity, but the results were inconclusive. However, with modifications to the experimental design, more meaningful results should be obtained.

The rapid increase in the activity of PEP carboxykinase which occurs after the withdrawal of insulin is comparable with the response of this enzyme in rat liver following physiological perturbations. In contrast, the response of pyruvate carboxylase is relatively slow, although the total increase in the activity of this enzyme is ultimately the greater.

The proportionality between the catalytic activity of pyruvate carboxylase and the immunochemically estimated enzyme protein demonstrates that the catalytic efficiency of the pyruvate carboxylase molecule, as measured *in vitro* under optimum conditions, has not been altered despite the wide variations in the levels of activity in liver extracts from sheep under different conditions. The changes in activity are actually determined by altered quantities of the enzyme protein in the liver. However, this finding does not exclude the possibility that control by transient changes in the catalytic efficiency per

enzyme molecule due to changes in the concentrations of various metabolites is also involved in the acute regulation of pyruvate carboxylase activity *in vivo*. The time course experiment suggests that the changes in the enzyme content occur relatively slowly. Thus, the control by inhibition or activation of the pyruvate carboxylase activity may play an important role when the rate of oxaloacetate synthesis must be adjusted rapidly, whereas the control by changes in the quantity of enzyme may make a greater contribution to the long term regulation.

It is interesting to note that there is no direct relationship between the plasma glucose concentration and the maximum assayable activity of pyruvate carboxylase in sheep treated with alloxan. In the stabilised diabetic animal the plasma glucose level was within the normal range, but the level of pyruvate carboxylaseactivity was a little higher than normal. On the other hand, in the immunochemical titration experiments one of the sheep had been treated with alloxan, and had a plasma glucose concentration of about 250 mg/100 ml, but the pyruvate carboxylase activity was only a little higher than in the normal animal. The plasma glucose concentration increased rapidly after the withdrawal of insulin from the stabilised diabetic sheep, and the relatively slow increase in the activity of pyruvate carboxylase which occurred is consistent with the lack of a direct relationship between the plasma glucose concentration and the hepatic activity of pyruvate carboxylase.

As an extension of this investigation, the mechanisms whereby the amount of immunochemically reactive pyruvate

carboxylase protein is increased should be determined. There are two means by which this increase could occur:

(1) As was discussed earlier (Section 5.3.(b)) the liver of normal sheep may contain apoenzyme which does not react with the antibody. From the evidence presented it is unlikely that apoenzyme lacking biotin is present, and unless pyruvate carboxylase exists as a proenzyme in another form, the increase in the activity of the enzyme cannot be accounted for by the activation of pre-existing enzyme.

(2) The more likely alternative is that the increased amount of enzyme protein is the result of changes in the rates of degradation or synthesis de novo of the enzyme protein.

In studies on acetyl-CoA carboxylase in rat liver it has been shown that the alterations in catalytic activity under various conditions are determined by changes in the quantities of enzyme protein (Majerus and Kilburn, 1969; Nakanishi and Numa, 1970). Furthermore, ^{14}C-leucine incorporation studies in vivo have indicated that in fasted and refed rats, or in rats suffering from diabetes, the change in activity is due to an increase or decrease, respectively, in the rate of synthesis of the enzyme. In fasted animals there is diminished enzyme synthesis as well as accelerated degradation. Nakanishi and Numa (1970) consider that their findings are consistent with the hypothesis that the control of enzyme content by changes in the rate of enzyme degradation may play an important role when the animal deviates from a steady state to adjust to a new environment. Once the new steady state has been achieved, the level of enzyme is maintained by an altered rate of synthesis

This view is supported by observations of Schimke (1964) on changes in the level of arginase in rats when the diet was changed from high to low protein content. Similarly, Rowe and Wyngaarden (1966) and Rechcigl (1968) have found that differences in the rate of synthesis, rather than in the rate of degradation, determine the steady state levels of xanthine oxidase and catalase when rats are maintained on diets that result in differing steady-state levels.

It would be interesting to know the roles of synthesis and degradation in determining the levels of pyruvate carboxylase in fasted and diabetic sheep where there is an increase in the activity, and in glucocorticoid treated sheep where the activity decreases (Section 3.3). The difficulties associated with the available techniques have already been mentioned briefly. Another problem arises with respect to the study of the effects of inhibitors of RNA and protein synthesis on the levels of pyruvate carboxylase, since it is a mitochondrial enzyme. It is presumably synthesised in the cytoplasm because the information content of the mitochondrial nucleic acids is considered to be rather limited (see Ashwell and Work, 1970) but some mitochondrial protein synthesis may be necessary for the incorporation of the enzymeinto the mitochondria. In a study of cytochrome oxidase, Chen and Charalampous (1969) followed haem protein synthesis and the development of enzymic activity during derepression in Saccharomyces. They found that the activities of both the cycloheximide-sensitive protein-synthesising system (cytoplasmic ribosomes) and the system sensitive to chloramphenicol and acriflavine (mitochondrial

ribosomes) were necessary for the formation of functional enzyme.

It has been demonstrated by the use of inhibitors that protein synthesis is involved in the increase in the activity of PEP carboxykinase in rat liver (Ray, Foster and Lardy, 1964; Lardy et al., 1964). Studies on the changes in the activity of this enzyme in sheep liver are complicated by the presence of significant levels of the enzyme in both the cytosol and mitochondria, with similar increases in activity occurring in both cell fractions.

CHAPTER 6

KINETICS OF THE PYRUVATE CARBOXYLASE REACTION WITH RESPECT TO PYRUVATE

6.1. INTRODUCTION

In previous chapters the long term control, or chronic adaptation of pyruvate carboxylase in sheep under conditions of perturbation has been discussed. However, acute adaptation may be equally important in the rapid regulation of the synthesis of oxaloacetate.

It has previously been established in this laboratory that sheep kidney pyruvate carboxylase may be subject to acute control by acetyl-CoA which exhibits positive homotropic cooperativity (Barritt, Keech and Ling, 1966) as does $MgATP^{2-}$, while Mg^{2+} shows positive heterotropic cooperativity with respect to $MgATP^{2-}$ (Keech and Barritt, 1967). The fact that acetyl-CoA is essential for the activity of vertebrate pyruvate carboxylases, and that the activation is an allosteric effect, has led to the hypothesis that the activity of the enzyme *in vivo* is controlled by the intracellular concentration of acetyl-CoA (Utter, Keech and Scrutton, 1964; Williamson, Browning and Olson, 1968).

The results described in this chapter indicate that the enzyme from sheep liver exhibits negative cooperativity (as defined by Levitzki and Koshland, 1969) with respect to the substrate, pyruvate. This property may also be significant in the acute control of the activity of the enzyme *in vivo*.

6.2. METHODS

(a) Enzyme

(i) The enzyme used in Sections 6.3.(a) and (b) was purified from sheep liver mitochondria as described in Section 2.10.(b) to a specific activity of 8.5 units/mg of protein (measured at 23°).

(ii) The enzyme used in Sections 6.3.(c) and (d) was purified as described in Section 2.10.(a) to a specific activity of 22 units/mg of protein (measured at 30°).

(b) Pyruvate

Either redistilled pyruvic acid neutralised with tris base immediately before use, or dimer free sodium pyruvate was used. Identical results were obtained with either preparation. The concentration of the solution was determined from the change in the extinction of NADH at 340 mμ in the presence of lactate dehydrogenase (Bergmeyer, Bernt and Hess, 1963).

(c) Enzymic activity

The reaction mixture (total volume 0.50 ml) contained in μmoles; tris-Cl (pH 8.4) 100; ATP, 1.25; $MgCl_2$, 4.0; $NaH^{14}CO_3$, 5.0 (6×10^5 counts per min. per μmole); acetyl CoA, 0.18; pyruvate, varied as required; and enzyme. After incubation at 30° for 4 min. the reactions were stopped by the addition of 0.05 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6 M HCl. Under these conditions the rate of product formation was linear for all of the pyruvate concentrations used. Radioactivity was determined as described in Section 2.6.(a) and the results were corrected for quenching by the channels ratio method.

6.3. RESULTS

(a) Kinetics of the pyruvate carboxylase reaction with respect to pyruvate

When the initial velocity of the reaction catalysed by sheep liver pyruvate carboxylase was measured and plotted as a function of the pyruvate concentration, a pronounced deviation from a rectangular hyperbola was consistently observed (Fig. 6.1). Double reciprocal plots (inset, Fig. 6.1) of these data were biphasic and the R_s value (i.e., the ratio of the pyruvate concentrations at 90% and 10% of the maximum velocity) of about 220 far exceeds the value of 81 which is expected for a classical Michaelis-Menten hyperbola. A Hill plot prepared from the initial velocity data presented in Fig. 6.1. is shown in Fig. 6.2. The slope of the curve varies markedly with pyruvate concentration. At very low levels of pyruvate the slope decreases from 1 to 0.7 and then to 0.4 in the region of transition from low to high levels of enzymic activity (1 mM pyruvate). The slope increases again and approaches a value of unity at high pyruvate concentrations. This system satisfies the criteria for negative cooperativity with respect to substrate binding as described by Levitzki and Koshland (1969), viz.,

(1) The plot of initial velocity against substrate concentration shows an intermediary plateau region.

(2) The double reciprocal plot of initial velocity against substrate concentration is biphasic with increasing slope at high substrate concentrations.

(3) The R_s value is greater than 81.

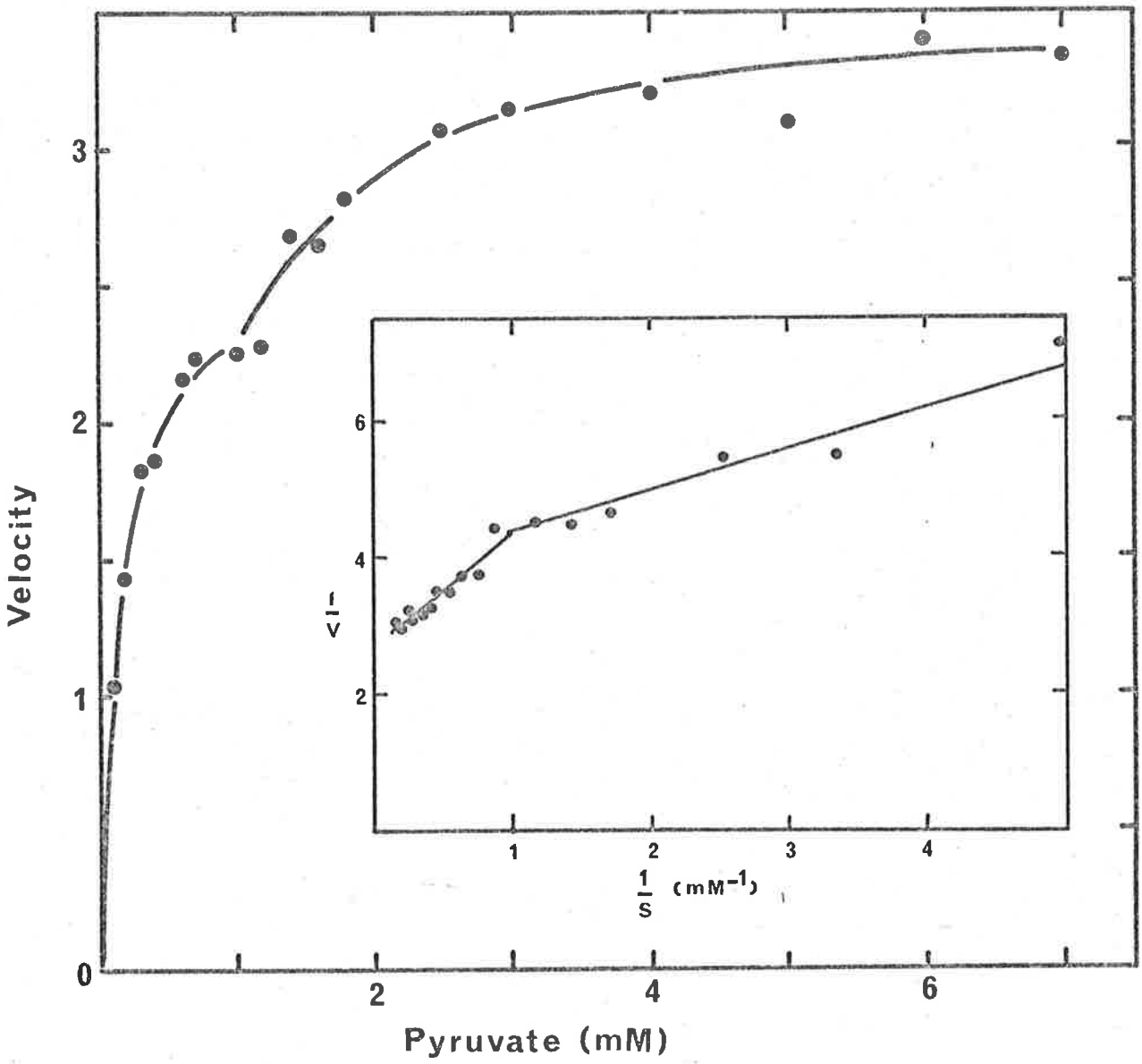


FIG. 6.1. Velocity plotted as a function of the pyruvate concentration. The points marked are experimental values. Inset: reciprocal of the velocity plotted as a function of the reciprocal of the pyruvate concentration. The curves were drawn from the kinetic constants obtained by fitting the data for separate parts of the biphasic plots to a computer programme for a rectangular hyperbola using the method of least squares (Wilkinson, 1961).

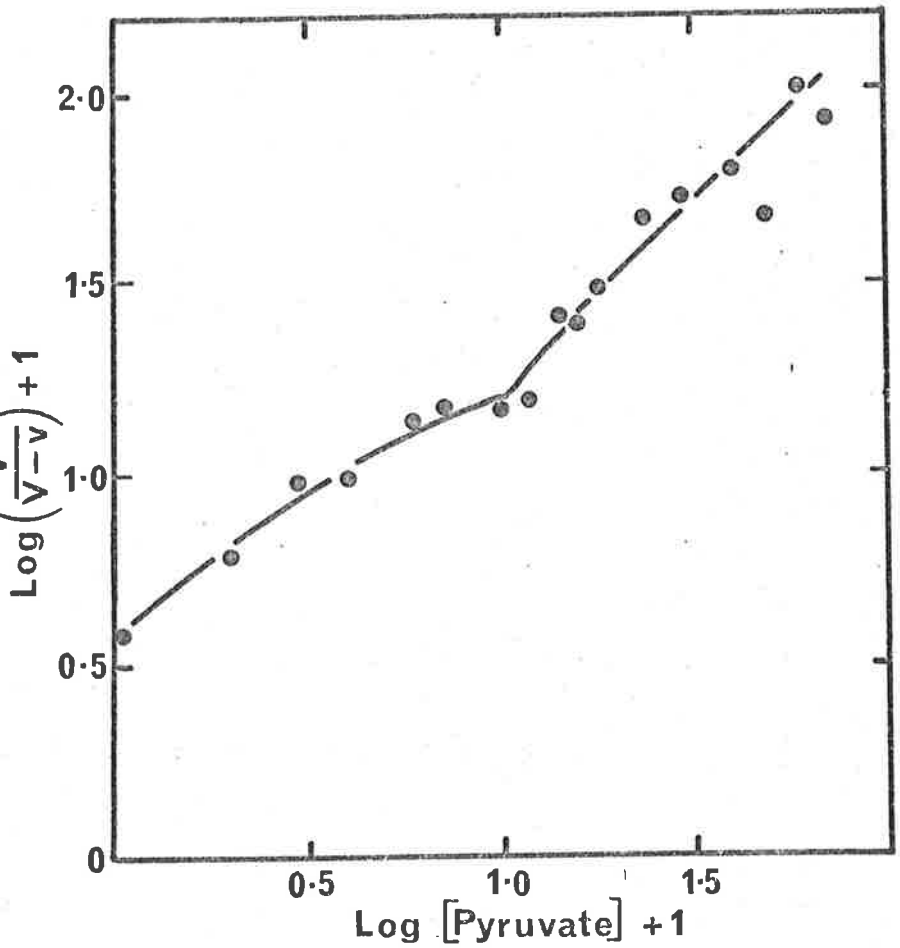


FIG. 6.2. Hill plot for the data presented in Fig. 6.1. The points marked are experimental values and the curves were drawn as described in Fig. 6.1.

(4) The Hill plot is nonlinear with an intermediate region of slope much less than unity.

There are several alternative explanations for the unusual kinetics of the pyruvate carboxylase reaction with respect to pyruvate:

(1) The enzyme preparation may contain two enzymes, one functional at low, and the other at high concentrations of pyruvate.

(2) The kinetic effects may be the result of inhibition of the enzyme by the product, oxaloacetate, as has been demonstrated for sheep kidney PEP carboxykinase (Barns, 1970).

(3) The observed kinetics may be a function of the particular pH and concentrations of substrates and effectors used in the normal assay system.

These possibilities were investigated by comparing the results obtained for the R_s value, the ratios of the two apparent K_m and V_{max} values, and the point of inflection of the double reciprocal plot under various conditions.

The first alternative was rejected on the basis of the data shown in Table 6.1. where it can be seen that the kinetic parameters remain constant throughout the purification procedure.

To test the possibility that oxaloacetate inhibition caused the unusual kinetics, pyruvate carboxylase was assayed at varying pyruvate concentrations with three different assay systems:

(i) uncoupled

(ii) coupled with malate dehydrogenase

(iii) coupled with glutamate-oxaloacetate transaminase.

TABLE 6.1: COMPARISON OF KINETIC PARAMETERS WITH RESPECT TO PYRUVATE AT VARIOUS STAGES OF THE PURIFICATION PROCEDURE

Stage of purification	Specific activity	n_H	High K_m (mM)	Low K_m (mM)	High K_m / Low K_m	High V_{max} / Low V_{max}	Inflection point of double reciprocal point (mM)
A.							
Polyethylene glycol step	1.4	239	1.01	0.18	5.7	1.7	0.94
DEAE-cellulose column, pH 6.5	3.5	276	1.04	0.18	5.91	1.7	0.93
DEAE-cellulose column, pH 7.5	5.1	294	0.94	0.15	6.3	1.6	1.11
G200 Sephadex column	8.5	222	1.10	0.19	5.8	1.9	0.84
B.							
DEAE-Sephadex column, pH 7.2	22	240	1.20	0.24	5.0	1.7	1.21

A. The enzyme was purified by the procedure described in Section 2.10.(b). The specific activity was measured at 23°.

B. The enzyme was purified by the procedure described in Section 2.10.(a). The specific activity was measured at 30°.

The kinetic parameters characteristic of negative cooperativity were still observed when oxaloacetate was converted either to malate or to aspartate (Fig. 6.3) even though NH_4^+ stimulation was shown with the coupled systems (see Section 2.7). The R_s value for pyruvate was 232 in the normal assay system and 215 in the coupled systems.

The third alternative was tested by varying the H^+ concentration and the concentrations of acetyl-CoA and $MgATP^{2-}$ in the assay system. These experiments are described in detail in the following section.

(b) Effect of varying components of the assay system

(1) Varying H^+ concentration

Initial velocities were determined as a function of pyruvate concentration at pH 9.0, 8.4, 7.9 and 7.4. The deviation from a Michaelis-Menten hyperbola appeared to become more pronounced as the pH was shifted away from the optimum (8.4) but when the curves were normalised with respect to the maximum velocity they were found to be very similar. The lack of effect of varying pH is apparent when the data in Table 6.2 are examined.

(2) Varying acetyl-CoA concentration

The apparent K_m of sheep kidney pyruvate carboxylase for acetyl-CoA is 0.041 mM (Barritt, Keech and Ling, 1966). When the pyruvate saturation kinetics were carried out at concentrations of 0.03, 0.07 and 0.25 mM acetyl-CoA, there was very little variation in the parameters even when the acetyl-CoA concentration was less than the apparent K_m.

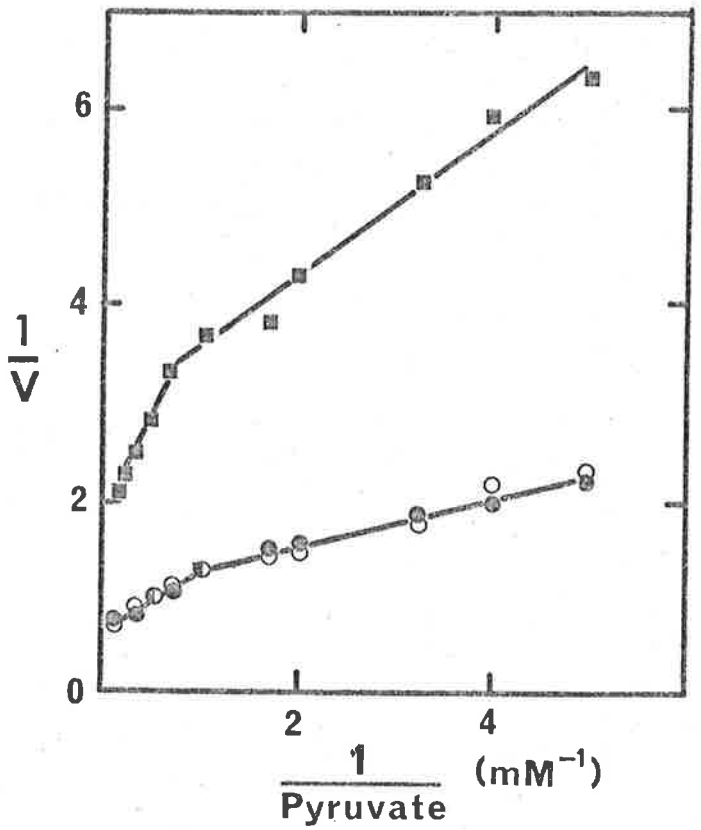


FIG. 6.3. Comparison of the coupled and uncoupled assay procedures. (■ ——— ■), uncoupled assay procedure; (● ——— ●), coupled with glutamate-oxaloacetate transaminase; (o ——— o), coupled with malate dehydrogenase. Assay mixture (total volume 0.5 ml) contained in μmoles: tris-Cl (pH 8.4), 100; ATP, 1.25; $MgCl_2$, 4.0; acetyl-CoA; 0.18; $NaH^{14}CO_3$, 5.0, pyruvate, varied as indicated; and enzyme. In addition, the coupled systems contained NADH, 0.12 μmoles and malate dehydrogenase, 2 units; or pyridoxal phosphate, 0.02 μmoles; glutamate, 10 μmoles and glutamate-oxaloacetate transaminase, 4.5 units. After incubation for 4 min. at 30° the coupled reaction was stopped by the addition of 0.25 ml of 10% (w/v) trichloroacetic acid, and the uncoupled reaction by the addition of 0.05 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6 M HCl. Radioactivity determinations were corrected for quenching by the channels ratio method.

TABLE 6.2. EFFECT OF VARIATION OF THE COMPONENTS OF THE ASSAY SYSTEM ON THE KINETIC PARAMETERS OF THE REACTION WITH RESPECT TO PYRUVATE

	R_s	High K_m (mM)	Low K_m (mM)	$\frac{\text{High } K_m}{\text{Low } K_m}$	$\frac{\text{High } V_{max}}{\text{Low } V_{max}}$	Intercept of double reciprocal plot (mM)
pH						
8.4	246	1.14	0.15	7.6	2.2	1.0
9.0	278	1.01	0.13	8.0	2.0	0.75
7.9	257	1.35	0.14	9.6	2.5	0.64
7.5	374	1.20	0.10	12.5	1.6	0.72
Acetyl-CoA						
0.25 mM	237	1.11	0.17	6.7	2.0	0.75
0.07 mM	280	1.28	0.16	8.3	2.1	0.86
0.03 mM	261	1.41	0.15	9.2	2.4	0.80
$MgATP^{2-}$						
2.4 mM	222	0.62	0.15	4.1	1.4	0.97
0.48	320	0.72	0.095	7.6	1.6	0.97
0.24	248	0.47	0.093	5.1	1.6	0.72

The enzymic activity was assayed in the system described in Section 6.2.(3) except that the concentrations of H^+, acetyl-CoA or $MgATP^{2-}$ were varied as indicated. The concentration of $MgATP^{2-}$ was varied in the presence of a constant level of 2.0 mM excess Mg^{2+}. The concentration of $MgATP^{2-}$ was calculated from the apparent stability constant for $MgATP^{2-}$ of 73,000 M^{-1} (O'Sullivan and Perrin, 1964).

(3) Varying $MgATP^{2-}$ concentration

Keech and Barritt (1967) have shown that the true substrate for sheep kidney pyruvate carboxylase is the $MgATP^{2-}$ complex rather than free ATP and that the apparent K_m of the sheep kidney enzyme for $MgATP^{2-}$ in the presence of excess Mg^{2+} is 0.32 mM. The experiment was carried out in the presence of excess Mg^{2+} at $MgATP^{2-}$ concentrations of 0.24, 0.48 and 2.4 mM. As was observed with varying H^+ or acetyl-CoA concentrations, there was no significant change in the kinetic response with respect to pyruvate concentration.

(c) The effects of pyruvate analogues

The effects of two substrate analogues, β-phenylpyruvate and p-hydroxypyruvate, which inhibit the enzyme were examined. Pyruvate saturation kinetics were studied at varying fixed levels of the inhibitors. At low concentrations of pyruvate the effects of the inhibitors were quite different (Fig. 6.4) but at high concentrations the curves for the two inhibitors were almost identical. The shape of the double reciprocal plots, particularly in the presence of β-phenylpyruvate, tended to curve markedly at high pyruvate concentrations instead of falling into two distinct straight line regions, and it was therefore impossible to calculate accurate R_s values for pyruvate in the presence of β-phenylpyruvate. The R_s value for pyruvate in the absence of inhibitor was 220, while in the presence of 3.0 and 10.0 mM β-phenylpyruvate values between 200 and 300 were obtained.

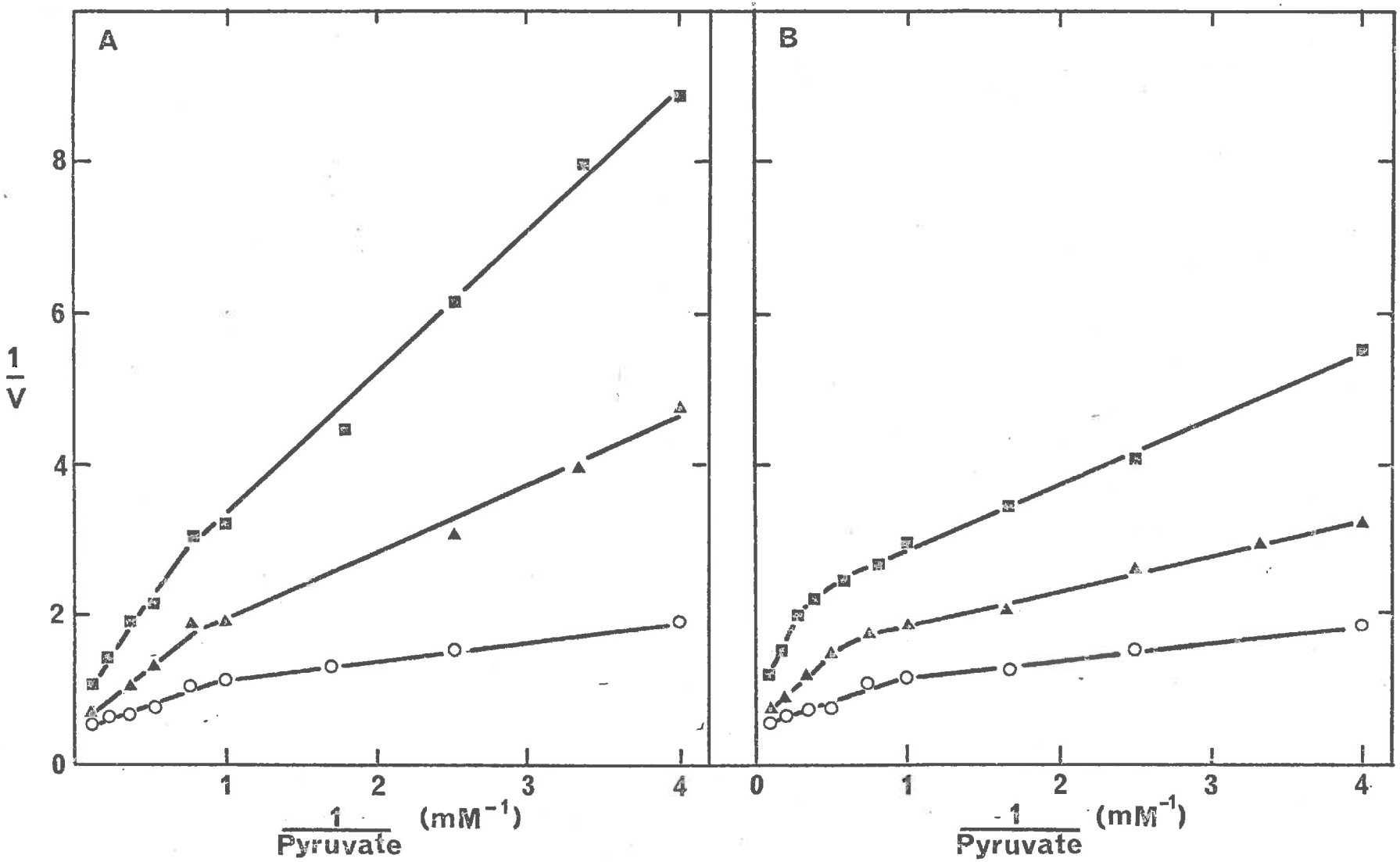


FIG. 6.4. Double reciprocal plots of initial velocity against pyruvate concentration. The standard reaction mixture described in Section 6.2.(c) also contained (A) p-hydroxypyruvate: (o ——— o), 0.0 mM; (▲ ——— ▲), 0.75 mM; (■ ——— ■), 2.0 mM; (B) β-phenylpyruvate: (o ——— o), 0.0 mM; (▲ ——— ▲), 3.0 mM; (■ ——— ■), 10.0 mM.

β-Hydroxypyruvate was the more efficient inhibitor and in the presence of 0.75 and 2.0 mM β-hydroxpyruvate, R_s values of 128 and 113, respectively, were observed, thus indicating that the negative cooperativity with respect to pyruvate had almost been abolished.

(d) Dissociation constants for the enzyme-pyruvate complex obtained by analysis of rates of inactivation by avidin

Mildvan, Scrutton and Utter (1966) have shown that the inactivation of chicken liver pyruvate carboxylase by avidin is accelerated by the presence of pyruvate and that the effect is a function of the pyruvate concentration. Scrutton and Utter (1965) derived the equation

$$\frac{V_a}{V_o} = \frac{k_2}{k_1} + K_d \frac{1 - V_a/V_o}{A} \quad \ldots (6.1)$$

where V_a and V_o represent, respectively, the pseudo-first order rate constants for the inactivation in the presence and absence of a, i.e., pyruvate; k_1 and k_2 are the fractional order rate constants for the inactivation by avidin (I) of free enzyme (Equation 6.2) and enzyme-pyruvate complex (Equation 6.3), respectively; A is the concentration of pyruvate, the potentiating agent; K_d is the dissociation constant for the enzyme-pyruvate complex (Equation 6.4).

$$E + nI \xrightarrow{k_1} EI_n \qquad \ldots (6.2)$$

$$EA + nI \xrightarrow{k_2} EAI_n \qquad \ldots (6.3)$$

$$E + A \xrightleftharpoons{K_d} EA \qquad \ldots (6.4)$$

When the ratio (V_a/V_o) of the pseudo first order rate constants for inactivation in the presence and absence of pyruvate is plotted against $(1 - V_a/V_o)/A$ the ordinate intercept represents k_2/k_1, the ratio of the fractional order rate constants for the reaction of the enzyme-pyruvate complex and free enzyme with the modifying agent. The slope of the line represents K_d.

The incubation system used by Mildvan, Scrutton and Utter (1966) contained $(NH_4)_2SO_4$ as well as tris-SO_4, avidin, enzyme and pyruvate. No reason was given for the inclusion of $(NH_4)_2SO_4$, but it was found during the investigation reported here that pyruvate potentiated the avidin inhibition only in the presence of $(NH_4)_2SO_4$. This may be related to the observation that chicken liver pyruvate carboxylase is protected against avidin inhibition by SO_4^{2-} (Madappally and Mistry, 1970b).

The curve presented in Fig. 6.5. is obviously biphasic, suggesting that at least two molecules of pyruvate are bound and that they have widely differing dissociation constants of 6.25 mM and approximately 0.1 mM. The region of discontinuity corresponds to a pyruvate concentration of approximately 0.8 mM which is consistent with the plateau region in the initial velocity experiments.

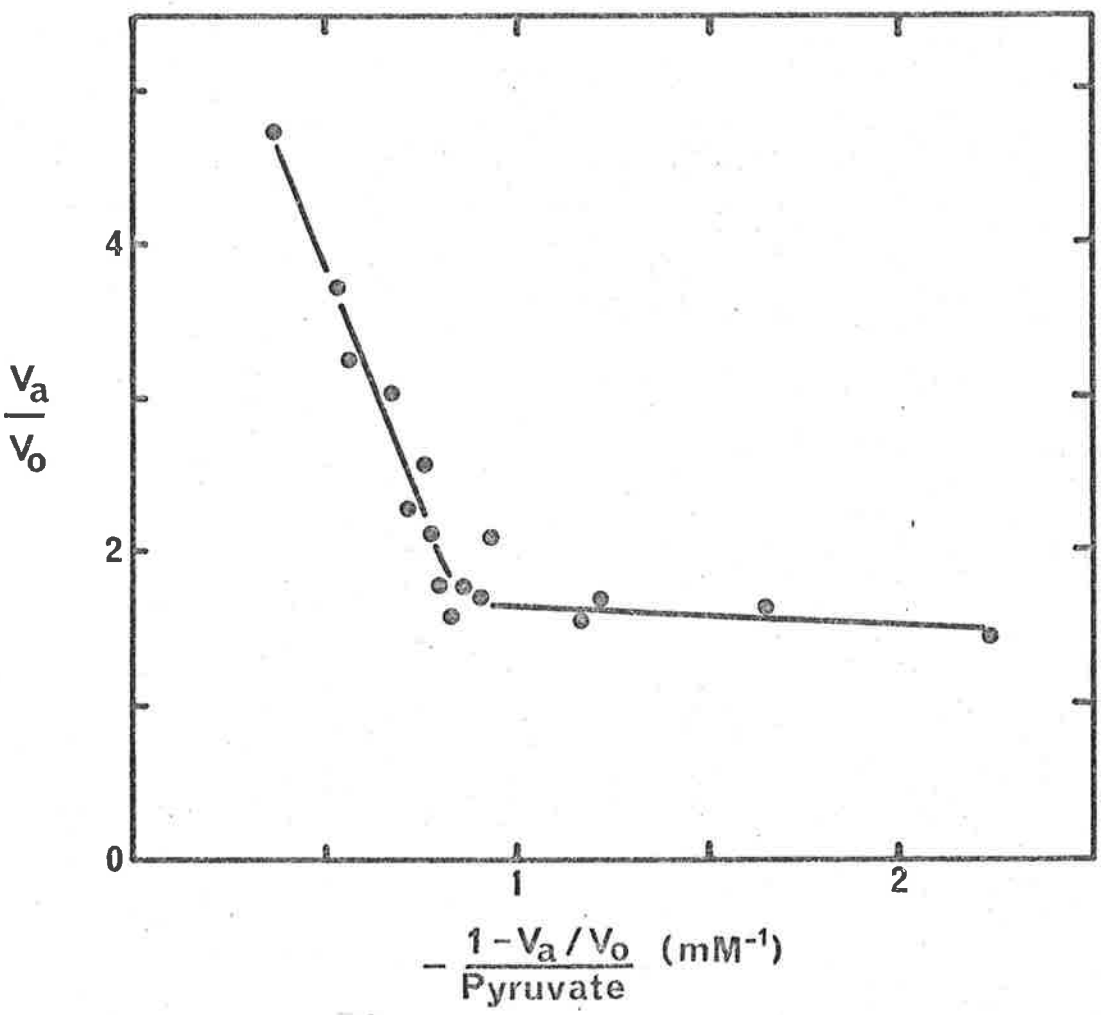


FIG. 6.5. Dissociation constant for pyruvate from its effect on the rate of avidin inactivation. The incubation system contained in 0.20 ml: 7 µmoles of potassium phosphate (pH 7.2); 10 µmoles of $(NH_4)_2SO_4$; 30 µg of avidin (10.3 units/mg); 0.05 units of pyruvate carboxylase, specific activity 22 units/mg of protein; and pyruvate, varied from 0 to 2.0 µmoles. After incubation of the mixture at 23° for 0, 1.5, 3.0 and 4.5 min. the residual enzymic activity was assayed in the system described in Section 6.2.(c). The V_o and V_a values were obtained from the slope of the pseudo-first order plots of the relationship between $\log_{10} E_t$ (residual enzymic activity) and t (min.) at various pyruvate concentrations.

6.4. DISCUSSION

(a) Initial velocity studies at varying pyruvate concentrations

This investigation demonstrates that the kinetics of the pyruvate carboxylase reaction with respect to pyruvate satisfy the criteria for negative cooperativity as defined by Levitzki and Koshland (1969). Alternative explanations for the unusual kinetics, such as the presence of two enzymes or inhibition by oxaloacetate, have been considered untenable. This is in contrast to observations made by Barns (1970) with sheep kidney PEP carboxykinase. PEP carboxykinase appears to satisfy the criteria for negative cooperativity with respect to IDP or PEP binding. The activity of the enzyme was assayed by the fixation of $^{14}CO_2$ to form oxaloacetate, in a system analogous to that used for the assay for pyruvate carboxylase activity. However, with PEP carboxykinase, it was found that a linear double reciprocal plot was obtained by the use of a coupled system involving either malate dehydrogenase or glutamate-oxaloacetate transaminase. This finding eliminates the presence of negative cooperativity and suggests instead that there is strong product inhibition by oxaloacetate, with this effector introducing new interactions in the binding of the substrate to the enzyme.

It was concluded that the pyruvate kinetics were independent of the pH of the assay system or of the concentrations of other components of the system, since the kinetic parameters remained relatively constant despite wide variations in pH and in the concentrations of $MgATP^{2-}$. a

substrate which exhibits positive cooperativity, and acetyl-CoA, a positive effector. Glutamate dehydrogenase exhibits negative cooperativity with respect to NAD^+ (LeJohn and Jackson, 1968) and if plots of velocity against NAD^+ concentration at various concentrations of glutamate are normalised, the curves become identical i.e., the R_s value remains constant. Levitzki and Koshland (1969) determined the initial velocity of CTP synthesis by CTP synthetase as a function of glutamine concentration in the presence of different fixed concentrations of GTP. They stated that at increasing concentrations of GTP the deviation from the Michaelis-Menten hyperbola became less pronounced but it appears that if one normalised the curves with respect to the maximum velocity they would be identical. Similarly, with sheep liver pyruvate carboxylase the deviation appeared to increase as the conditions became less optimal although the R_s value and the other parameters remained relatively constant under all of the conditions tested.

Nielsen (1970) has demonstrated that pyruvate carboxylase from sheep kidney exhibits pyruvate kinetics analogous to those for the sheep liver enzyme. The apparent K_m values of the two enzymes are similar, but the point of inflection of the double reciprocal plot for the kidney enzyme occurs at 2-3 mM pyruvate, which contrasts with the value of 1 mM found for the liver enzyme. It has also been reported that the double reciprocal plot for initial velocity versus pyruvate concentration is biphasic for both the overall reaction (Mildvan and Scrutton, 1967) and the pyruvate-oxaloacetate exchange reaction (Scrutton, Keech and Utter, 1965) catalysed

by chicken liver pyruvate carboxylase. McClure (1969) and Wimhurst and Manchester (1970) have found that the enzyme isolated from rat liver exhibits Michaelis-Menten kinetics, but the data presented by Wimhurst and Manchester (1970) indicated that the pyruvate concentrations used did not exceed 3 mM.

(d) K_d values for enzyme-pyruvate complex

From the potentiation of avidin inhibition by pyruvate it is concluded that at least two molecules of pyruvate are bound by the enzyme. The value for the K_d at high pyruvate concentrations (in excess of 1 mM) agrees well with that reported for the chicken liver enzyme (Mildvan, Scrutton and Utter, 1966). Low concentrations of pyruvate were not included in the study with the chicken liver enzyme. Mildvan and Scrutton (1967) have attempted to explain the discrepancy between the K_d value and apparent K_m by suggesting that during the normal enzymic reaction pyruvate binds to the E-biotin-CO_2 intermediate after dissociation of ADP and P_i, but that at high concentrations of pyruvate, binding to the E-biotin may become kinetically significant. This argument is based on the observation that in the presence of non-saturating concentrations of ATP the apparent substrate activation is more marked and gives an apparent K_m for pyruvate which is in the range of 2-5 mM. Under these conditions the K_m approximates the K_d for pyruvate determined from proton relaxation rate analysis and from the potentiation of avidin inhibition (Mildvan, Scrutton and Utter, 1966). However, with neither the sheep liver enzyme nor the sheep kidney enzyme was there

any change in the apparent K_m values for pyruvate at low concentrations of $MgATP^{2-}$.

This study of the potentiation of avidin inhibition also confirms that the manifestations of negative cooperativity are solely a property of the interaction of pyruvate with the enzyme. Therefore, it should be possible to demonstrate the binding of two or more molecules of pyruvate to the enzyme by binding studies with radioactive pyruvate. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Conway and Koshland, 1968) and E. coli alkaline phosphatase (Simpson and Vallee, 1970) have both been shown to possess the catalytic features diagnostic for negative cooperativity. Furthermore, these enzymes exhibit negative cooperativity with respect to the binding of the substrate. In the former case four moles of NAD^+ are bound with progressively decreasing affinity, while alkaline phosphatase binds two moles of PP_i, the first more readily than the second. From a theoretical consideration of negative cooperativity Teipel and Koshland (1969) have concluded that more than two substrate binding sites are necessary for the generation of saturation curves with intermediary plateau regions. They restricted their treatment to a rapid random situation and the fact that the binding of only two moles of PP_i to alkaline phosphatase can be detected may be an illustration of the limited application of this treatment, or it may be a shortcoming of the method used to detect the binding.

Attempts were made to study the binding of pyruvate to pyruvate carboxylase by the method of Colowick and Womack (1969). It was found necessary to reduce the porosity of the dialysis

membrane by acetylation (Craig, 1967) but even when this problem had been overcome, no significant binding of pyruvate was detected when 40 mg of pyruvate carboxylase of specific activity 22 units per mg of protein was placed in the upper chamber of the dialysis cell in a volume of 1.0 ml. Because of the relatively large quantities of purified enzyme required it was not considered practicable to characterise the system fully under a variety of conditions e.g., with varying salt concentrations. Other methods available for detecting the binding of substrate to an enzyme, such as gel filtration (Hummel and Dreyer, 1962) or ultracentrifugal techniques (Velick, Hayes and Harting, 1953) also require large amounts of purified enzyme and hence have not been employed with pyruvate carboxylase.

(c) Pyruvate analogues

The observed kinetic properties of pyruvate carboxylase with respect to pyruvate may be explained by the occurrence of negative homotropic interactions between binding sites or by the existence of mutually independent sites with different intrinsic properties. From the theoretical arguments of Levitzki and Koshland (1969) it is possible that the binding of pyruvate at one site leads to an interaction with another binding site causing the affinity for the next site to be reduced. The effects observed with the substrate analogues may also be explained on this basis. β-Phenylpyruvate and p-hydroxypyruvate, being substrate analogues, probably bind to the enzyme at the same site as pyruvate, and on binding could possibly produce the interaction normally elicited by pyruvate.

Therefore, the subsequent interaction produced by pyruvate itself is decreased, as indicated by the decreased R_s value for pyruvate. β-Hydroxypyruvate inhibits the enzyme at a much lower concentration than β-phenylpyruvate and presumably has a higher affinity for the enzyme than β-phenylpyruvate. The greater efficiency of the inhibition by β-hydroxypyruvate is particularly evident at low concentrations of pyruvate where it competes more strongly with pyruvate than does β-phenylpyruvate.

The effects of the inhibitors could also be explained in terms of two different binding sites for pyruvate, such that the pyruvate analogues react differently with one site but similarly with the other. However, it has not yet been demonstrated that any of the enzymes exhibiting the characteristics of negative cooperativity possesses two different binding sites.

At the present time insufficient information is available to allow any more than speculation about the relationship of the observed pyruvate kinetics to the structure of the enzyme. Chicken liver pyruvate carboxylase consists of four subunits, each of which contains more than one polypeptide chain (Valentine et al., 1966). Furthermore, each mole of the enzyme contains four moles of biotin and four gram atoms of manganese (Scrutton, Utter and Mildvan, 1966). Results obtained from proton relaxation rate studies have been interpreted to indicate that the manganese participates in the binding of pyruvate by the formation of an enzyme-manganese-pyruvate bridge complex, and hence that each major

subunit possesses one catalytic site (Mildvan and Scrutton, 1967). Manganese can provide six ligand binding positions, three of which are probably occupied by the enzyme, the other three being occupied by water molecules. During the binding of pyruvate one of these is displaced. This argument is difficult to reconcile with the recent discovery that active pyruvate carboxylase containing bound magnesium is isolated from the liver of manganese-deficient chickens (Griminger and Scrutton, 1970). Magnesium has a coordination number of two, and it seems unlikely that it can perform the same function as manganese. Nevertheless, if there are in fact four binding sites for pyruvate per mole of enzyme, these binding sites may be identical or different, depending on the structure of the subunits, which has yet to be elucidated. On the other hand, the subunits may function as two dimers, which could explain the two values obtained for the dissociation constants. Thus, although the observed pyruvate kinetics may be the result of negative homotropic cooperativity, it is quite feasible that there could be two distinct binding sites with different intrinsic properties.

(d) Physiological significance of the observed kinetics

It is becoming increasingly evident that negative cooperativity is a fairly widespread property amongst enzymes and is not restricted to a few isolated examples. Although explanations other than negative homotropic interactions exist for the occurrence of this phenomenon, its functional significance is not affected by the mechanism by which it occurs. As a

result of these kinetics, it appears that pyruvate carboxylase is insulated from fluctuations in activity over a wide range of pyruvate concentrations. The apparent K_m value for pyruvate in the lower activity range is 0.2 mM which is approximately three-fold higher than the reported physiological levels of pyruvate in the ruminant liver (Ballard, Hanson and Kronfeld, 1968; Baird et al., 1968). If, as has been assumed by Williamson (1969) and Krebs and Veech (1969), the pyruvate is distributed evenly throughout the cell water, it would seem that the activity represented by the upper portion of the initial velocity curve would be used only when a large influx of pyruvate precursors occurred, e.g., lactate after vigorous exercise.

CHAPTER 7. GENERAL DISCUSSION

The most significant conclusions which have been drawn from this investigation are:

1. The activity of pyruvate carboxylase varies markedly in the livers of sheep under conditions of physiological perturbation whereas the activity of PEP carboxykinase increases only in the diabetic sheep.

2. Under all of the conditions investigated, the hepatic activity of pyruvate carboxylase is confined to the mitochondria *in vivo*. The activity of PEP carboxykinase is distributed between the mitochondria and cytosol, with similar increases in activity occurring in both fractions in diabetes.

3. The increase in the activity of pyruvate carboxylase in fasted and diabetic sheep is associated with a proportionate increase in the liver content of immunochemically reactive enzyme protein.

4. Pyruvate carboxylase from sheep liver exhibits negative cooperativity with respect to the substrate, pyruvate.

The observations leading to these conclusions, together with their possible implications, have already been discussed in the relevant chapters. In this chapter some of those lines of experimentation which suggest themselves as an extension of this investigation will be considered. These future studies could be directed at solving three main problems which have arisen:

(a) the physiological significance of the changes in the activities of the enzymes, particularly pyruvate carboxylase

(b) the factors causing the changes in the activities

(c) the mechanism by which the activities are altered, or more specifically, in the case of pyruvate carboxylase, the mechanism by which the quantity of enzyme protein is altered.

In most instances it would be advantageous to pursue studies along these lines with pyruvate carboxylase and PEP carboxykinase, concurrently, as was done in most of the experiments reported in this thesis.

The elucidation of these problems is hindered in many respects by the lack of a suitable small laboratory animal. Although there are reports in the literature of substantial increases in the activity of pyruvate carboxylase in the liver of rats, results obtained in this laboratory with animals of various species suggest that the marked response of pyruvate carboxylase to fasting and diabetes is unique to ruminants. However, because of its size, the sheep is obviously not a very suitable experimental animal for studying the mechanism and cause of changes in the activities of enzymes. In some instances, the cost of the necessary quantities of chemicals and drugs has been prohibitive, and in others technical difficulties have arisen. Attempts to develop a system of dissociated cells from sheep liver have so far been unsuccessful, and cell cultures have not been considered because few cells when cultured retain the differentiated regulatory properties

characteristic of those of the intact animal. With these limitations in mind it may be constructive to consider some of the experimental approaches which could be used.

Although a considerable amount of information has now been accumulated about the changes in the activities of pyruvate carboxylase and PEP carboxykinase in the sheep under various conditions of physiological perturbation, very little is known about the relevance of these observations to the situation *in vivo*. The increase in the activity of PEP carboxykinase in the liver of the diabetic sheep is probably associated with an increased rate of hepatic gluconeogenesis, but the reasons for the wide responses in the activity of pyruvate carboxylase are less clear. Crossover studies should provide information for the assessment of the physiological relevance of the observed changes in enzyme activity. The transfer of labelled carbon from lactate or pyruvate to glutamate and glucose could also be followed to complement the measurements of enzymic activities by providing a comparison of the amounts of pyruvate converted to acetyl-CoA and oxaloacetate under the different conditions (Freedman and Graff, 1958; Hill, Hobbs and Koeppe, 1958; Black, Luick, Moller and Anand, 1966). The importance of alanine in rats and humans suggests that alterations in the metabolism of alanine may be associated with the increased activity of pyruvate carboxylase found in the livers of starved and diabetic sheep. However, it is possible that the activity of pyruvate carboxylase increases not to cope with an increased supply of substrate, but rather to ensure that any available substrate is converted to oxaloacetate instead of being

oxidised to acetyl-CoA. Studies in this laboratory are currently in progress to determine the plasma concentrations of amino acids in the sheep under conditions of perturbation, and it should also be profitable to measure the turnover rates of pyruvate precursors, especially alanine.

The whole animal is not ideally suitable for studying the factors causing the changes in the enzymic activities. It appears that the increase in pyruvate carboxylase occurs relatively slowly, and this enhances the possibility that after administration of compounds to the animal secondary effects could arise during this time period. The perfused liver has been widely used in studies of this nature in the rat. It is possible that a more rapid increase in the activity of the enzyme may be effected in the perfused liver, and another advantage is that a certain amount of control can be exercised over the composition of the perfusion medium. Again, in an animal the size of the sheep there are certain technical problems and it has not been possible to undertake liver perfusions during the course of this investigation.

Results obtained from immunochemical experiments strongly suggest that the increased activity of pyruvate carboxylase is due to an altered rate of degradation or synthesis de novo of the enzyme protein. It has not yet been demonstrated conclusively that pyruvate carboxylase does not exist in the liver of normal sheep as a proenzyme or apoenzyme, but evidence gained so far indicates that this is unlikely. Some of the means of determining the relative roles of synthesis and degradation have already been discussed. Although the use

of inhibitors has implicated an increased rate of synthesis as the mechanism for the increased activity of PEP carboxykinase observed in rats, the enzyme has not been studied immunochemically or with respect to the effects of inhibitors in the sheep. The situation here is complicated by the occurrence of the enzyme in two cell compartments with similar increases in activity in both. Ideally, the cytosol and mitochondrial activities should be examined separately. The mitochondrial activities of pyruvate carboxylase and PEP carboxykinase are presumably synthesised in the cytoplasm. It is becoming increasingly evident that the various components of the mitochondria have widely differing rates of turnover, but it is not known how increased quantities of certain enzymes are incorporated in the mitochondria under conditions of stress.

The occurrence of significant proportions of PEP carboxykinase activity in the two cell compartments poses the problem of the functional significance of this distribution. Johnson, Ebert and Ray (1970) have reported that in the rabbit, where 75% of the activity is mitochondrial, oxaloacetate seems to follow the same gluconeogenic pathway as that utilised in the rat where most of the activity is in the cytosol. It may be interesting to carry out analogous studies with isolated sheep liver mitochondria to determine whether labelled carbon in pyruvate emerges from the mitochondria as PEP or as malate, citrate, aspartate and fumarate. On the basis of kinetic evidence obtained with purified sheep kidney mitochondrial PEP carboxykinase, Barns (1970) has suggested an anaplerotic role for this enzyme, but this needs to be supported by additional information before it can be extrapolated to the situation in vivo

There are some very marked differences in the enzymatic aspects of gluconeogenesis at the three-carbon level in the liver and kidney. It is not known whether the liver and kidney enzymes are isoenzymes, controlled by different factors, or whether they are identical, with the differences in activity being due to their different environments. Ouchterlony double diffusion analysis indicates that the pyruvate carboxylases isolated from sheep liver and kidney are identical, but other confirmatory experiments have not yet been attempted.

A facet of this investigation which remains almost completely unexplored is that of the enlarged mitochondria seen in liver sections from fasted and diabetic sheep. Preliminary observations suggest that this phenomenon occurs to such a marked degree only in the liver and that the mitochondria of the livers of other species are less susceptible to such changes. As was discussed earlier, these alterations may be due to the infiltration of the liver with lipids in fasting and diabetes. This could perhaps be tested by infusing a normal sheep with fatty acids. There are some indications in the literature that the population of mitochondria in the rat liver is heterogeneous. It is conceivable that if a similar situation exists in sheep liver, some mitochondria may swell more readily than others, and that increased amounts of enzymes may be incorporated preferentially into certain types of mitochondria. Unfortunately, with conventional techniques it is impossible to isolate mitochondria

from the livers of fasted and diabetic sheep without the disruption of a large percentage of the enlarged mitochondria, as evidenced by the results described for the cell fractionation experiments. Mitochondria have been isolated successfully from adipose tissue (Patel and Hanson, 1970) and muscle (Bullock, Carter and White, 1970) by the use of enzymatic rather than mechanical means for the disruption of the cell membrane. These methods may prove more satisfactory than homogenisation for the isolation of the enlarged mitochondria from sheep liver. If this could be achieved, the mitochondria could be examined by zonal centrifugation, and significant differences may also be detectable in their composition.

Thus, during the course of this project, some interesting observations have been made about the behaviour of pyruvate carboxylase and PEP carboxykinase in the liver and kidney of the sheep, and several lines of investigation worthy of further attention have been revealed. As this discussion has indicated, there remains a great deal to be elucidated about the significance and mechanisms of the changes in the activities of these enzymes, and such information may add to our general knowledge of the control of enzymic activities. Furthermore, the observations are interesting on a comparative basis, both in the one species when the liver and kidney are compared, and between species where there appear to be marked differences in the behaviour of the hepatic enzymes. It is often through differences such as these that a greater insight into the understanding of the mechanism

and control of a metabolic process is gained, as has been the case in studies of healthy and diseased tissues.

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