

**STUDIES IN THE**

**CARBOHYDRATE METABOLISM**

**OF THE LIVER**

I wish to express my thanks to Professor Sir  
Hans A. Krebs, F.R.S., for inspiration, supervision and  
friendly guidance at all times in the course of these studies.

All the members of the Medical Research Council Unit for  
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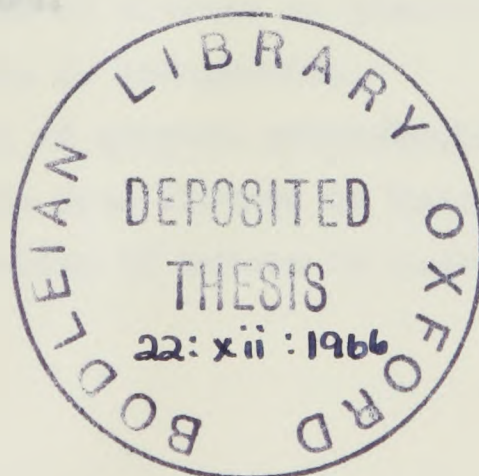
**DOCTOR OF PHILOSOPHY**

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A B S T R A C T

The carbohydrate metabolism of the liver is subject to large physiological variations both in the rates and the direction of the overall transformations. Pigeon-liver homogenates carry out the entire sequence of gluconeogenic reactions from lactate and other precursors at rates equivalent to those observed in intact animals (Krebs, Dierks & Gascoyne, 1964), and can also degrade carbohydrate in the absence of such precursors. They form a convenient material for detailed studies of some control mechanisms of the opposing pathways of glycolysis and gluconeogenesis; such studies must take into account the important differences between tissue homogenates and intact cells with respect to specific structural organization, enzyme concentrations and the activation of autolytic enzymes.

Washed pigeon-liver mitochondria are capable of synthesizing phosphoenolpyruvate at high rates from a variety of precursors, including pyruvate (Bartley & Avi-Dor, 1955). In view of the significant species differences that have been described for the intracellular distribution of key enzymes catalysing this process (Nordlie & Lardy, 1963), a detailed study of the mechanism of phosphoenolpyruvate synthesis in pigeon liver was undertaken, with particular reference to the distribution, activity and properties of the relevant enzymes; and to the control of their activity in situ.

In order to place in perspective the studies with pigeon-liver



homogenates and isolated mitochondria described in this thesis, some of the factors controlling the carbohydrate metabolism of an organ such as the liver are discussed. These include availability of substrates, the effects of non-substrates on the activity of rate-limiting enzymes, inter-conversion of enzyme forms and variations in enzyme concentrations.

Since low concentrations of bicarbonate ions decrease both the rates of carbohydrate synthesis from lactate and of glucose degradation in pigeon-liver homogenates, the oxygen consumption was measured in the presence of "carbon dioxide buffers", generally as described by Warburg & Krippahl (1960). A procedure was elaborated for the generation of alkali in closed manometer vessels by mixing mercuric oxide and a solution of sodium iodide, in order to measure the equilibrium carbon dioxide pressures above concentrated potassium carbonate and bicarbonate mixtures containing carbonic anhydrase, capable of acting as "carbon dioxide buffers".

The effects of added adenine nucleotides on carbohydrate metabolism in pigeon-liver homogenates were investigated, after suitable experimental conditions had been established; these included the addition of inorganic phosphate (20 mM),  $\text{NAD}^+$  (0.5 mM) and bicarbonate ions (5 to 25 mM). Lactate addition increased the oxygen consumption to a much greater extent than could be accounted for by the net synthesis of glucose, and kept the concentration of ATP high and that of AMP low, apparently by the increased provision of respiratory substrate. Added AMP (1.25 to 5 mM) raised the net rate of carbohydrate removal and inhibited the net formation of carbohydrate from lactate, as well as the incorporation of lactate into



glucose. These effects were accompanied by a fall in the concentrations of hexose 6-phosphates and a rise in those of fructose 1,6-diphosphate and triose phosphates. Experiments with  $^{14}\text{C}$ -glucose indicated that "cycling" of hexose phosphates and free glucose occurred in the homogenates, and that this was inhibited at high concentrations of AMP or in the presence of lactate. When the activity of glyceraldehyde 3-phosphate was limited experimentally by a low concentration of  $\text{NAD}^+$  or when it was blocked with iodoacetate, the accumulations of fructose 1,6-diphosphate and triose phosphates were large and accounted for most of the carbohydrate degraded in the presence of AMP. AMP addition also inhibited the conversion of pyruvate into phosphoenolpyruvate. Data on the concentrations of pyruvate, phosphoenolpyruvate and intermediates of the tricarboxylic acid cycle, as well as of isotope distribution, suggest that the effect was due to inhibition of phosphoenolpyruvate carboxykinase. The concentrations of adenine nucleotides were always such as to indicate that equilibrium between them was maintained by adenylate kinase.

Fructose diphosphatase of pigeon liver was cytoplasmic, and was qualitatively similar to that of rat liver (Underwood & Newsholme, 1965) with respect to inhibition by AMP and fructose 1,6-diphosphate.

Malate accumulating in pigeon-liver homogenates during incubations with lactate was formed by carboxylation of pyruvate and not by forward operation of the tricarboxylic acid cycle. The incorporation of  $[3-^{14}\text{C}]$ -lactate into carbohydrate and  $\alpha$ -glycerophosphate was lower in homogenates of livers from fed birds than in those from starved birds. Increasing



concentrations of lactate were associated with changes in the concentrations of gluconeogenic intermediates such as to suggest that lactate addition produced an enhancement of the net rate of fructose 1,6-diphosphate hydrolysis.

Citrate (in the presence of an excess of  $Mg^{2+}$  ions) stimulated the formation of glucose from fructose by a more than additive amount, and increased the incorporation of  $^{14}C$ -fructose into glucose four-fold; a smaller but significant effect of the latter kind was also exerted by lactate addition. Citrate was a powerful inhibitor of the "cycling" activity catalysed by fructose diphosphatase and 6-phosphofructokinase, while part of the similar effect of lactate was due to an increase in the citrate concentration. Citrate addition did not increase the rate of gluconeogenesis from lactate, because the formation of fructose 6-phosphate was not the rate-limiting step in this overall process except in the presence of raised AMP concentrations. The formation of carbohydrate from malate was limited by the availability of NADH: most of the oxidation of this substrate occurred within the mitochondria.

The results obtained with pigeon-liver homogenates support the concept that fructose diphosphatase and 6-phosphofructokinase catalyse reactions that are among those that can limit the rates of hepatic glycolysis and gluconeogenesis, and that the concentrations of intracellular "modifiers", especially adenine nucleotides and citrate, regulate the activity of these enzymes and also the direction of glucose metabolism. The possible significance of ATP-utilizing "cycles" is discussed from the



point of view of the control of metabolism and the effects of hormones. The role of adenine nucleotides, citrate and acetyl coenzyme A in the co-ordinated control of carbohydrate metabolism during the oxidation of fatty acids is also discussed.

Pyruvate carboxylase activity in pigeon-liver homogenates or mitochondria could not be assayed by  $^{14}\text{C}$ -bicarbonate incorporation because of the occurrence of a rapid carbon dioxide-oxaloacetate exchange catalysed by phosphoenolpyruvate carboxykinase. A similar method involving  $[1-^{14}\text{C}]$ -pyruvate was satisfactory; the activity was 10 - 12  $\mu\text{Moles/min./g. dry weight at } 25^{\circ}$ , and was mainly mitochondrial in location.

Malate enzyme was present exclusively in the soluble fraction and the activity varied between 57 (48 hour-starved pigeons) and 200  $\mu\text{Moles/min./g. dry weight at } 25^{\circ}$  (well-fed birds).

Phosphoenolpyruvate carboxykinase was present principally in the mitochondria and showed "latency" phenomena in that mitochondrial disruption increased the activity 6 - 8 times. The activity was 27 - 33  $\mu\text{Moles/min./g. dry weight at } 25^{\circ}$  and did not vary during starvation and refeeding. The activity was not affected by adenine nucleotides and a variety of other metabolites. The affinity for GTP ( $K_m$  0.4 mM) was higher than that for ITP ( $K_m$  1.6 mM) but the  $V_{max}$  was lower. GDP inhibited competitively with the nucleoside triphosphate substrate ( $K_i$  0.4 mM). The kinetic data suggest that the GTP/GDP ratio is an important determinant of the activity of phosphoenolpyruvate carboxykinase activity in situ. The differences between pigeon and rat liver with regard to the mechanism



of phosphoenolpyruvate synthesis are emphasized.

Pyruvate kinase was found in the "soluble" fraction, but well-washed mitochondria were always significantly contaminated. Pyruvate kinase and enolase activity did not change during starvation and refeeding, (50 - 70, and 150 - 200  $\mu\text{Moles/min./g. dry weight at } 25^{\circ}$ , respectively). Pigeon-liver pyruvate kinase was inhibited by ATP and  $\text{Ca}^{2+}$  ions, and activated by  $\text{K}^{+}$  and  $\text{Mg}^{2+}$  ions and by the substrates; a number of other metabolites tested did not affect the enzyme activity.

Nucleoside diphosphokinase was present primarily in the mitochondria and was "latent". The activity was 250 - 300  $\mu\text{Moles/min./g. dry weight at } 25^{\circ}$ . Kinetic studies showed that the reaction mechanism was of the "double displacement" type;  $K'_S$  values for GTP and ADP were  $8 - 9 \times 10^{-5}\text{M}$  and  $4 - 5 \times 10^{-5}\text{M}$  respectively. ADP inhibited at concentrations above 0.1 mM, competitively with GTP. The results suggest that the mitochondrial GTP/GDP ratio is continuously "geared" to that of ATP/ADP.

Previous studies of phosphoenolpyruvate synthesis in liver mitochondria are reviewed, with particular attention to species differences, compartmentation of phosphate transfer in the mitochondria and the problem of "turnover" of phosphoenolpyruvate.

Pure washed pigeon-liver mitochondria formed phosphoenolpyruvate from oxaloacetate, malate, fumarate, pyruvate + bicarbonate,  $\alpha$ -oxo-glutarate, succinate and citrate (in order of effectiveness). Inorganic phosphate (5 - 15 mM) and added adenine nucleotides (1 - 5 mM) were stimulatory. The accumulation was linear with time only if  $\text{Mg}^{2+}$  ions were not added.



Contaminant pyruvate kinase was partly responsible for "turnover" of phosphoenolpyruvate which occurred in the presence of  $Mg^{2+}$ , but a fall in the ATP/ADP ratio (due to the concerted action of  $Mg^{2+}$ -dependent adenylate kinase and adenosine triphosphatase) was of greater importance, since phosphoenolpyruvate formation was inhibited in the presence of low concentrations of  $Ca^{2+}$  ions (0.5 mM), dinitrophenol and oligomycin, even when pyruvate kinase activity was totally suppressed due to the absence of added  $Mg^{2+}$  ions. A fall in the ATP/ADP ratio was associated with an increase in the formation of citrate and  $\alpha$ -oxo-glutarate, which was not related to changes in the concentration of oxaloacetate. Experiments with atractyloside showed that phosphoenolpyruvate carboxykinase and nucleoside-diphosphokinase were situated within the atractyloside-sensitive "barrier" to the mitochondrial metabolism of added adenine nucleotides. Phosphoenolpyruvate synthesis was not functionally related to the rate of  $\alpha$ -oxo-glutarate oxidation, suggesting that there was no compartmentation of phosphate transfer between the substrate-level phosphorylation and phosphoenolpyruvate carboxykinase.

Synthesis of phosphoenolpyruvate from pyruvate only occurred in the presence of 5 - 10 mM bicarbonate. A progressive fall in the ATP/ADP ratio due to addition of dinitrophenol or  $Mg^{2+}$  ions depressed the synthesis of phosphoenolpyruvate before it affected the formation of malate. The greater part of the pyruvate carboxylase activity was also found to lie within the atractyloside-sensitive "barrier".

The studies indicate that the regulation of phosphoenolpyruvate



and citrate synthesis in situ in pigeon-liver mitochondria is critically dependent on the phosphorylation potential of the mitochondrial adenine nucleotides. Various molecular mechanisms for this effect are discussed. A second and possibly associated factor is the availability of oxaloacetate.

Both the mitochondrial synthesis of phosphoenolpyruvate and the net formation of fructose 6-phosphate in the cytoplasm are subject to "steady-state" control by the energy status of the respective compartments. The evidence for the physiological importance of such control mechanisms is discussed, with special reference to present concepts on the metabolism of free nucleotides within liver cells.

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A B B R E V I A T I O N S

The following abbreviations have been used:

|                     |                                                            |
|---------------------|------------------------------------------------------------|
| AMP                 | Adenosine 5'-monophosphate                                 |
| ADP                 | Adenosine 5'-diphosphate                                   |
| ATP                 | Adenosine 5'-triphosphate                                  |
| GDP                 | Guanosine 5'-diphosphate                                   |
| GTP                 | Guanosine 5'-triphosphate                                  |
| UDP                 | Uridine 5'-diphosphate                                     |
| UTP                 | Uridine 5'-triphosphate                                    |
| CDP                 | Cytidine 5'-diphosphate                                    |
| CTP                 | Cytidine 5'-triphosphate                                   |
| IDP                 | Inosine 5'-diphosphate                                     |
| ITP                 | Inosine 5'-triphosphate                                    |
| CoA                 | Coenzyme A                                                 |
| NAD <sup>+</sup>    | Nicotinamide adenine dinucleotide, oxidised form           |
| NADH                | Nicotinamide adenine dinucleotide, reduced form            |
| NADP <sup>+</sup>   | Nicotinamide adenine dinucleotide phosphate, oxidised form |
| NADPH               | Nicotinamide adenine dinucleotide phosphate, reduced form  |
| Glucose 1-P         | D-Glucose 1-phosphate                                      |
| Glucose 6-P         | D-Glucose 6-phosphate                                      |
| Hexose 6-P          | Combined D-hexose 6-phosphates                             |
| 6-P gluconate       | D-6-phosphogluconate                                       |
| Fructose 6-P        | D-Fructose 6-phosphate                                     |
| FDP                 | D-Fructose 1,6-diphosphate                                 |
| Triose-P            | Combined triose phosphates                                 |
| $\alpha$ -Glycero-P | L- $\alpha$ -glycerophosphate                              |



|                |                                      |
|----------------|--------------------------------------|
| 1,3-diPGA      | D-1,3-diphosphoglycerate             |
| 3-PGA          | D-3-phosphoglycerate                 |
| 2-PGA          | D-2-phosphoglycerate                 |
| PGA            | Combined D-2 and 3-phosphoglycerates |
| PEP            | Phosphoenolpyruvate                  |
| UDPG           | Uridine diphosphoglucose             |
| Pi             | Inorganic orthophosphate             |
| PPi            | Inorganic pyrophosphate              |
| EDTA           | Ethylene diamine tetra-acetate       |
| TCA            | Trichloroacetic acid                 |
| "Tris"         | Tris (hydroxymethyl) aminomethane    |
| DNP            | 2,4-Dinitrophenol                    |
| Glucose 6-Pase | D-Glucose 6-phosphatase              |
| FDPase         | D-Fructose diphosphatase             |
| PEPCK          | Phosphoenolpyruvate carboxykinase    |
| PFK            | 6-Phosphofructokinase                |
| PK             | Pyruvate kinase                      |
| NuDiKi         | Nucleoside diphosphokinase           |
| conc'n         | concentration                        |
| wt.            | weight                               |



## CHAPTER 1

### GENERAL INTRODUCTION

From the point of view of metabolic regulation, the carbohydrate metabolism of the liver has particular interest because it is subject to large physiological variations both in the rates and direction of the overall transformations. Thus the organ is capable both of degrading carbohydrate by glycolytic reactions and of resynthesizing it from non-carbohydrate precursors (gluconeogenesis). Significant advances have been made in the elucidation of the enzymic steps involved in both these pathways (see Krebs, 1964, ☐), but rather less is known of the co-ordinated mechanisms by which control of the multi-enzyme systems is effected.

Pigeon-liver homogenates have been the only cell-free preparation hitherto described in which the entire sequence of gluconeogenic reactions from lactate and other precursors is carried out at rates equivalent to those observed in intact livers (Krebs et al., 1964/ ). This system was considered suitable for detailed studies of the control mechanisms of hepatic glycolysis and gluconeogenesis, since the homogenates can also degrade carbohydrate in the absence of glucogenic precursors. Recent studies suggest that the special properties of rate-limiting enzymes might be important in the regulation of carbohydrate metabolism in the



liver; the homogenates form a convenient experimental material for examining further whether the activations and inhibitions found with isolated enzymes may operate in the multi-enzyme system of the liver cells.

Washed pigeon-liver mitochondria are capable of synthesizing PEP at high rates from a variety of precursors (Bartley & Avi-Dor, 1955). Although rat liver has been intensively studied with respect to the path of carbon from pyruvate to PEP, and to the properties, distribution and activities of the relevant enzymes, the significant species differences that exist in this area of metabolism (Nordlie & Lardy, 1963,b) suggested that valuable information might be gained from a detailed study of the mechanism and control of PEP synthesis in pigeon liver.

To place in perspective the studies with pigeon-liver homogenates and mitochondria reported in this thesis, a brief review is necessary of some of the factors that are important in the control of enzyme activity in an organ such as the liver:

(i) Availability of substrates. The liver may at any time be presented with large amounts of lactate (severe exercise), glucose (large carbohydrate meal), ethanol, triglycerides (fatty meal) or free fatty acids (states of fatty acid mobilization from adipose tissue). Since the liver appears to be freely permeable to all these substances (Hohorst et al., 1959; Cahill et al., 1958; French, 1963; Gordon, 1957), dramatic changes in the activity of many enzymes may be expected in each case as a result of increased saturation with substrates. This



necessitates an elaborate system of metabolic signals to permit the occurrence of a controlled and co-ordinate transition from one metabolic pattern to another.

Substrate availability ("availability control") within the network of reactions can be a potent regulatory factor, e.g. at branching points, or when there is competition for shared cofactors (Krebs, 1959, 1964,b). There is increasing evidence for the regulation of individual multi-substrate reactions by "steady-state control" involving the ratios of nucleotide or other substrates as the regulatory factors (Klingenberg, 1964). This may express itself at the active site, depending on the detailed mechanism of catalysis, or it may involve more complex "second-site" phenomena of enzyme control (see (ii)). The biological efficiency of this type of control is potentially much higher than simple "availability control" of enzyme reactions. An example of the difference between the two concepts is the control of mitochondrial respiration by ADP, irrespective of the ATP concentration (Chance & Williams, 1956), and the control by the "phosphorylation potential" ( $[ATP] / [ADP] \cdot [Pi]$ ) which is based on the concept of the reversibility of oxidative phosphorylation (Klingenberg, 1964).

(ii) Non-substrates that affect enzyme activity. Many specific and non-specific inhibitors and activators of hepatic enzymes are known. Relatively few substrate analogues have hitherto been considered to have physiological significance; "allosteric" or "second-site" effects (Monod et al., 1963), on the other hand, are widely accepted as agents in the



control of metabolic pathways. The theory of "allosteric control" of rate-limiting enzymes was based primarily on certain characteristic properties encountered in enzymes involved in feed-back regulation of synthetic pathways. Such properties are:

a) Specific inhibition by compounds usually not sterically related to the substrates of the individual reactions.

b) Unusual inhibition- and substrate-kinetics; often the relationship of substrate or inhibitor concentration to the velocity is sigmoid, which is ascribed to "co-operative" effects of the binding of these molecules on the enzyme activity.

c) Mild denaturation often removes the effects of the "allosteric" inhibitor without decreasing the rate of catalysis ("desensitization").

It was postulated that binding of the "allosteric effector" occurs at a site other than the active catalytic site, and that the effect on the enzyme activity is mediated by reversible conformational changes in the protein. The analogy with haemoglobin suggested that "allosteric" enzymes might be oligomers; more recently, Monod et al., (1965) have proposed a general model for "allosteric" systems in which the enzymes exist in two conformational states (with different substrate kinetics), which are in equilibrium. The effects of activators or inhibitors is supposed to depend on their affinities for the two forms such that their binding affects the equilibrium. Gerhart & Schachman, (1965) have reported evidence for two types of sub-units in the bacterial enzyme aspartate transcarbamylase, one of which bears the catalytic active site



and exhibits "normal" kinetic behaviour, while the other (the regulatory sub-unit) contains the binding site of the "allosteric" inhibitor CTP. Ferdinand (1966) has recently proposed a kinetic explanation for the properties of PFK in relation to at least two of its effectors, ATP and fructose 6-P.

It would appear that no general model may account for the special features of the "allosteric" effects that are undoubtedly exerted on many enzymes, including mammalian enzymes. Nucleotides and other specific metabolites are commonly involved, and a number of hepatic enzymes appear to exhibit such effects under a variety of conditions. The attractive features of the "allosteric" theory from the point of view of the regulation of metabolism, are the efficient control of the activity of rate-limiting enzymes, arising from the sigmoid nature of the substrate- or inhibitor-velocity relationship (such as is decisively important in the physiological function of haemoglobin) and the possibility of control of metabolic pathways by sterically-unrelated metabolites produced by separate reaction sequences. At the present time, the evidence for a true regulatory role of many of these interactions is somewhat sparse. This is partly due to technical difficulties encountered in the measurement of the concentrations of intermediates in intra-cellular compartments of intact tissues, and the fact that many of the special properties may be different in the intracellular milieu, with its high protein concentration and intricate structural organization. The study of metabolic control in homogenates and in isolated particles bridges the gap between



experiments with purified enzymes and those with intact tissues, in that conditions and concentrations can be manipulated to some extent, decreasing the need for purely extrapolative conclusions.

(iii) Inter-conversions of enzyme forms. A well-known example of this kind of control is afforded by the glycogen phosphorylase system. The molecular basis of the inter-conversion of the more active phosphoenzyme and the less active dephosphophosphorylase (which is activated by AMP) is fairly well understood, and the independent enzymes catalysing it have been studied in relation to the actions of hormones like adrenaline and glucagon (Wosilait & Sutherland, 1956). Glycogen synthetase appears to be present in rat liver in two forms (Steiner, 1964); preliminary evidence for an inter-conversion has been reported (Hizukuri & Larner, 1963). Such systems provide the means whereby hormones can override the endogenous cellular control mechanisms by converting enzymes into forms not decisively subject to feedback control by intracellular metabolites.

In the case of a number of enzymes, transitions of a different kind, at present ill-understood, may have regulatory significance. Some of these involve reversible "binding" of enzymes to particles or membranes, e.g. liver glycogen phosphorylase (Tata, 1964), or conformational changes, such as those described by Steiner et al., (1965) for glycogen synthetase and by Foster et al., (1966) in the case of rat-liver PEPCK.

(iv) Enzyme concentrations. Many hepatic enzymes vary in their overall tissue activities when diets are changed or certain hormones administered. Both substrate supply and hormonal effects ("induction



permissive effects") appear to be involved in the control of enzyme concentrations, e.g. the induction of glucokinase in rat liver by carbohydrate feeding in the presence of insulin (Salas et al., 1963). Control of the enzyme concentration is effected by changes in the rate of enzyme biosynthesis or degradation, or both (see Schimke et al., 1965). In many cases it has proved difficult to assign true primary regulatory significance to the increased capacity of enzymes so affected (see Fitch & Chaikoff, 1960), although it is probable that the changes in enzyme concentration, once established, exert a powerful "coarse" control on metabolic events.

The pathways of glycolysis and gluconeogenesis in the liver are depicted in SCHEME A, with special reference to reactions that may have regulatory significance because of their strategic position, or because they are catalysed by enzymes with low activities or special properties.

The regulation of gluconeogenesis in the liver. Krebs (1964,a) has reviewed the physiological importance of gluconeogenesis and has stressed the large variations in the rates of this major biosynthetic pathway according to the requirement of the animal for a constant supply of glucose, and the need to remove products of metabolism such as lactate derived from muscles during severe exercise.

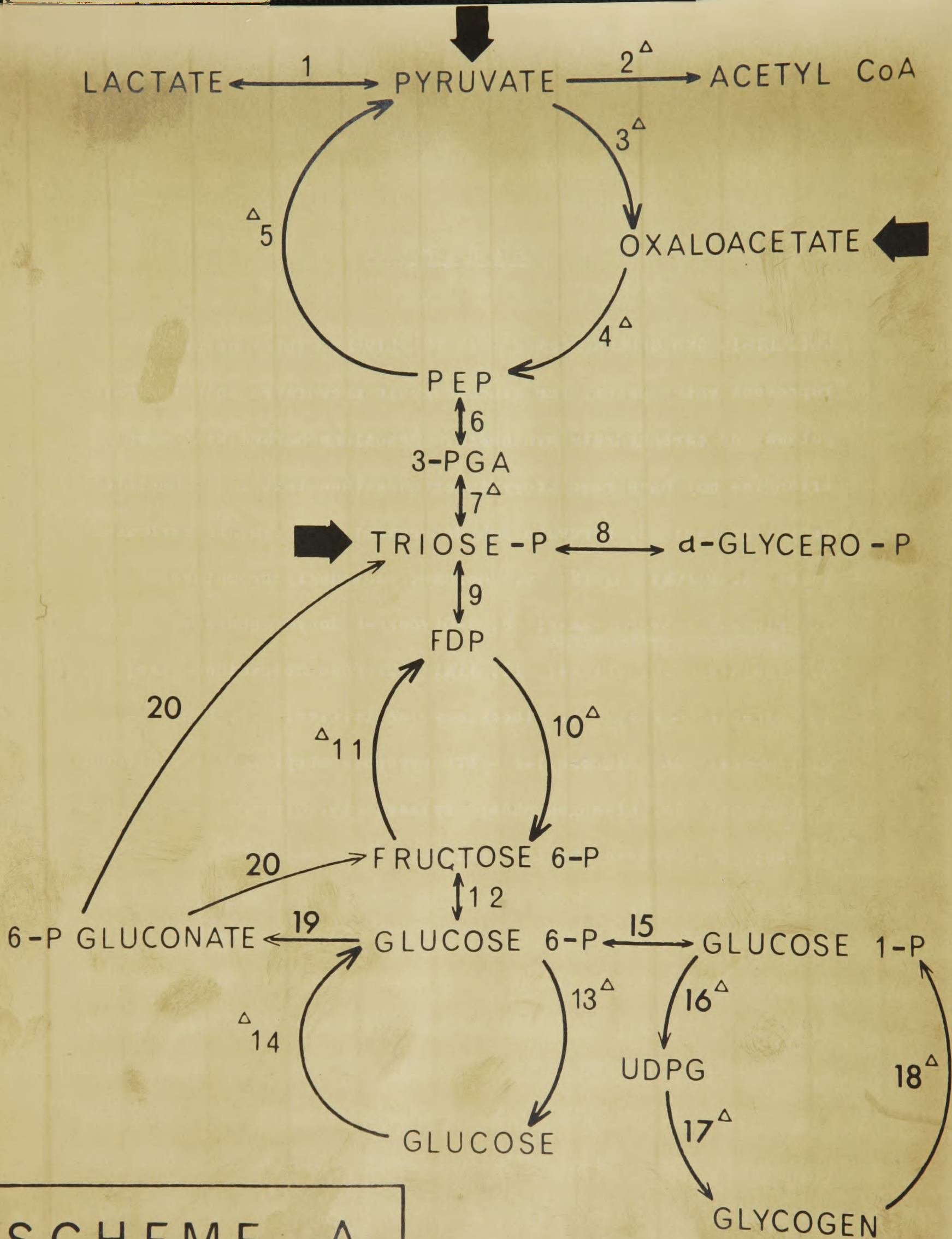
The rate of gluconeogenesis from amino acids and a number of other precursors depends to a certain extent on the availability of substrates; thus the early glycogenesis which followed administration of



## S C H E M E A

GLYCOLYSIS AND GLUCONEOGENESIS IN THE LIVER: Thick arrows represent entry points for gluconeogenic precursors into the main pathway of carbohydrate synthesis. Reactions marked with open triangles may have regulatory importance (see text). 1. Lactate dehydrogenase; 2. Pyruvate dehydrogenase; 3. Pyruvate carboxylase; 4. PEPCK; 5. PK; 6. Enolase, phosphoglyceromutase; 7. Triose-P dehydrogenase; } 8.  $\alpha$ -Glycero-P dehydrogenase;  
phosphoglycerate kinase }  
9. Aldolase; 10. FDPase; 11. PFK; 12. Phosphohexoisomerase; 13. Glucose 6-Pase; 14. Glucokinase activities; 15. Phosphoglucomutase; 16. Glucose 1-P - UTP pyrophosphorylase; 17. Glycogen synthetase; 18. Glycogen phosphorylase; 19. Glucose 6-P dehydrogenase; 20. Pentose shunt reactions.





SCHEME A



glucocorticoids to rats was associated with increased hepatic free amino acid levels before any other changes were detectable (Weber et al., 1965,c). Hornbrook et al., (1965) observed a rapid increase in liver glycogen after injections of large amounts of lactate, while the tissue content of practically all the intermediates of carbohydrate metabolism was increased. Another factor of importance is the activity of the enzymes initiating the substrate degradation. Thus Krebs (1964,a) showed that the rates of carbohydrate synthesis from a number of precursors in rat kidney-cortex slices were lower than those from early intermediates. The activities of a number of amino-acid degrading enzymes are increased in the liver under physiological conditions where the rate of gluconeogenesis is increased (see Knox & Greengard, 1965).

Most of the precursors are converted into the key intermediates pyruvate, oxaloacetate and triose-P. At each of these stages (and at some others) there is a choice of reactions leading to different end-products. This requires the operation of mechanisms to direct the metabolites into the pathway leading to glucose formation, as well as mechanisms to control the actual rate of flux.

The control of pyruvate metabolism. Competing reactions for pyruvate are carboxylation to oxaloacetate (pyruvate carboxylase), oxidative decarboxylation to acetyl CoA (pyruvate dehydrogenase) complex and transamination to alanine. Some aspects of the control of pyruvate metabolism in rat liver have been studied recently by Haslam & Krebs (1963)



and Berry (1965). The subject is further discussed in Chap. 4.

The control of oxaloacetate metabolism. This key intermediate is a reactant for the initiation of the tricarboxylic acid cycle (citrate synthase) as well as for gluconeogenesis (synthesis of PEP by PEPCK). It can also undergo reduction by NADH to malate (NAD<sup>+</sup>-linked malate dehydrogenase) or transamination to form aspartate. Some of the mechanisms regulating the metabolism of oxaloacetate in the liver are discussed in Chap. 4, with special reference to the pigeon.

The control of PEP metabolism. Gluconeogenesis from oxaloacetate requires that the removal of PEP by pyruvate kinase be limited. The mechanism for this appears to differ in various species and organs. In yeast (Ruiz-Amil et al., 1965) and in rat liver (Krebs & Eggleston, 1965) the enzyme concentration varies greatly in an inverse relation to the rate of gluconeogenesis, while the last-named authors have failed to demonstrate similar changes in rat kidney-cortex. (See Chap. 4, Section D).

The control of triose-P dehydrogenase in relation to gluconeogenesis. The enzyme reversibly converting 1,3-DiPGA into glyceraldehyde 3-phosphate in rat liver is very active (v. Fellenberg et al., 1962) in comparison with other glycolytic enzymes. The NAD<sup>+</sup>-NADH system in the cytoplasmic compartment is highly oxidised (Hohorst et al., 1959), whilst very little is known about the metabolic behaviour in this compartment of Pi, another reactant. The detailed catalytic mechanism (of the rabbit-muscle enzyme)



is very complex (Velick & Furfine, 1963). All these factors have tended to obscure the possible regulatory significance of this step in carbohydrate synthesis, which is discussed in Chap. 3.

The control of the conversion of FDP into fructose 6-P. This step is catalysed by FDPase, which is a  $Mg^{2+}$ -dependent, highly specific phosphatase present primarily in gluconeogenic tissues (Gomori, 1943), although it has recently been found also in striated muscle (Krebs & Woodford, 1964). Pogell & McGilvery (1952) demonstrated that the enzyme was active at neutral pH as well as at pH 9 and suggested that the enzyme was important in gluconeogenesis (a possibility previously referred to by Lardy, 1949, and Olson, 1951). They also observed an activation by autolysis and papain treatment, which indicated that there might be two forms of the enzyme.

Taketa & Pogell (1963, 1965) described a specific inhibition of rat- and rabbit-liver FDPase by AMP at low concentrations, and also reported that the enzyme was inhibited by substrate concentrations higher than 0.1 mM, although the  $K_m$  for FDP was very low (less than 5  $\mu M$ ). They drew attention to the relatively low activity of FDPase in the liver, pointing to a rate-limiting role for certain metabolic transformations. These observations were confirmed in essence by Mendicino & Vasarhely (1963) for the kidney enzyme, and by Newsholme (1963) and Underwood & Newsholme (1965,a) for rat-liver FDPase. Of the large number of metabolites tested by the last-named authors, only AMP, FDP and  $Mg^{2+}$  ions affected



the activity to a significant extent. These studies were carried out at neutral pH in the presence of 2-mercaptoethanol, which was markedly stimulatory. EDTA also increased the enzyme activity, an effect apparently not due to the chelation of added metal ions.

Taketa & Pogell (1965) reported that papain digestion removed the inhibitory effects of AMP long before the catalytic activity was lost. Luppis et al., (1964) found preliminary evidence suggesting two interconvertible forms of rabbit-liver FDPase, based on the effects of pre-incubating the purified enzyme at pH 7.4; the activating effect was blocked by FDP. Pontremoli et al., (1965) discussed the possibility that an -SH group, not situated at the active site, might regulate the activity of FDPase depending on whether it was ionized or "blocked". This conclusion was reached after studies on the effect of dinitrophenylation of purified rabbit-liver FDPase. The studies of Rosen & Rosen (1966) on FDPase from *Candida utilis*, have revealed the existence of two separate reactive sites for FDP and AMP, both containing two pairs of specific lysyl and tyrosyl groups involved in binding or the maintenance of conformation. Desensitization of the enzyme was possible by using 2M urea or by carrying out dinitrophenylation in the presence of FDP. The enzyme from *Candida* appears not to consist of sub-units, although the mammalian enzyme has been dissociated into at least 4 sub-units (Ellis et al., 1966).

It is evident from the above discussion that liver FDPase is a complex enzyme with "allosteric" properties. It catalyses an irreversible



reaction overcoming one of the principal thermodynamic barriers to the reversal of glycolysis (Krebs, 1954) and it is a site of metabolic regulation. Evidence for this is discussed in Chap. 3.

The control of glycogen synthesis. The transglucosylation from UDPG to glycogen, catalysed by glycogen synthetase (Leloir & Cardini, 1957) is probably the rate-limiting step in glycogen synthesis in the liver (Steiner, 1964), although the experimental evidence for this is not complete. Hornbrook et al., (1965) observed a "cross-over point" (Chance & Williams, 1956) between UDPG and glycogen in rat livers after hydrocortisone administration. The activity of the enzyme is controlled in part by the relative proportions of the glucose 6-P-dependent (D) and glucose 6-P-independent (I) forms, although the inter-conversion between the two forms described by Hizukuri & Larner (1963) has not yet been confirmed (Steiner et al., 1965). The glucose 6-P concentration, transitions between particle-bound and soluble forms (Tata, 1964) and variations in the total amount of enzyme (Steiner & King, 1964) are other factors that may have regulatory importance. The decisive role of insulin in determining the concentration of active enzyme has been emphasized (Steiner, 1964). Of particular interest is the recent report (Steiner et al., 1965) that hepatic glycogen synthetase is markedly inhibited by UDP, competitively with UDPG. The activating effect of glucose 6-P is partly due to an antagonism of this inhibition. Kornfeld (1965) has shown that rat-liver UTP-glucose 1-P pyrophosphorylase is inhibited by UDPG (competitively with respect to UTP) and by AMP,



such that increasing concentrations of glucose 1-P are associated with progressive substrate inhibition in the presence of AMP. Both these reports are in accord with the observation of Nigam & Fridland (1964) and Zancan & Hers (1965) of the requirement of UDPG and glycogen synthesis in pigeon-liver homogenates for concomitant energy metabolism. Thus the UTP/UDP ratio, which is related to the ATP/ADP ratio by the NuDiKi reaction, and also to the AMP concentration by the action of adenylate kinase, might be expected to affect the rate of the conversion of glucose 1-P into glycogen. This would constitute another example of "steady-state control" by nucleotides, which was previously described for the control of respiration and which expressed itself in a different way (also involving AMP) at the level of the FDP-fructose 6-P inter-conversion (see Chap. 3) and in the synthesis of PEP in pigeon liver mitochondria (see Chap. 4).

The regulation of glycolysis in the liver. One of the important physiological functions of hepatic glycolysis is the conversion of excess carbohydrate supplied to the liver, into fats (lipogenesis). Glycolysis provides both  $\alpha$ -glycero-P and  $C_2$  units derived from pyruvate, as well as some of the reducing power for the synthesis of fatty acids (Wise & Ball, 1964). The rate of lipogenesis in the liver varies greatly; it is decreased during fasting and in diabetes, and is enhanced when animals are fed high carbohydrate diets or when refeeding is begun after starvation (see Fritz, 1961; Masoro, 1962). In all these states, the



gluconeogenic capacity of the liver is obversely affected.

There is evidence that when starved rats are refed, the "switch-off" of gluconeogenesis has a slow onset period, so that a great deal of glycogen immediately laid down may originate from non-carbohydrate precursors (Friedmann et al., 1963). At a certain stage of saturation with glycogen, a "switch" to lipogenesis occurs with cessation of gluconeogenesis.

The regulation of glycolysis, like that of gluconeogenesis, involves a number of key intermediates for which there is competition, and a number of rate-limiting enzymes, most of which do not participate in the direct pathway of carbohydrate synthesis. This permits independent control of the direction and rate of flow.

The regulation of glucose phosphorylation. At least 2 glucose-phosphorylating enzymes are present in the liver. Glucokinase has a high specificity (for glucose and mannose) but a low affinity for its substrate, such that the  $K_m$  for glucose is higher than the plasma glucose concentration; the enzyme is also not subject to inhibition by glucose 6-P. The activity varies in different nutritional and hormonal states, and insulin is required for induction (see Walker, 1966 for review). A group of less specific "animal hexokinases", with high affinity for substrate (Crane & Sols, 1954), is also present. These are inhibited by glucose 6-P, a fact which has regulatory importance for glycolysis in other tissues, e.g. brain (Lowry & Passonneau, 1964) and



rat heart (Newsholme & Handle, 1961 ). This may apply also to the liver, especially in those species where the quantitative contribution of hexokinase to the total phosphorylating activity is large, e.g. the guinea-pig (Walker, 1963) and pigeon (unpublished work).

The regulation of glycogenolysis. Although hormonal control of glycogenolysis is of paramount importance in the liver, the activating effect of AMP on dephosphophosphorylase (Wosilait & Sutherland, 1956) is entirely in keeping with the effects of the same metabolite on PFK and FDPase.

The regulation of PFK in the liver. Hepatic PFK has been studied by Passonneau & Lowry (1964) and Underwood & Newsholme (1965,b). The results were broadly similar to those described for PFK preparations from other tissues, such as skeletal muscle (Passonneau & Lowry, 1962) and sheep heart (Mansour, 1963). Partially-purified PFK from rat liver is strongly inhibited at low concentrations of ATP (below 0.5 mM) and the inhibition is potentiated by citrate, also at low concentrations (less than 0.5 mM).  $Mg^{2+}$  ions are also inhibitory, although the Mg-ATP complex is less inhibitory than free ATP. A number of metabolites de-inhibit the ATP- or citrate-inhibited enzyme, e.g. FDP, AMP, ADP and Pi, in order of potency.

Exact kinetic studies have been hampered by the tendency of the properties to change when less dilute enzyme solutions are used. A number of complex aggregation-disaggregation phenomena have been



encountered in studies of purified muscle PFK; these take place without change in activity as measured subsequently at high dilution (e.g. Ling et al., 1965). A second transformation is a rapid inactivation process under various conditions, such as pre-incubation at low pH, which has been found by Mansour (1965) to consist also of a monomer-polymer transformation. In this case the monomeric form is inactive. Vinuela et al., (1964) have described an interconversion of two forms of PFK in yeast, which resembles the b to a transformation of glycogen phosphorylase. A further development has been the discovery of the association of nucleic acid with PFK from skeletal muscle (Pette & Hofer, 1966). All these reports emphasize the great complexity of PFK and its importance as a regulatory enzyme, also in the liver. It is apparent that the net rate of fructose 6-P phosphorylation will depend on the activity of FDPase. The control of these two enzymes, and their importance as rate-limiting stages of carbohydrate synthesis and degradation, has been investigated in pigeon-liver homogenates (see Chap. 3).

Other steps in glycolysis that may have regulatory importance.

Woods & Burk (1965) have produced evidence in liver slices that glycolysis is inhibited at the stage of triose-P dehydrogenation when NADH is allowed to accumulate. A similar explanation could account for the stimulatory effect of aerobic pre-incubation on the rate of lactate formation from glucose in liver slices (Negelein & Noll, 1963). Soling et al., (1966) have failed to find evidence for a "block" in glycolysis at triose-P dehydrogenase in diabetic rat liver, based on the measurements



of intermediates in perfused livers and in the livers of intact animals. Such a "block" had previously been postulated by Lamprecht & Trautschold (1958). (See Chap. 3).

Pyruvate kinase activity is an important determinant of the fate of PEP (see Chap. 4).

#### Special features of avian hepatic carbohydrate metabolism.

Hazelwood (1965) has reviewed the subject of carbohydrate metabolism in birds, with special reference to chickens and pigeons. He has stressed that avian metabolism differs from that of mammals not in kind but in quantity. Some special features are the high blood glucose concentrations (150 - 250 mg./100 ml.) and the special sensitivity to glucagon, which is thought to play a central role in glucose homeostasis in birds (Hazelwood & Lorenz, 1957,a). A "diabetic syndrome" does not follow pancreatectomy or the administration of alloxan, as regards carbohydrate metabolism, and there appears to be a special degree of tolerance to doses of insulin that would be lethal in other animals. This may be related to the high activity of insulinases in the livers of avian species. Disturbances of fat metabolism do occur after pancreatectomy (Stamler et al., 1950) but the manifestations are not severe and ketosis does not occur. The adrenal glands have been shown to play a role in the stimulation of gluconeogenesis during hypoglycaemia induced by large doses of insulin, giving rise to "rebound" hyperglycaemia and glycogen synthesis (Hazelwood & Lorenz, 1957,b). This effect was ascribed to the action of



adrenaline rather than steroid hormones (see Exton & Park, 1966, for an effect of adrenaline on gluconeogenesis from lactate in perfused rat liver). Pigeon livers contain very active lipogenic systems and are rich in malate enzyme (Ochoa et al., 1950) and fatty acid synthetase (Wakil et al., 1966). High rates of gluconeogenesis from lactate were obtained only in pigeon-liver homogenates, and not in those of many other species of mammals or birds (Krebs et al., 1964). These active anabolic systems may be an expression of the need for large amounts of storage materials during food deprivation for long periods of flight, as well as the requirement rapidly to remove lactate accumulating during severe exertion.



## CHAPTER 2

### METHODS AND MATERIALS

Chemicals. Except as stated below, all chemicals were of analytical grade; usually those supplied by British Drug Houses Ltd., Poole, Dorset were used. The following were laboratory reagent grade chemicals: D-fructose, L-aspartic acid, L-alanine, L-glycine, L-glutamic acid, imidazole, sodium n-butyrate, oxamic acid, propionic acid, isobutyric acid, iodoacetic acid, hydrazine hydrate and nicotinamide.

NUCLEOTIDES: The sodium salts of ATP, ADP and AMP were products of C.F. Boehringer u. Soehne G.m.b.H., Mannheim, Germany. By enzymatic analysis there was a small ( $< 1\%$ ) cross-contamination of all 3 adenine nucleotides even in fresh solutions. GDP and GTP were obtained as lithium salts from the same source. In view of their instability, they were used within a month of arrival and the percentage purity was determined by enzymatic means at each use.  $\text{NADP}^+$ , NADPH, Coenzyme A,  $\text{NAD}^+$  and NADH were also products of C.F. Boehringer u. Soehne. The AMP content of NADH samples was kept low by using only fresh solutions, while in a special case (enzymatic determination of ADP and AMP) solutions were treated with bacterial alkaline phosphatase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) as suggested by Lowry et al., (1964). GMP, ITP, IDP, IMP, UDP, UTP, and cyclic 3',5'-AMP were obtained from Sigma Chemical



Co., St. Louis, Mo., U.S.A.

**GLYCOLYTIC INTERMEDIATES:** The following were products of C.F. Boehringer u. Soehne: D-glucose 6-P, D-fructose 6-P, FDP, 3-PGA and pyruvate (all sodium salts); PEP (potassium salt) and 2-PGA (barium salt). The last-named was converted into sodium 2-PGA by dissolving it in 0.1N-HCl; a slight excess of  $\text{Na}_2\text{SO}_4$  was added, followed by centrifugation. The supernatant was neutralized and stored at  $-15^\circ$  after standardization by enzymatic analysis. L- $\alpha$ -glycero-P was obtained as the tricyclohexyl-ammonium salt from Calbiochem., Los Angeles, Calif., U.S.A. Crystalline L-lactic acid was prepared by Mr. T. Gascoyne according to Krebs (1961) and was stored in a desiccator at  $2^\circ$ . The sodium salt was used.

**OTHER METABOLITES:** Oxaloacetic acid was prepared according to the method of Wohl & Claussner (1907) by Mr. T. Gascoyne. It was stored dry at  $-15^\circ$ . Freshly-prepared solutions were brought to pH 7 with NaOH immediately before experiments were due to begin. Sodium fumarate, sodium succinate and sodium  $\alpha$ -oxoglutarate were from C.F. Boehringer u. Soehne, while L-malic acid was obtained from Koch-Lights, Colnbrook, Bucks. Octanoic and oleic acids were gifts of Dr. M.B. Enser. Palmityl-Coenzyme A was from the Sigma Chemical Co. Acetyl-Coenzyme A was prepared by the method of Ochoa (1955).

**OTHER CHEMICALS:** DNP was twice recrystallized from water and was a gift of Dr. M.W. Whitehouse. It was made up in 0.01M  $\text{NaHCO}_3$  as a stock solution which was stored at  $2^\circ$  and renewed every 2 weeks.



Oligomycin was a product of Mann Research Laboratories Inc., New York 6, N.Y., U.S.A., and was dissolved in ethanol. It was a gift of Dr. D.E. Griffiths, as was atractyloside, which was dissolved in water.

D,L-Carnitine was obtained from Calbiochem. Bovine serum albumen (fraction V) was from the Armour Pharmaceutical Co., Ltd., Chicago, U.S.A. and was used without further purification. Tris(hydroxymethyl)-amino-methane ("tris") and triethanolamine hydrochloride were from C.F. Boehringer u. Soehne.

Solutions. All solutions were made with glass-distilled water and were neutralized where necessary using a glass electrode or an external indicator. Solutions of labile compounds were stored at  $-15^{\circ}$  and were re-analysed or discarded whenever there was a risk of hydrolysis or other decomposition.

Enzymes. A crude preparation of carbonic anhydrase (EC 4.2.1.1.) was obtained from bovine erythrocytes by the method of Meldrum & Roughton (1933). The lyophilized buff-coloured powder was stored dry at  $2^{\circ}$  and was stable for more than 2 years. The yield was about 15 g. of powder from 12 L. of fresh blood. Aconitase (EC 4.2.1.3.) was prepared by Miss K. Taylor from pig heart by the method of Siebert (1963). Glucose oxidase, type II, was purchased from the Sigma Chemical Co. and "diazyme" was "Takamine" from the Miles Chemical Co., Clifton, N.J., U.S.A.

All other enzymes used in this work were products of C.F. Boehringer u. Soehne. They were stored at  $2^{\circ}$ . In certain cases where it was



necessary to free the enzyme suspensions of ammonium ions, dialysis for 24 hr. against 10 changes of distilled water was carried out in dialysis tubing previously treated by prolonged boiling and rinsing. When such enzymes were subsequently stored at  $-15^{\circ}$  frequent checks of activity were done because of the reduced stability.

Pigeons. Adult pigeons supplied by D. Harradine, Esq., 5, Whittlesea Road, March Cambridge, were used throughout this work. They were domestic pigeons of mixed strain and included both males and females. No attempt was made to study sex differences in metabolism or the effects of seasonal or diurnal variations (see Hazelwood, 1965).

DIETS: The pigeons were maintained on a diet of commercial "chicken-corn", consisting primarily of maize, barley and wheat corn. When they were fasted, food was withheld at 9 a.m., water being freely available. Using this regime, starvation for 48 hr. was sufficient to reduce the total carbohydrate content of the liver to less than 100  $\mu$ Moles/g. dry wt. in every case.

Tissue preparations and incubation techniques. HOMOGENATES of pigeon livers were prepared as described by Krebs et al., (1964) with modifications. The standard procedure was as follows: A pigeon was decapitated and blood allowed to drain from the neck for a minute. The liver was gently but rapidly excised. The organ was perfused with 15 ml. ice-cold 0.154M-KCl (pH 7.4) through the orifices of the portal and



hepatic veins, to remove blood and to assist in rapid cooling. The liver was blotted on hard filter paper, cleaned of adhering connective tissue and fat, weighed on a torsion balance and snipped into small pieces with scissors. Homogenization in chilled medium followed in an ice-cold stainless-steel homogenizer (Potter-Elvehjem type). This had an internal diameter of 2.5 cm. and a clearance of 30  $\mu$ . 10 up-and-down movements of the barrel, with the piston rotating at 2000 r.p.m., sufficed to give a smooth homogenate that could be pipetted easily. The preparation was kept cold and used within 3 min. of homogenization to avoid alkalization due to loss of  $\text{CO}_2$ . Phase contrast microscopy showed that there were no intact cells left.

Media: The standard medium was prepared by mixing 100 ml. 0.154M-KCl, 21 ml. 0.154M- $\text{NaHCO}_3$ , 4 ml. 0.2M- $\text{KH}_2\text{PO}_4$  pH 7.4, 2 ml. 0.1M- $\text{MgCl}_2$  and 0.4 ml. 0.1M-disodium EDTA. In certain experiments the content of Pi was increased 4.5-fold, and in others bicarbonate was reduced or omitted.

The homogenates (final dilution 1 : 15, w/v) were incubated at 40° in conical manometer vessels at shaking rates of 120-130/min. Other additions made to the vessels will be described in individual experiments. The final volumes were 4 ml. The gas space was  $\text{CO}_2 + \text{O}_2$  (5 : 95) or 100%  $\text{O}_2$  as required; in the latter case, 0.3 ml. of 2N-NaOH were placed in the centre wells.

To minimize changes in the concentrations of rapidly-reacting metabolites during manipulations after completion of incubations, these



were terminated in all cases by tipping 0.4 ml. of 20%  $\text{HClO}_4$  from a side-arm at the same time as the vessels were rapidly removed from the bath and immersed, with swirling, in an ethanol-dry ice mixture ( $-78^\circ$ ) for 15 sec., after which they were transferred to a tray of crushed ice. After 15 min. (sufficient for the extraction of the soluble glycogen and all enzymically-active proteins) the material was centrifuged at low temperature. A measured sample of the supernatant was neutralized with KOH (30%, saturated with KCl) in the presence of 2 drops of universal indicator, and the precipitate of  $\text{KClO}_4$  was removed by centrifugation after standing in ice for 2 hr. The supernatant was diluted to known volume and stored at  $-15^\circ$ .

The dry weight of each homogenate was determined by drying a known volume to constant weight at  $110^\circ$ , and correcting for the content of added salts.

PIGEON-LIVER MITOCHONDRIA were prepared, following generally the procedures of Bartley & Davies (1954). Modifications were devised, bearing in mind the fact that pigeons have no gall-bladders, necessitating storage of bile in the intra-hepatic system of bile-ducts. Since bile may damage mitochondria (Christ & Hulsmann, 1962) it was considered important to remove as much bile as possible before intracellular structures were exposed to it by homogenization of the liver.

The standard procedure was as follows: 2 pigeons were decapitated and exsanguinated. Livers were removed and kept for 2 min.



in polythene bags buried in crushed ice. After blotting on hard filter-paper and cutting away connective tissue, each liver was divided into 5-7 pieces which were placed on filter-paper and cut with a razor-blade at 1-2 mm. intervals in a criss-cross fashion. The pieces were now washed to remove as much bile as possible, by repeated inversion (6 times) in 5 volumes of freshly-prepared ice-cold 0.25M-sucrose, also containing 0.01M-"tris" and 0.0002M-EDTA, all at pH 7.4. Mincing in a Fischer mincer (Jouan, Paris) followed. A weighed portion of mince was homogenized in 4 volumes of sucrose solution by the same technique as that described for standard pigeon-liver homogenates. The homogenate was centrifuged at  $2^{\circ}$  in 15 ml. conical centrifuge tubes at 450g for 5 min. The supernatants were carefully decanted into thick-walled test-tubes ( $5 \times \frac{5}{8}$ " ) so that none of the reddish material (consisting of erythrocytes, nuclei and debris) was included. A portion of the mitochondrial fraction was lost in this procedure, as no absolutely sharp separation could be achieved by varying the gravitational force - the speed selected gave the largest yield of pure mitochondria. The tubes containing the supernatant were centrifuged at  $2^{\circ}$  for 10 min. at 2500g. The supernatants were decanted and the fatty deposits on the sides of the tubes thoroughly removed with absorbant paper. The mitochondria, now appearing as buff-coloured pellets, were re-suspended in 7 volumes of sucrose solution using a teflon pestle, rotating at 1000 r.p.m. Centrifugation at 2500g was repeated. The washing procedure was carried out again and the particles were finally suspended in 0.154M-KCl containing 0.01M-"tris",



at pH 7.4 and 0%. They were used immediately for incubations or assays of enzyme activity, or were frozen and stored at  $-15^{\circ}$ . Phase contrast microscopy showed that the preparation consisted of pure mitochondria of normal size and appearance.

Incubations were carried out in manometer vessels (gas phase 100%  $O_2$  or air) in a water-bath at  $30^{\circ}$ , with shaking. The final fluid volume was 4 ml./cup and 18-32 mg. of TCA-precipitable material (dry weight) were added to each cup. Other additions will be described in the text. The centre wells contained 0.3 ml. 2N-KOH. Incubations were terminated by rapid freezing and acidification as earlier described, and neutral extracts were prepared for analysis.

Known volumes of mitochondrial suspensions were treated in previously weighed tubes with (successively) 4%, 2% TCA and water, with centrifugation at 3000 r.p.m. between each mixing, and were dried to constant weight at  $110^{\circ}$ .

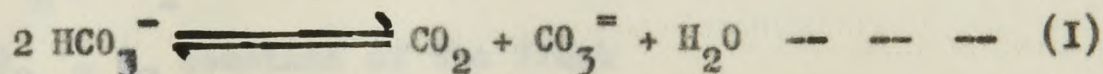
Measurement of gas exchanges. Since low concentrations of bicarbonate ions decrease the rate of gluconeogenesis in pigeon liver homogenates (Krebs et al., 1964) as well as the rate of glucose oxidation (see later) the  $O_2$  consumption was measured according to the principle of Warburg & Krippahl (1960). This permits the measurement of  $O_2$  uptake in the presence of a range of concentrations of  $CO_2$  and bicarbonate, each of which is kept constant by the use of " $CO_2$  buffers".

THEORY: Warburg (1919) used dilute carbonate-bicarbonate



buffering systems as part of incubation media in order to provide low constant pressures of  $\text{CO}_2$ . Theoretical considerations underlying the use of amines as " $\text{CO}_2$  buffers" for manometric work were discussed by Fardee (1949) and Krebs (1951, ). Warburg & Krippahl (1960) and Warburg et al., (1962) described the use of concentrated  $\text{K}_2\text{CO}_3$ - $\text{KHCO}_3$  mixtures, incubated in separate compartments of manometer vessels, to provide  $\text{CO}_2$  pressures up to 1000 mm. Brodie's fluid. This represented a great advance in the range of the method to include physiological  $\text{CO}_2$  pressures for mammalian systems.

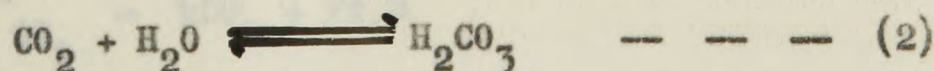
Aspects of the theory relevant to the practical use of the method are as follows: Mixtures of  $\text{K}_2\text{CO}_3$  and  $\text{KHCO}_3$  in solution generate or absorb  $\text{CO}_2$  according to reaction (I)



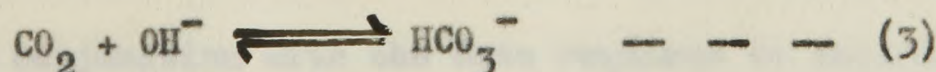
The Equilibrium constant K is given by

$$K = \frac{[\text{HCO}_3^-]^2}{[\text{CO}_3^{=}] \cdot [\text{CO}_2]}$$

The  $\text{CO}_2$  pressure thus depends on the ratio of the concentrations of  $\text{HCO}_3^-$  and  $\text{CO}_3^{=}$  ions, and is further critically influenced by the total concentration of ions and by the temperature. In the presence of carbonic anhydrase, which catalyses reaction (2)



and also reaction (3) at alkaline pH,



such mixtures maintain a constant  $\text{CO}_2$  pressure in a confined space above



them as long as diffusion from and into the solution is not limiting. (This can be ensured by rapid shaking and by the inclusion of small glass beads to increase turbulence.) However, the "CO<sub>2</sub> buffers" are not perfect absorbers of CO<sub>2</sub> and a correction factor ("Retention") has to be measured. This can be done experimentally (see below).

According to Warburg (1924), when 2 gases (e.g. O<sub>2</sub> and CO<sub>2</sub>) disappear or are evolved in a constant volume manometer, then

$$x.O_2 = h. \left[ \frac{kCO_2 \cdot kO_2}{kCO_2 + y \cdot kO_2} \right] = h. K.O_2$$

where  $x.O_2 = \mu l. O_2$  taken up

$h$  = mm. net pressure change

$kCO_2$  = CO<sub>2</sub> constant of the flask

$kO_2$  = O<sub>2</sub> constant of the flask

$y$  = Respiratory Quotient of the tissue

$KO_2$  = O<sub>2</sub> constant of the flask when both O<sub>2</sub> and CO<sub>2</sub> are involved in gas exchanges simultaneously.

Through retention,  $kCO_2$  increases. If  $k_{CO_2}^R$  is the CO<sub>2</sub> constant of the flask when retention of CO<sub>2</sub> by the buffer is taken into account and  $V_f$  is the volume of the buffer, then

$$k_{CO_2}^R = kCO_2 + V_f.R$$

In this way, knowledge of the R value of the buffer used in a particular experiment, in conjunction with the data required to calculate the gas constants of each vessel, makes it possible to calculate the true O<sub>2</sub>



exchange ( $x'.O_2$ ) from the corrected  $O_2$  constant ( $K'O_2$ ) as follows:

$$x'O_2 = h. \left[ \frac{k^R_{CO_2} \cdot kO_2}{k^R_{CO_2} + y.kO_2} \right] = h.K'O_2$$

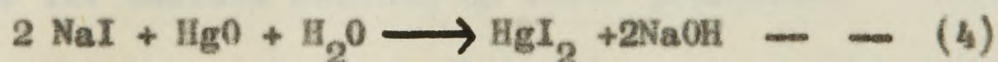
provided a reasonable estimate of the Respiratory Quotient can be made.

**PREPARATION OF BUFFERS:** In preparing the buffer solutions according to the specifications of Warburg & Krippahl (1960), it has to be borne in mind that commercial  $K_2CO_3$  invariably contains some  $HCO_3$  and moisture, while  $KHCO_3$  contains a variable amount of  $CO_3$ . The stock solutions were therefore analysed by titration by the method of Kolthoff & Sandell (1952).  $K_2CO_3$  was dissolved at room temperature, titrated for its content of  $CO_3^{=}$  and  $HCO_3^{-}$  ions, and finally brought to exactly 3M with respect to total concentration of the two ions.  $KHCO_3$  was dissolved in water by rapid stirring at  $30^{\circ}$  and was then gassed with 100%  $CO_2$  for 2 hr. Titration and standardization at 3M were carried out as for the other solution. Mixtures of the two solutions were made before experiments with exact knowledge of the ionic composition.

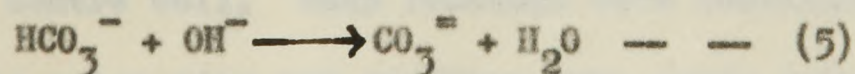
**DETERMINATION OF  $CO_2$  PRESSURES:** In order to calibrate the composition of 3M  $K_2CO_3$ - $KHCO_3$  mixtures in terms of the equilibrium  $CO_2$  pressures maintained in a confined space above them at given temperatures, a method was devised based on a modification of the principle of Krebs (1930) for determining the  $CO_2$  content of gas mixtures.  $HgO$  (432 mg.) and carbonic anhydrase (20 mg.) were placed in the main compartments of



conical Warburg vessels of about 25 ml. capacity, fitted with 2 side-arms. 1 ml. of 4M-NaI was pipetted into one side-arm and 1 ml. of the test buffer into the other immediately before attaching the flask to the manometer. A control vessel contained 1 ml. of 4M-NaCl instead of the test buffer, as well as 0.3 ml. 2N-KOH in the centre well. This was to measure the gas pressure changes (less than 5 mm.) due to changes in the vapour pressure. A thermobarometer contained water. The three manometers were equilibrated briefly in a water-bath at the required temperature. The taps were closed after the menisci had been adjusted to permit a large increase in gas pressure, and the buffer was emptied into the main compartment. This caused a rapid output of  $\text{CO}_2$ . Equilibrium was established in about 5 - 10 min. - the time taken was used as a rough test of the activity of the carbonic anhydrase. The anions present did not appear to inhibit the enzyme to a significant extent (see Roughton & Booth, 1946 ). The NaI was now tipped into the main compartment. NaOH was generated by reaction (4):



The  $\text{CO}_2$  of the gas space was rapidly and quantitatively absorbed. At the same time, by virtue of reaction (5),



generation of  $\text{CO}_2$  by the buffer is stopped, permitting exact estimation of the  $\text{CO}_2$  of the system at the time of tipping. The concentration of NaI chosen gave the smallest vapour pressure change on mixing with the test buffer in the absence of HgO; a similar test led to the choice of



the amount of NaCl used in the control.

In fig. 1 equilibrium  $\text{CO}_2$  pressures generated by a range of 3M mixtures are plotted, based on measurements at  $40^\circ$ . With such data, it is possible to calculate the required molar  $\text{HCO}_3^-$  concentration of media for any required pH and  $\text{pCO}_2$ .

DETERMINATION OF RETENTION FACTOR "R"; The capacity of a known volume of " $\text{CO}_2$  buffer" to absorb a known amount of  $\text{CO}_2$  released by acid from a solution of  $\text{NaHCO}_3$ , in a separate compartment of a manometer cup at a particular temperature, was assessed in Dickens-Simer vessels as follows: 3 ml. of 0.154M- $\text{NaHCO}_3$  were placed in the outer ring, and exactly 0.5 ml. of 0.02M-citric acid in the adjoining side-arm. The centre well contained the test buffer and 5 small glass beads; in its adjoining side-arm there were 20 mg. carbonic anhydrase. The centre well in a control vessel was empty. The gas phase was filled with  $\text{CO}_2 + \text{O}_2$  (5 : 95) for buffers in equilibrium with  $\text{CO}_2$  pressures of 300 - 500 mm. Brodie, and air for buffers setting up lower pressures. A thermobarometer contained water. The vessels were shaken in a manometer bath at the required temperature for 3 min. The carbonic anhydrase was added to the buffer in the centre well. When readings were constant, the acid was tipped into the outer ring and readings were again taken until they were constant (5 - 15 min. when 20  $\mu\text{Moles}$   $\text{CO}_2$  were released). The activity of carbonic anhydrase was stable for 1 hr. in this alkaline milieu (pH 9.8) as judged by the rate of  $\text{CO}_2$  absorption at different times after mixing.



$p\text{CO}_2$  (mM Brodie)

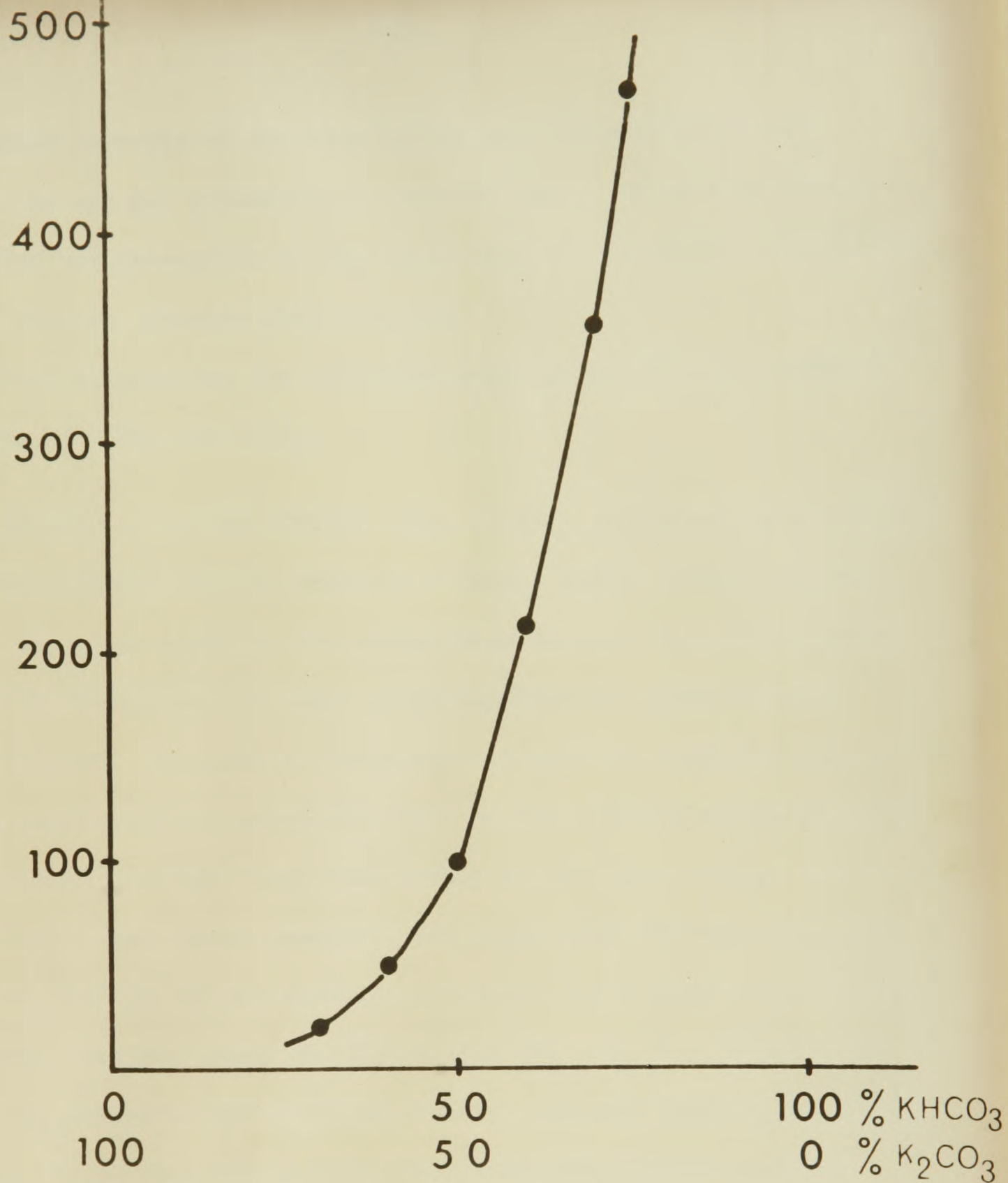


Fig. 1. Equilibrium  $\text{CO}_2$  pressures set up in an enclosed space above 3M- $\text{K}_2\text{CO}_3$ - $\text{KHCO}_3$  mixtures containing carbonic anhydrase, at  $40^\circ$ , determined as described in METHODS.



The retention capacity of the test buffer was estimated from the difference in net gas production in the two cups. "R" was derived as representing the absorption of  $\text{CO}_2$  in  $\mu\text{l.}$  per ml. of buffer solution for every mm. rise in pressure that occurred.

Fig.2 illustrates the retention capacity at  $40^\circ$  of a series of 3M mixtures of  $\text{K}_2\text{CO}_3$  and  $\text{KHCO}_3$ .

APPLICATION OF THE METHOD: Some of the incubations were carried out in Dickens-Simer vessels (Dickens & Simer, 1931); later, manometer cups more directly suited to the method were designed in collaboration with Mr. F.S. Rolleston and made in the departmental glass blowing workshop (Fig.3). Features of these vessels were the inverted lips of the large centre wells, preventing spill of the alkaline contents, and extra stoppered openings permitting access to the outer rings. 1 - 2 ml. of tissue preparation were placed in the outer ring of such a cup, and 3 ml. of  $\text{CO}_2$  buffer, 20 mg. carbonic anhydrase and 5 glass beads in the centre well. The gas space was filled with  $\text{CO}_2 + \text{O}_2$  (5 : 95) for the range of  $\text{CO}_2$  pressures 300 - 500 mm. Brodie, and with 100%  $\text{O}_2$  for lower pressures. To shorten the period of equilibration before accurate readings of  $\text{O}_2$  uptake could be taken, in standard experiments - where a  $\text{CO}_2$  pressure of 500 mm. Brodie was required in conjunction with a bicarbonate concentration in the medium of 25 mM - the required mixture was saturated with  $\text{CO}_2 + \text{O}_2$  (5 : 95) at  $40^\circ$  for 1 hr. before use.

The  $\text{O}_2$  uptake of a standard pigeon-liver homogenate metabolizing



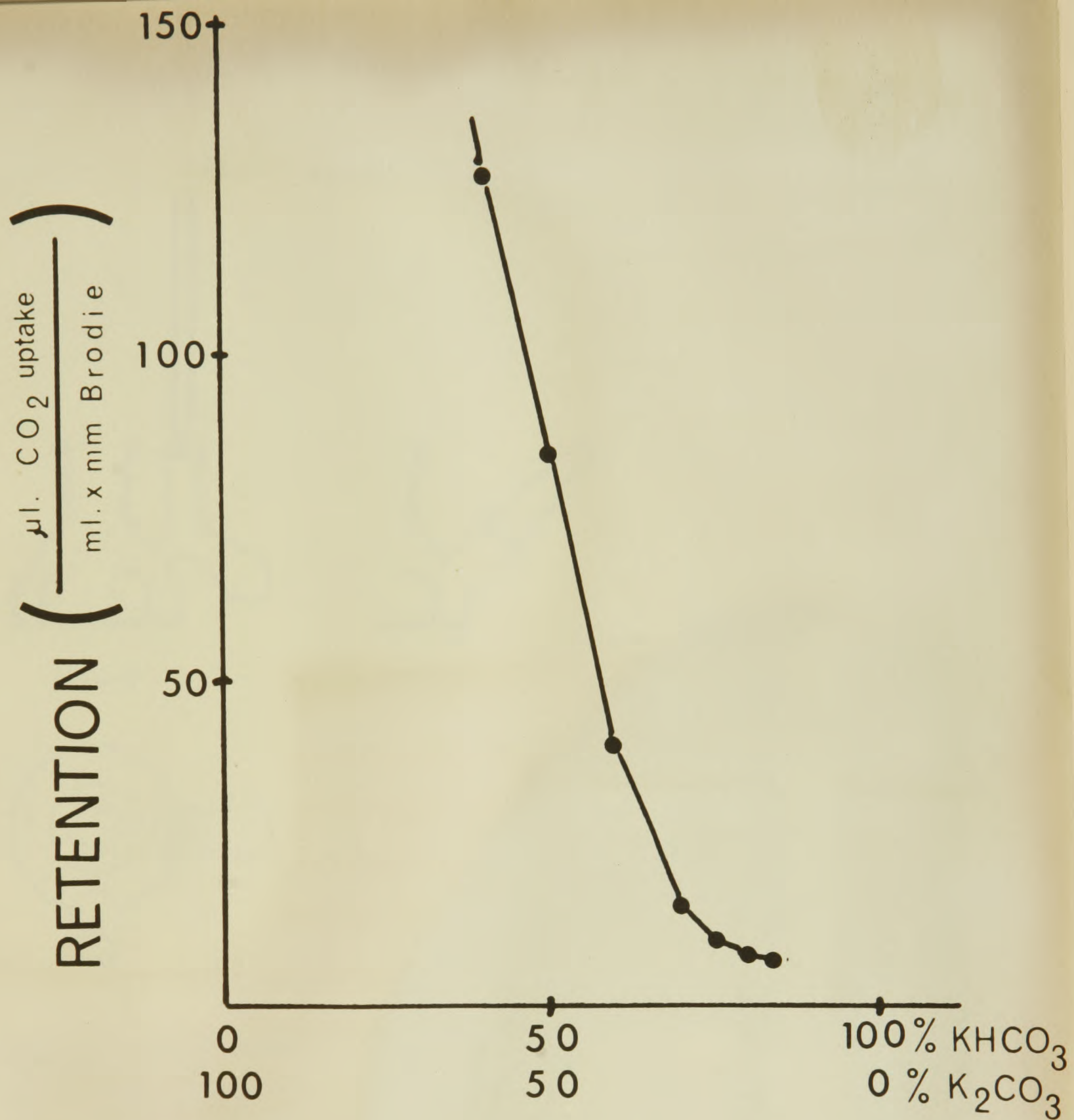
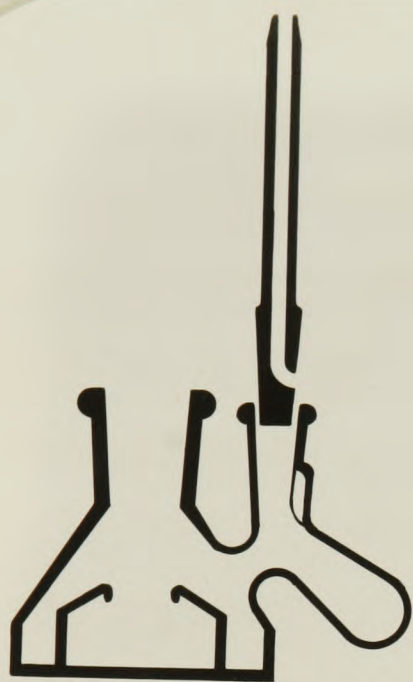
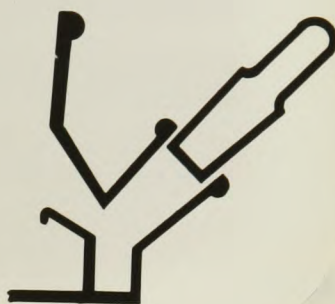
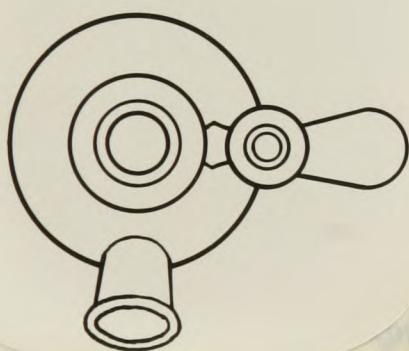


Fig. 2. Retention capacities of 3M- $\text{K}_2\text{CO}_3$ - $\text{KHCO}_3$  mixtures containing carbonic anhydrase, at  $40^\circ$ , determined as described in METHODS.

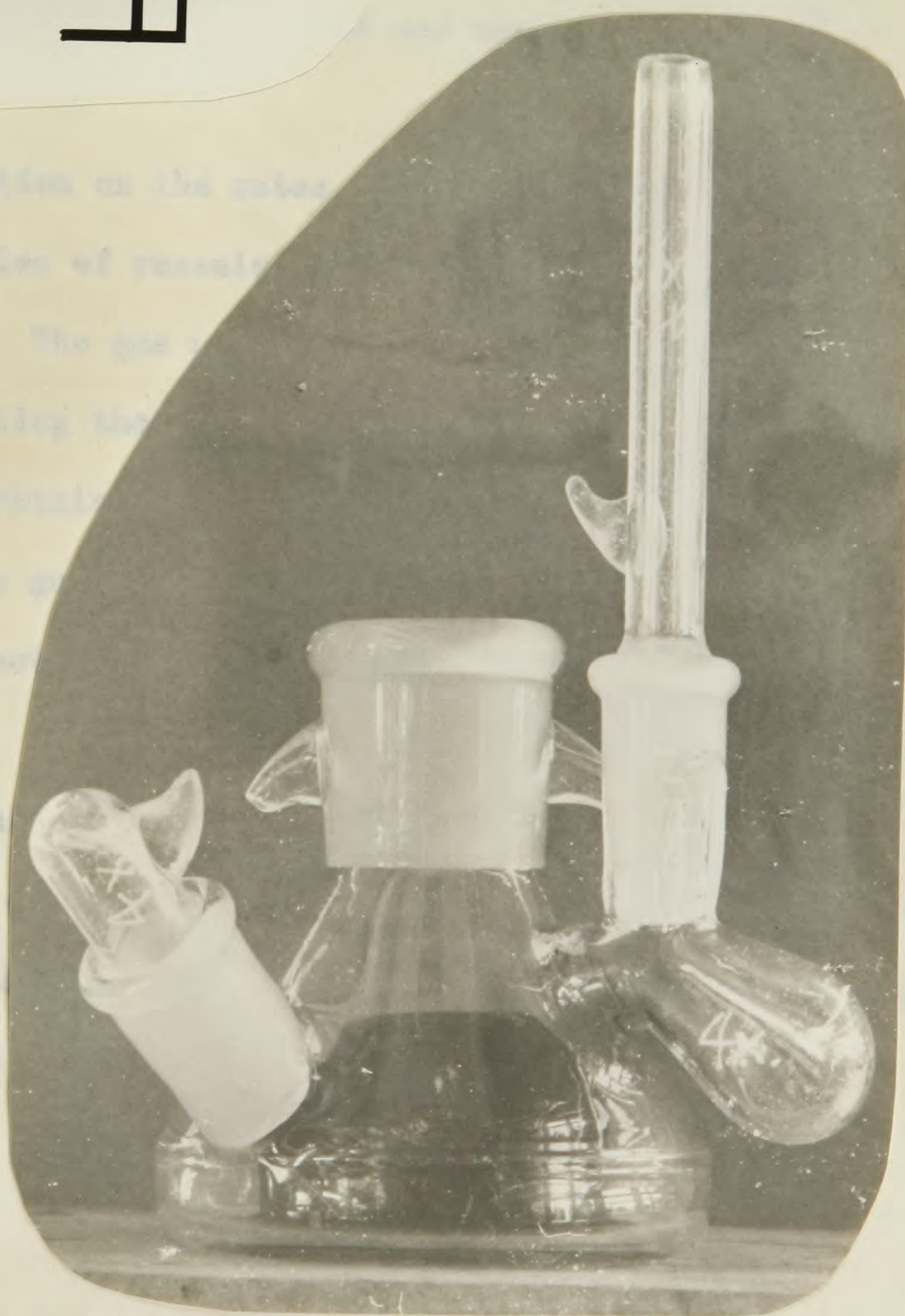




2 cm



**Fig. 3.** Special manometer cups used to measure  $O_2$  uptake in the presence of " $CO_2$  buffers". Average volume 23 - 25 ml.



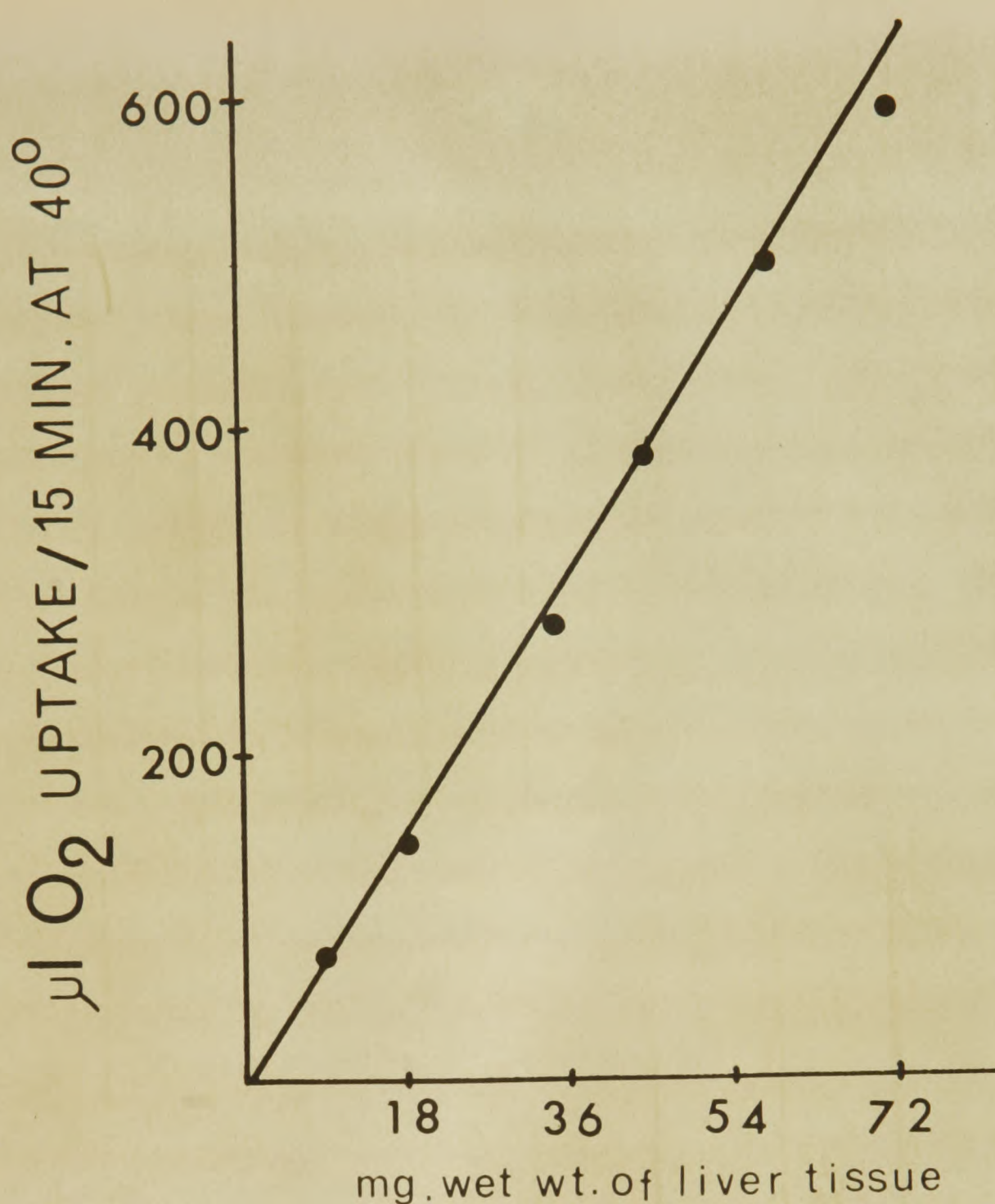


L-lactate at  $40^{\circ}$  was measured by this method over a wide range of tissue content/cup (Fig.4). Accurate results were obtained at rates of gas consumption up to  $2000 \mu\text{l./hr.}$  provided all necessary corrections were made. Rates were linear 5 min. after immersion of the cups in the water-bath, a period not longer than that required for routine thermal equilibration. Experiments illustrating the use of " $\text{CO}_2$  buffers" to provide a range of constant  $\text{CO}_2$  pressures in homogenates are described in Chap. 3. Before such experiments, the constancy of pH in media containing all additions except tissue was tested visually by including phenol red.

To obtain information on the rates of  $\text{CO}_2$  production of standard homogenates, a second series of vessels was incubated, with  $\text{CO}_2 + \text{O}_2$  (5 :95) in the gas space. The gas pressure changes in these vessels were recorded as representing the sum of  $\text{O}_2$  uptake and  $\text{CO}_2$  output. Using the values for  $\text{O}_2$  uptake obtained by the use of " $\text{CO}_2$  buffers", the net production of  $\text{CO}_2$  into the gas phase could readily be calculated. (Note: This was not corrected for retention of  $\text{CO}_2$  by the incubation medium.)

In some experiments, the  $\text{O}_2$  uptake and  $\text{CO}_2$  formation were measured by the principle of Warburg & Yabusoe (1924). A phosphate-buffered saline with low bicarbonate concentrations (up to 5 mM) was used.





**Fig. 4.** Measurement of  $O_2$  uptake in the presence of " $CO_2$  buffers" - linearity with tissue concentration.

Samples of a pigeon-liver homogenate, prepared as described in METHODS, were added to a series of manometer cups (Fig. 3) each containing in the centre well 3 ml. " $CO_2$  buffer", 5 glass beads and 20 mg. carbonic anhydrase.  $CO_2 + O_2$  (5 : 95) was the gas phase. Incubation was at  $40^\circ$  with rapid shaking, and the pressure changes were recorded for the period 5 - 20 min., during which time they were constant. Calculations to determine the  $O_2$  uptake were carried out as described in the text, including all appropriate corrections.



Analytical procedures. The following metabolic intermediates were measured enzymatically: D-Glucose and "glycogen" (i.e. D-glucose set free by enzymatic hydrolysis of oligo- and polysaccharides) (Krebs et al., 1964). D-Glucose 6-P and D-fructose 6-P (Hohorst, 1963,a). FDP and combined triose-P (Bucher & Hohorst, 1963). L- $\alpha$ -Glycero-P (Hohorst, 1963,b). 3-PGA, 2-PGA and PEP (Czok & Eckert, 1963). Pyruvate (Bucher et al., 1963). L-Lactate (Hohorst, 1963,e). L-Malate (Hohorst, 1963,d). Fumarate was measured as L-malate after addition of fumarase. Citrate and isocitrate (Siebert, 1963).  $\alpha$ -Oxo-glutarate (Bergmeyer & Bernt, 1963). Oxaloacetate (Hohorst & Reim, 1963). L-Aspartate (Pfleiderer, 1963). L-Glutamate and L-glutamine (Bernt & Bergmeyer, 1963).  $\text{NAD}^+$  (Klingenberg, 1963).

Purine nucleotides: ATP was estimated by the method of Lamprecht & Trautschold (1963). This determination fails to distinguish between ATP and ITP, but the latter is present in very low concentrations in liver tissue (Heldt & Klingenberg, 1965). Consistently higher ATP values were obtained when incubations were terminated by rapid acidification and instantaneous freezing, than when acid was added to cups cooled slowly in ice. GTP was determined by subtracting the value for ATP + ITP from the value for ATP + GTP + UTP + ITP obtained by the method of Adam (1963,a). This procedure was valid only when guanine nucleotides had been added to incubated material, as no ready correction was otherwise possible for UTP content.

Combined ADP + GDP + UDP + CDP were measured by the method of



Adam (1963,b), in conjunction with the determination of AMP. The method was modified to reduce interfering blanks. These were traced to contamination of ATP samples with ADP, and a variable but significant occurrence of AMP in all commercial NADH preparations tested. The former difficulty was overcome by adding ATP (required in the procedure for the adenylate kinase-catalysed conversion of AMP into ADP) after ADP had been determined, and the second by treating solutions of NADH with alkaline phosphatase (see "Chemicals" section).

In most experiments where adenine nucleotides were measured, the lack of specificity of the analytical methods was rendered unimportant by the large excess of the added adenine nucleotides over the endogenous non-adenosine nucleoside phosphates.

Isotopic techniques. All radioactive materials were obtained from the Radiochemical Centre, Amersham. Bucks.

[U-<sup>14</sup>C]-D-glucose was dissolved in water containing carrier glucose to give a final specific activity of 0.25  $\mu\text{C.}/\mu\text{Mole}$ . [U-<sup>14</sup>C]-D-fructose was similarly treated to yield the same specific activity. The two samples were chromatographed in the two-dimensional system described below, followed by autoradiography, which showed that there were no radioactive impurities.

[U-<sup>14</sup>C]-L-lactate is not commercially available. [U-<sup>14</sup>C]-sodium pyruvate was used to prepare pure [U-<sup>14</sup>C]-sodium L-lactate by reducing it with NADH in the presence of lactate dehydrogenase, while NADH was regenerated with ethanol and alcohol dehydrogenase. An ampoule containing



23.6  $\mu$ Moles (0.1 mC.) of labelled pyruvate was opened and kept at 0° while 0.75 ml. 0.02M- $\text{KH}_2\text{PO}_4$  (pH 8.0), 0.1 ml. 0.01M- $\text{NAD}^+$ , 0.05 ml. ethanol, 500  $\mu$ g. lactate dehydrogenase and 1000  $\mu$ g. alcohol dehydrogenase were added (both crystalline stock suspensions in 2.8M- $(\text{NH}_4)_2\text{SO}_4$ ). After mixing, the ampoule was incubated at room temperature for 30 min. This was followed by immersion in boiling water for 2 min. and centrifugation at 3000 r.p.m. for 10 min. The supernatant was chromatographed for 4 hr. on Whatman No. 1 paper (previously thoroughly washed with water and dried) using the ascending technique. The solvent was diethyl ether-formic acid (90 : 60, v/v). The paper was dried in a stream of cold air for 24 hr. and was then placed on Kodirex Xray film (Kodak Ltd., London, W.C.2) for 48 hr. An intense band of radioactivity, (about 4 cm. broad) was found ( $R_f$  0.8), as well as a pale, thin band with  $R_f$  0.6. On analysis of eluates these were found to be L-lactic acid and pyruvic acid, respectively. Elution of the principal band was with 2 ml. distilled water over 2 hr. The resulting solution was freed of  $\text{NH}_4^+$  ions (introduced with the enzymes in the preparative reaction) by alkalization to pH 12 with NaOH and vigorous aeration with  $\text{N}_2$  at room temperature, until nesslerization of a small sample showed that  $\text{NH}_4^+$  ions were absent. The solution was brought to pH 7 and stored at -15°. Two-dimensional chromatography (as described below) and autoradiography of a sample showed that all the radioactivity was located in one spot, representing L-lactate; enzymatic analysis enabled the specific activity to be determined. The yield of the whole procedure was 70% of the initial labelled pyruvate as L-lactate.



After incubations of liver homogenates with labelled compounds, duplicate 100  $\mu$ l. samples of the final neutral extracts were spotted on Whatman No.1 paper, and descending chromatograms in two directions were carried out at room temperature, using the "Semi-stench" of Crowley et al., (1962) and butan-1-ol-propionic acid-water (47 : 22 : 31, v/v) as solvents. After thorough drying, the papers were placed on Kodirex Xray film for 3 weeks. A further series of 0.4 ml. samples were added to "diazyme" (0.2% in 0.01M-sodium acetate buffer, pH 4.4), incubated for 2 hr. at 50° and then heated in a boiling water-bath for 5 min. After centrifugation, 200  $\mu$ l. samples of the supernatants were chromatographed as described.

Areas of radioactivity revealed by autoradiography were identified as follows: i. by their topographic relation to the predicted positions of compounds tested (as pure solutions) in this particular solvent system by Crowley et al., (1962). ii. by enzymatic analysis of eluates. iii. by one- or two-dimensional co-chromatography with suspected marker substances, followed by autoradiography and the use of appropriate sprays for amino acids (Ninhydrin, 0.2% in ethanol, w/v), aldoses (Partridge, 1949), ketoses (Forsyth, 1948), carboxylic acids (Buch, et al., 1952) and phosphorylated compounds (Hanes & Isherwood, 1949). iv. by location of ultraviolet light-absorbing areas.

The radioactivity of areas was measured directly on the paper with an end-window Geiger-Muller tube and a Panax Scaler (Panax Equipment Ltd., Redhill, Surrey). Both sides of the paper were counted to a



minimum of 10,000 counts, corrected for background activity and dead time, and the mean was taken to be the number of counts/min. in the spot. Differences between the two sides were less than 1%. There were losses of certain volatile compounds during the procedures e.g. pyruvate >80% and L-lactate >30%, as checked by enzymatic analysis. Acetoacetate and acetate were largely lost. Most other compounds of interest were stable throughout chromatography, although this was tested critically only for L-malate,  $\alpha$ -oxo-glutarate, L- $\alpha$ -glycero-P and glucose.

The initial radioactivity of materials added was determined by spotting a series of diluted samples on to paper and counting as above. The radioactivity of  $\text{CO}_2$  formed was measured as follows: The manometer was connected with rubber tubing to a suction system and the  $\text{CO}_2$  of the gas phase and medium, including the bicarbonate, was collected (Sakami, 1955) at the end of incubation in 3 ml. 2N-KOH. Known volumes of the alkali were spotted in duplicate on Whatman No.1 paper, previously impregnated with 0.1 ml. 10% barium acetate solution. After drying, the areas were counted as above. Tests showed that there was no quenching due to the presence of alkali under these conditions, provided that a total of less than 50  $\mu\text{l}$ . were added.

Assays of enzyme activity. Several enzymes involved in carbohydrate metabolism were assayed in pigeon liver extracts. In each case the wet weight of the liver, and the dry weight of thrice-TCA-washed materials, were used as reference standards for expression of enzyme activities.



PYRUVATE KINASE (PK) (ATP-pyruvate phosphotransferase, EC 2.7.1.40.). This was assayed by two methods. The first was based on the procedure of Bucher & Pfleiderer (1955). A second assay system was developed to test the effects of pyruvate concentration on PK. This was a sampling method based on the measurement of GTP produced by phosphorylation of GDP by PK. ADP could not be used as acceptor because of interference by adenylate kinase and ATP ases. GTPases were insignificant in these extracts.

A series of test-tubes were set up as follows: All but two contained 1 ml. of medium consisting of  $\text{KH}_2\text{PO}_4$  (66 mM),  $\text{MgCl}_2$  (10 mM), KCl (50 mM), potassium PEP (1 mM) and lithium GDP (2 mM) - all at pH 7.4 and 25°. One blank contained no PEP (to measure the rate of GTP synthesis independent of PK) and another contained 0.25  $\mu\text{Moles}$  of GTP (to estimate GTPase activity). The reaction was started by adding the freshly-diluted enzyme. Samples were removed at 0, 5, 10 and 20 min. by adding 0.1 ml. of 20% perchloric acid and transferring to crushed ice. Neutral extracts free of protein and perchlorate ions were prepared and analysed for GTP.

The reaction rate was linear for 30 min. and the calculated activity was 60% of that found in the same extract by the previous assay method using ADP, and equal to that using GDP. There was no endogenous GTP synthesis.

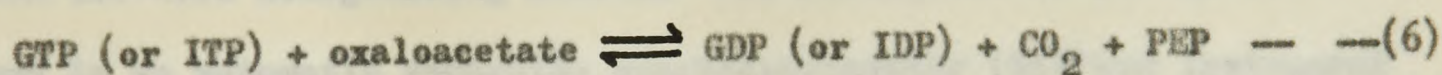
ENOLASE (D-2-phosphoglycerate hydro-<sup>y</sup>lase, EC 4.2.1.1.). This assay was according to Delbrück et al., (1959).



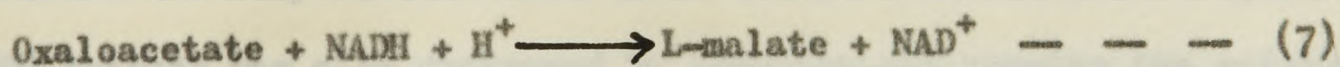
FRUCTOSE DIPHOSPHATASE (D-fructose 1,6-diphosphate 1-phospho-  
hydrolase, EC 3.1.3.11.) was assayed by the method of Underwood &  
Newsholme (1965<sup>a</sup>).

MALATE ENZYME (L-malate:NADP oxidoreductase, decarboxylating,  
EC 1.1.1.40.). The measurement was in the direction of L-malate  
decarboxylation according to the method of Rutter & Lardy (1958).

PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) (GTP: oxaloacetate  
carboxy-lyase, transphosphorylating, EC 4.1.1.32.). This enzyme was  
assayed in pigeon liver in both directions of the catalytic reaction (6):



i) A spectrophotometric method measuring the rate of oxaloacetate  
synthesis from PEP and based on the principle of Stickland (1959,c)  
was suggested by Mr. L.V. Eggleston. The method was modified in view  
of the observed "latency" of the mitochondrial enzyme (Chappell &  
Greville, 1963; see also Chap. 2). The indicator reaction was that  
catalysed by malate dehydrogenase (7):



After killing a pigeon, the liver was kept cold and dry in ice  
for 2 min. It was blotted and cleaned, and passed through a Fischer  
mincer. A weighed quantity of mince was homogenized in 3 volumes of  
ice-cold medium containing 0.25M-sucrose, 0.01M-"tris", 0.0002M-EDTA,  
and 0.01M-2-mercaptoethanol, all at pH 7.4 and freshly prepared. 2 ml.  
of the homogenate were now sonicated for two separate periods of 60 sec.



in an M.S.E. ultrasonic disintegrator (60 watt, probe  $\frac{3}{8}$ " (M.S.E. Equipment Ltd., Spenser Street, London, S.W.1) to give the final extract for assay.

3 ml. of the medium at 25° contained the following: "tris" (40 mM),  $\text{MnCl}_2$  (1 mM), sodium PEP (1 mM), sodium IDP (1.3 mM, freshly dissolved), NaCN (0.2 mM), NADH (0.15 mM), 2-mercaptoethanol (10 mM), dialysed malate dehydrogenase (4  $\mu\text{g.}$ ) and  $\text{NaHCO}_3$  (15 mM, saturated with  $\text{CO}_2$ ), which was the only constituent omitted in the blank. The reaction was started by adding suitable amounts of extract, diluted just before use with ice-cold homogenizing medium. The absorption at 340 m $\mu$  was followed in an automatic recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio, U.S.A.). A steady state was reached in 1 - 2 min. and the reaction was then linear for 60 min. Activity was proportional to extract concentration over the measurable range of rates. Cyanide was added to inhibit the considerable blank NADH oxidase activity activated in homogenates by sonication. This blank was reduced from 30% of the total activity to less than 8% in the presence of 0.2 mM cyanide. The NADH oxidase was also decreased by freezing and thawing the extract (less than half remained after 48 hr. at -15°) or by centrifugation at 100,000g for 45 min. (70% decrease). 2-Mercaptoethanol, when included throughout the procedures, increased the activity by 20 - 30%; when it was included in the assay only, the increase was still significant and varied between 10 - 20%. Reduced glutathione (1.66 mM) could be used with similar results. "Tris" was the best buffer, although triethanolamine



could also be used. The concentrations of other reactants in the standard assay were optimal (see Chap. 4 for details).

A significant blank activity is caused by PK reacting in conjunction with lactate dehydrogenase. These two enzymes are plentiful in the whole homogenates and PK contaminates all commercial malate dehydrogenase preparations (L.V.Eggleston, personal communication). The interference of this blank was reduced as follows:

a) The use of IDP instead of GDP decreased the activity of PK, which when tested in the assay system previously described reacted with ADP, GDP and IDP in the ratio 100 : 60 : 15.

b) The medium was kept free of  $K^+$  and  $NH_4^+$  ions (both activators of PK - Kachmar & Boyer, 1953) by using sodium salts of reactants, and by dialysing the malate dehydrogenase suspension until it was free of  $NH_4^+$  ions.

c) For kinetic studies (see Chap. 4) of the mitochondrial enzyme, an initial purification was achieved by using washed, sonicated mitochondria as starting material. The lactate dehydrogenase and PK activity of such extracts was very low (2% of the unfractionated homogenate).

When the initial homogenization was carried out in distilled water, the rates were very low before sonication; even after sonication they failed to exceed 50% of those obtained in the standard procedure. The activity was very low in standard "sucrose" homogenates before sonication (see Chap. 4, Table 25), and the application of repeated



freezing and thawing cycles (Bandurski & Lipmann, 1956; Nordlie & Lardy, 1963,b) was less effective than sonication in releasing "latent" activity; in fact, increasing degrees of inhibition were observed after 3 such cycles. Furthermore, the enzyme previously released by sonication was unstable to repeated freezing and thawing during storage at  $-15^{\circ}$  (40% decrease after 4 cycles over 2 months) although the effect was less marked than when unfractionated homogenates were subjected to rapid cycles of this kind.

ii) A sampling method similar to that of Nordlie & Lardy (1963,b) was used to measure the rate of phosphoenolpyruvate synthesis from oxaloacetate and ITP or GTP. The extracts were prepared as for the first method, and similar findings relating to "latency" were made.

A number of tubes were set up at  $25^{\circ}$ , containing the following in a volume of 1.5 ml.: "tris" (50 mM), NaF (13 mM),  $MgSO_4$  (13 mM), sodium ITP or lithium GTP (3 mM), sodium oxaloacetate (5 mM) and 2-mercaptoethanol (10 mM) - all at pH 8 or 7.4. The reaction was started by mixing in samples of extract; to the zero-time sample 0.15 ml. of 20%  $HClO_4$  were immediately added, while other samples were removed at intervals over 20 min. Neutral extracts free of protein and  $ClO_4^-$  ions were prepared as rapidly as possible (as earlier described for incubations involving pigeon-liver homogenates). Pyruvate and PEP were now estimated in the extracts by an enzymatic procedure (see Analytical Procedures). An important feature of this analysis was the adherence to a strict time schedule during the setting up of cuvettes and addition of

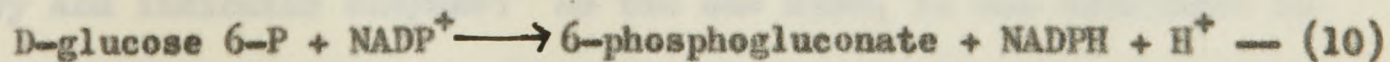
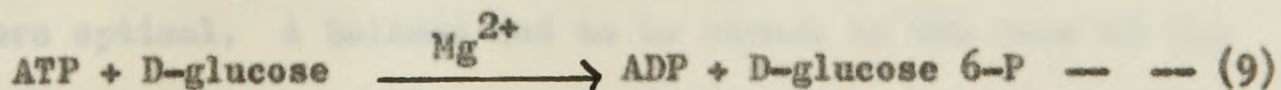
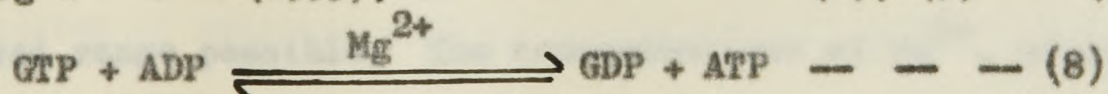


enzymes; this enabled an accurate time course of the absorption changes to be plotted, so that the PEP concentrations could be estimated by graphical corrections for the constant rates of oxaloacetate decarboxylation (to pyruvate) occurring during the procedure. This was checked with standards.

The reaction rate by this sampling method was linear for 20 min. and was of the same order as that observed in the opposite direction by the other method of assay. For kinetic work, the same precautions described above were taken to reduce blank PK activity. The addition of oligomycin as an inhibitor of ITPases (Holten & Nordlie, 1965) had no effect on the rates obtained in this system.

ADENYLATE KINASE (ATP : AMP phosphotransferase, EC 2.7.4.3.). This was assayed according to Oliver (1955).

NUCLEOSIDE DIPHOSPHOKINASE (NuDiKi) (ATP : nucleosidediphosphate phosphotransferase, EC 2.7.4.6.). This was assayed by a method derived from Berg & Joklik (1953), based on reactions (8), (9) and (10):



Extracts were prepared exactly as described for the assay of PEPCK, since "latency" phenomena were found, very similar to those observed with that enzyme.



2.1 ml. of assay medium at 25° constituted the "basic mixture": triethanolamine (75 mM),  $\text{MgCl}_2$  (5 mM), D-glucose (10 mM),  $\text{NADP}^+$  (0.5 mM), hexokinase (10  $\mu\text{g.}$ ) and glucose 6-P dehydrogenase (10  $\mu\text{g.}$ ) - all at pH 7.4 or 8.0. After mixing, the absorption at 340 m $\mu$  was followed by automatic recording. As soon as the base-line had been recorded, 2  $\mu\text{Moles}$  of ADP were mixed in. An initial rapid reduction of  $\text{NADP}^+$  followed, due to contamination of ADP with ATP, but a low, constant blank rate was soon attained (2 - 3 min.). 2  $\mu\text{Moles}$  of GTP were now added. The constant, slightly increased activity was due to NuDiKi contaminating the ancillary enzymes. Finally, a sample of freshly-diluted extract was added. A steady state was reached in 1 - 2 min., after which the rate was linear for 30 min. or as long as sufficient  $\text{NADP}^+$  was present. This rate was corrected for the blank rate immediately before addition of the test extract. A separate blank was set up to assess the contribution of adenylate kinase to the recorded activity. In this case, enzyme extract was added to the basic mixture first, followed by ADP.

NuDiKi activity was proportional to extract concentration over the widest range possible. The concentrations of  $\text{Mg}^{2+}$ , glucose and  $\text{NADP}^+$  were optimal. A balance had to be struck in the case of the auxillary and indicator enzymes: on the one hand, it was desirable to keep as low as possible the amount of NuDiKi and  $\text{NH}_4^+$  contaminating these enzymes; on the other, a sufficient excess of their activity had to be permitted to allow accurate kinetic studies on the test enzyme to be carried out. Tests showed that the concentrations used in the



standard procedure were optimal. The concentrations of ADP and GTP were based on studies described in Chap. 4.

This method of measuring NuDiKi is rather more suitable for tissue extracts (such as pigeon liver extracts) where NuDiKi activity is high relative to adenylate kinase, and procedures are available for physical separation of the two activities, such as differential centrifugation. Preparation of washed mitochondria resulted in considerable purification of NuDiKi and reduced the amount of adenylate kinase by 55%.

PYRUVATE CARBOXYLASE (Pyruvate :  $\text{CO}_2$  ligase (ADP), EC 6.4.1.1.). The rate of  $\text{CO}_2$  incorporation into dicarboxylic acids (and other intermediates of metabolism) in pigeon-liver homogenates and mitochondria was measured with an isotopic technique developed in this laboratory and assumed to be a measure of pyruvate carboxylase activity in crude tissue preparations capable of carrying out oxidative phosphorylation (Krebs, Eggleston & Lund, 1966 - in preparation). The method depends on incubation of extracts with high concentrations of pyruvate and  $^{14}\text{C-Na}_2\text{CO}_3$ . Oxaloacetate formed by the action of pyruvate carboxylase (using ATP generated by oxidative phosphorylation, as well as some added ATP) is reduced to L-malate in the presence of highly-active malate dehydrogenase and NADH (the latter fortified in the system by addition of  $\text{NAD}^+$  or NADH). A large pool of unlabelled L-malate is added to "trap" the labelled molecules, so that losses of incorporated  $^{14}\text{C}$  by secondary



reactions of L-malate and oxaloacetate are reduced to a minimum. Knowledge of the initial specific activity of ( $\text{CO}_2 + \text{HCO}_3^- + \text{CO}_3^{=}$ ) in the system, and of the total incorporation of label into acid-soluble non-volatile material, allows the calculation of the net rate of  $^{14}\text{CO}_2$  incorporation. Since significant differences in the behaviour of homogenates of pigeon liver and those of other tissues (rat liver and kidney, bovine liver and rabbit skeletal muscle) were found, a detailed investigation of the pigeon-liver system was carried out. A description of the basic procedures is given here; further studies will be described in Chap. 4.

1 g. of fresh pigeon liver was homogenized in 6.5 ml. of a saline medium prepared by mixing 40 ml. 0.154M-KCl, 9 ml. 0.1M- $\text{KH}_2\text{PO}_4$  (pH 7.4), 1 ml. 0.05M- $\text{MgCl}_2$  and 10 ml. 0.2M- $\text{NaHCO}_3$ , saturated with  $\text{CO}_2 + \text{O}_2$  (5 : 95) before use. 0.5 ml. were placed in the main compartments of small conical manometer vessels (capacity 7 - 8 ml.) each already containing  $\text{MnCl}_2$  (0.5  $\mu\text{Moles}$ ), ATP (0.5  $\mu\text{Moles}$ ) and  $\text{NAD}^+$  (0.25  $\mu\text{Moles}$ ), as well as sodium pyruvate (20  $\mu\text{Moles}$ ) and sodium L-malate (20  $\mu\text{Moles}$ ) in a final volume of 0.45 ml. The side-arms contained 0.05 ml.  $^{14}\text{C-Na}_2\text{CO}_3$  (2.5  $\mu\text{C.}$ , 0.17  $\mu\text{Mole}$ ) and the gas space  $\text{CO}_2 + \text{O}_2$  (5 : 95). At zero-time, the contents of the flasks were mixed and the incubations were carried out at  $25^\circ$  in a water-bath with shaking. After a suitable period (e.g. 7 min.) the flasks were removed, 0.25 ml. of 15% trichloroacetic acid was added and after centrifugation, the radioactivity of small samples of the supernatants was determined as described under "Radioactive techniques",



as was the initial radioactivity of the  $^{14}\text{C-Na}_2\text{CO}_3$ . The  $\text{CO}_2$  content of the homogenate at the start of incubations was determined by a number of tipping experiments and was found to be constant enough to use an average value ( $16 \mu\text{Moles/cup}$ ) in further work done under the same conditions. In certain experiments, washed mitochondria were suspended in the above homogenizing saline and incubated as described.

which carbohydrate synthesis from lactate and glyceraldehyde-3-phosphate in pigeon-liver homogenates. This was the first time that the rate of glucose synthesis was found to be constant in a cell-free liver preparation and the system was regarded as suitable for the detailed investigation of the regulatory mechanisms of hepatic carbohydrate metabolism.

The homogenates perform the complete series of metabolic reactions from lactate to glucose, as well as the complete glycolytic pathway. Throughout these studies, however, it has been necessary to recognize clearly that in many important respects the homogenates are fundamentally different from intact cells with respect to:

- i) the breakdown of all cell structures and of the associated loss of the intracellular structural organization;
- ii) the dilution of the "metabolic components" by the incubation buffer, without corresponding dilution of various enzymes located within organelles, and
- iii) the activation of endogenous enzymes.



### CHAPTER 3

#### REGULATION OF CARBOHYDRATE METABOLISM IN

#### PIGEON-LIVER HOMOGENATES

Krebs et al., (1964) have described some of the conditions under which carbohydrate synthesis from L-lactate and certain other precursors occurs in pigeon-liver homogenates. This was the first recorded instance where rates of net glucose synthesis approximating those found in vivo were obtained in a cell-free liver preparation, and the system was regarded as suitable for the detailed investigation of some control mechanisms of hepatic carbohydrate metabolism.

The homogenates perform the complete series of reactions leading from lactate to glucose, as well as the required energy-supplying processes. Throughout these studies, however, it has been necessary to recognize clearly that in many important respects the homogenates as incubated differed from intact liver cells because of

i) the break-down of all cell membranes and of an undefined part of the intracellular structural organization,

ii) the dilution of the "soluble" cytoplasm by 15 times in a saline buffer, without corresponding dilution of enzyme systems within intact organelles, and

iii) the activation of autolytic enzymes.



The results have therefore been correlated as far as possible with those obtained by workers using other preparations such as liver slices and isolated perfused livers. In general, agreement has been good enough to suggest that many physiologically important control mechanisms operated in the disrupted cells, and were in them more amenable to direct study than in the other systems.

Besides active carbohydrate synthesis from lactate, pigeon-liver homogenates also metabolize added glucose, or utilize endogenous carbohydrate, on incubation without gluconeogenic precursors. Since the problem of the direction of carbohydrate metabolism is one peculiar to this organ (and to the kidney), the homogenates have been studied with reference to the regulation of both degradative and synthetic pathways. In particular, some of the mechanisms that may control "switches" from one to the other have been investigated. The homogenates form a convenient system for examining whether the activations and inhibitions found with isolated enzymes may operate in the functional multi-enzyme systems of the liver cells.



SECTION A

GENERAL PROPERTIES OF PIGEON-LIVER HOMOGENATES:

CARBOHYDRATE SYNTHESIS: The optimal conditions for maximal rates of carbohydrate formation from lactate have been defined by Krebs et al., (1964). These were confirmed in the present work and may be summarised as follows:

i) Essential components were both  $\text{Na}^+$  and  $\text{K}^+$  ions, EDTA (0.25 mM),  $\text{Mg}^{2+}$  ions (1.25 mM), Pi (4 - 20 mM),  $\text{HCO}_3^-$  (25 mM) (see below) and  $\text{NAD}^+$  (0.125 - 0.5 mM).

ii) High concentrations of lactate were required (20 - 40 mM) because of rapid rates of utilization, about a quarter of the lactate removed being converted into free glucose (rather than glycogen).

iii) The rates were much higher in liver homogenates from pigeons starved for 48 hr. than in those from well-fed birds or birds refed after such a period of starvation.

iv) Both the particulate and the "soluble" fractions of the homogenates were required.

v) The rates were approximately linear for 45 min., after which there was a decline in lactate removal,  $\text{O}_2$  consumption and carbohydrate synthesis.

vi) Synthesis from a variety of other precursors (fumarate, glutamate,  $\alpha$ -oxo-glutarate, glycerol and  $\alpha$ -glycero-P) did occur but was much slower



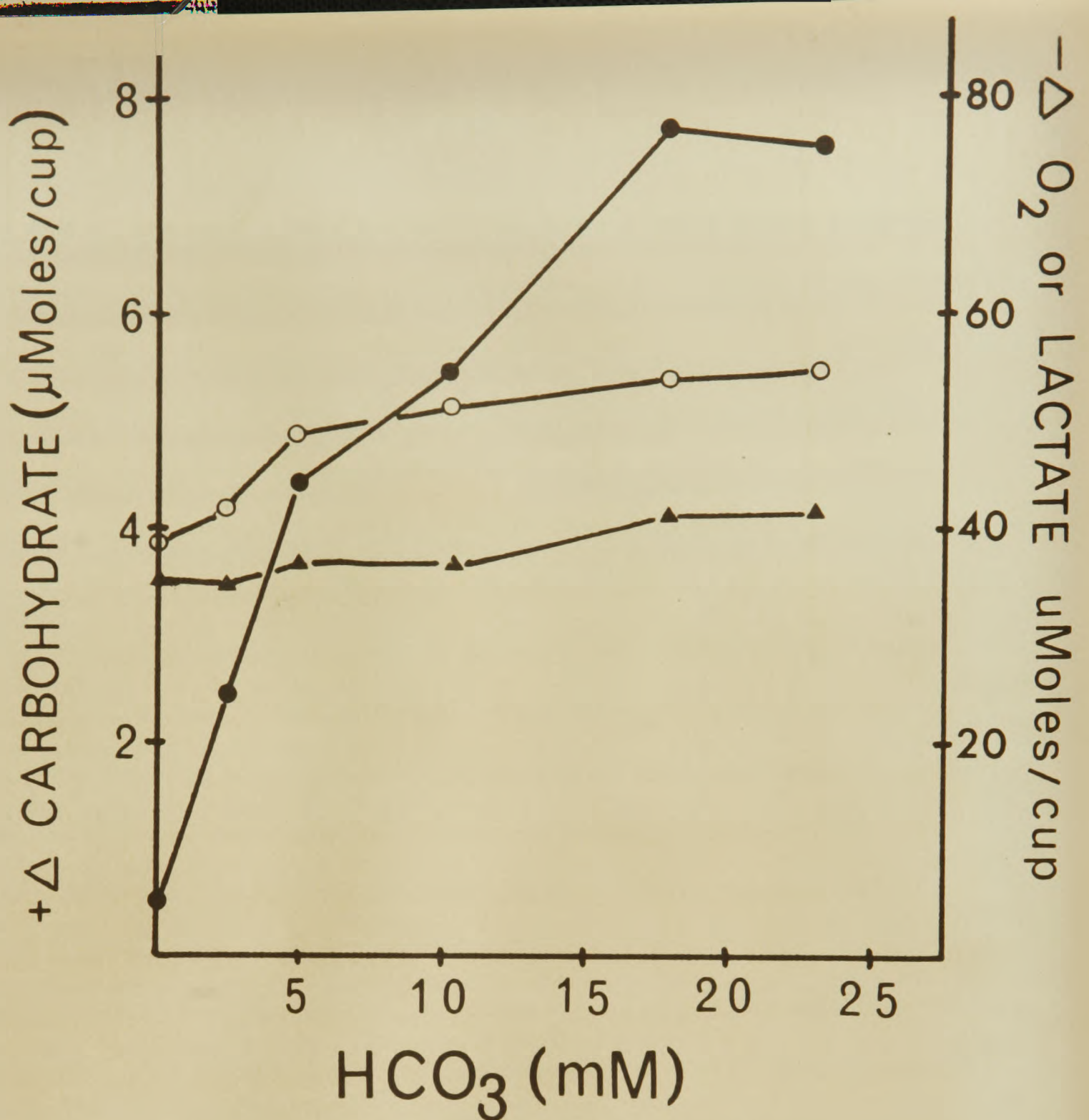
than from lactate.

vii) AMP and ATP addition drastically decreased the net synthesis of carbohydrate from lactate.

Effect of  $\text{HCO}_3^-$  concentration on carbohydrate formation from lactate: This was tested using the manometric procedures described in METHODS; the results are shown in Fig. 5. Both the rate of lactate utilization and  $\text{O}_2$  consumption increased only slightly with increasing  $\text{HCO}_3^-$  concentrations, while carbohydrate synthesis was stimulated over 3 fold by raising this concentration from 3 to 25 mM. In the phosphate buffer (20 mM) without  $\text{HCO}_3^-$ , net formation of carbohydrate hardly occurred.  $\text{HCO}_3^-$  is known to be a reactant in gluconeogenesis at the stage of pyruvate carboxylation. The  $K_m$  value of pyruvate carboxylase for  $\text{HCO}_3^-$  is about 1 mM (Utter & Keech, 1963) while that of malate enzyme is 35 mM (Utter, 1963). The possibility is raised that part of the conversion of pyruvate into PEP proceeded via malate enzyme, malate dehydrogenase and PEPCK; the experiments with isolated mitochondria in Chap. 4 show this to be unlikely. The observed effects may be partly due to inadequate buffering (in the absence of  $\text{HCO}_3^-$ ) in the face of rapid production of alkali when lactate is oxidised to  $\text{CO}_2$  or converted to carbohydrate.

CARBOHYDRATE DEGRADATION: Fresh liver homogenates prepared from well-fed pigeons contained much endogenous carbohydrate, mainly glycogen; starvation for 48 hr. as described in METHODS depleted this store by





**Fig. 5.** Effects of  $\text{HCO}_3^-$  concentration on  $\text{O}_2$  uptake, lactate removal and carbohydrate synthesis in pigeon-liver homogenates (starved bird).

A standard liver homogenate (not containing  $\text{HCO}_3^-$ ) was prepared as in METHODS and 2 ml. portions were incubated for 45 min. at  $40^\circ$  in special manometer cups (Fig. 3) containing 3 ml. of " $\text{CO}_2$  buffer" in the centre wells, together with 5 glass beads and 20 mg. carbonic anhydrase. The composition of the " $\text{CO}_2$  buffers",  $\text{HCO}_3^-$  conc'ns. in the medium and  $\text{CO}_2$  pressures in the gas phases were selected following on calculations described in detail in METHODS. The homogenates in each cup contained 20 mM Pi and 0.5 mM  $\text{NAD}^+$ , and their dry wt. was 42 mg./cup. ●: net carbohydrate synthesis; ○: lactate removal; ▲:  $\text{O}_2$  uptake.



over 90% on average. When homogenates from starved pigeons were incubated, 5 mM glucose was added to saturate the capacity to metabolize carbohydrate. Since more accurate measurements of net changes could be made, most of the studies of optimal conditions for carbohydrate removal were done with this system.

In the standard saline of Krebs et al., (1964) glucose was removed at 60 - 80  $\mu$ Moles/hr./g. dry wt. at 40°. Very little glycogen or oligosaccharide was formed - less than 10% of the hexose removed. The uptake was linear for 45 min. When  $K^+$  ions were completely replaced with  $Na^+$  ions, glucose uptake was reduced by 40% (see Ashmore et al., 1957). This may have been due to the activating effect of  $K^+$  ions on 3 important glycolytic enzymes - hexokinase (Perez et al., 1964), PFK (Passonneau & Lowry, 1964) and PK (Kachmar & Boyer, 1953). The optimal concentration of  $Mg^{2+}$  was 1 - 3 mM, as both omission of  $Mg^{2+}$  and concentrations higher than 4 mM were inhibitory.  $Mg^{2+}$  ions inhibit PFK (Passonneau & Lowry, 1964) and activate FDPase (Underwood & Newsholme, 1965,a). Omission of EDTA had no effect on the overall rate of carbohydrate removal. Increasing the concentration of Pi from 5 to 20 mM increased the rates by 15%, while omission of Pi halved them (see Uyeda & Racker, 1965). When  $HCO_3^-$  was not included and Pi was 20 mM, the glucose uptake was only 75% of that which occurred when 5 mM  $HCO_3^-$  was added or the standard saline was used, in agreement with Hastings & Fanestil, (1963).  $NAD^+$  addition (0.25 - 0.5 mM) stimulated glucose removal by 25 and 40% respectively, as found by Wenner et al., (1953).



In each case there was a proportionate increase in adenine nucleotides at the end of incubation, including AMP, and part of the effect was probably due to pyrophosphorolysis of  $\text{NAD}^+$  (Haugaard et al., 1960).

## SECTION B

**GAS EXCHANGES IN PIGEON-LIVER HOMOGENATES:** In standard homogenates (starved pigeons) the  $\text{O}_2$  consumption in the absence of lactate but presence of added glucose was 800 - 1000  $\mu\text{Moles/hr./g. dry wt. at } 40^\circ$ , while in the presence of 40 mM lactate it was 50 - 60% higher, though less well sustained (e.g. Table 9). (The corresponding values in phosphate-buffered homogenates were 10% lower.) The amounts of  $\text{CO}_2$  formed were such that the respiratory quotients were 0.85 - 0.88 in the absence of lactate, and 0.55 - 0.60 when it was added. This latter low value is due to the fact that  $1/3$  of the respiratory  $\text{CO}_2$  formed from lactate appeared as  $\text{HCO}_3^-$ , while the conversion of lactate into carbohydrate formed alkali which converted free  $\text{CO}_2$  into  $\text{HCO}_3^-$ .

On the basis of the ATP requirements and assuming an average P/O ratio of 3 for oxidative phosphorylation, an uptake of 1  $\mu\text{Mole}$  of  $\text{O}_2$  would be needed for the synthesis of 1  $\mu\text{Mole}$  of glucose. The observed ratio

extra  $\text{O}_2$  used on addition of lactate

glucose equivalents formed



was much higher, e.g. 5 - 7. This high  $O_2$  uptake was probably due to other energy-requiring processes taking place, or to the fact that the efficiency of mitochondrial phosphorylation was lower than the above estimate.

The  $O_2$  consumption of homogenates from well-fed pigeons was rather lower (500 - 600  $\mu$ Moles/hr./g. dry wt. at  $40^\circ$ ) and the increase caused by lactate addition was smaller.

### SECTION C

THE EFFECTS OF ADENINE NUCLEOTIDES ON CARBOHYDRATE METABOLISM IN PIGEON-LIVER HOMOGENATES: The addition of adenine nucleotides to these homogenates greatly increased the rate of carbohydrate removal in the presence and absence of lactate. Apart from being reactants in kinase reactions (hexokinase, glucokinase, PFK, phosphoglycerokinase, PK) and in oxidative phosphorylation, adenine nucleotides are known to affect the activity of at least two key enzymes of hepatic carbohydrate metabolism. PFK is inhibited by ATP and deinhibited by ADP and AMP (Passonneau & Lowry, 1964; Underwood & Newsholme, 1965,b). FDPase is inhibited by AMP (Taketa & Pogell, 1963; Underwood & Newsholme, 1965,a). As these activations and inhibitions were taken to be of importance in the regulation of carbohydrate metabolism (Krebs, 1964,a) the effects of added adenine nucleotides, mainly AMP, were examined in detail.



Stability of added nucleotides: Preliminary experiments

indicated that added adenine nucleotides rapidly disappeared in pigeon-liver homogenates prepared by the method of Krebs et al., (1964). Most of the added nucleotides were lost within 10 - 20 min. at 40°, the degradation being mainly due to 5'-AMP deaminase and 5'-nucleotidase (Dresel, 1953; see also Haugaard et al., 1960; Tiedemann & Born, 1963; Berry, 1965). Both these enzymes are known to be inhibited by Pi (Kizer et al., 1963; Segal & Brenner, 1960), and, as expected, increasing the Pi concentration in the incubation medium from 4.7 to 20 mM greatly diminished the loss of nucleotide, especially in the presence of lactate (Table 1). The results shown in Table 1 indicate that the protective effect of lactate at 20 mM-Pi was associated with the maintenance of a high concentration of ATP and a low concentration of AMP, the adenine nucleotide most liable to enzymic hydrolysis. Because of its stabilizing effect, 20 mM-Pi was used in most subsequent experiments. Lactate increased the rate of respiration (Section B) and the maintenance of higher ATP/AMP ratios in the presence of added lactate was probably related to the provision of substrate for coupled respiratory-chain phosphorylation (State 2 to 3 transition of Chance & Williams, 1955).

NAD<sup>+</sup> rapidly disappeared during the preparation of the homogenate (see Krebs & Hems, 1964) and on incubation both endogenous and added NAD<sup>+</sup> were extensively degraded under standard conditions. Nicotinamide at 20 mM inhibited this process. When NAD<sup>+</sup> (0.5 mM) was added to the medium, almost half was lost in 45 min. in the absence of nicotinamide,



Table 1. *Effect of the concentration of  $P_i$  on the stability of adenine nucleotides in pigeon-liver homogenates*

The data refer to 4 ml. of pigeon-liver homogenate prepared and incubated as described in the text. The pigeons were starved for 48 hr. Glucose (5 mM) was added in all experiments. AMP was added as a 0.1 M solution of the disodium salt from the side arm after 20 min. and incubation was continued for another 20 min. The reaction was stopped by rapid cooling (see the text) and the addition of  $\text{HClO}_4$ . 'Preformed' adenine nucleotides refers to the sum of AMP, ADP and ATP found at zero time.

| Expt.<br>no. | Concn. of<br>$P_i$<br>(mM) | Concn. of<br>added<br>lactate<br>(mM) | Added<br>AMP<br>( $\mu\text{moles}/$<br>cup) | Preformed<br>adenine<br>nucleo-<br>tides<br>( $\mu\text{mole}/$<br>cup) | Adenine nucleotides found<br>at end of incubation<br>( $\mu\text{moles}/\text{cup}$ ) |      |      |       | Change of<br>nucleo-<br>tides<br>( $\mu\text{moles}/$<br>cup) | $\frac{[\text{AMP}][\text{ATP}]}{[\text{ADP}]^2}$ |
|--------------|----------------------------|---------------------------------------|----------------------------------------------|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------|------|------|-------|---------------------------------------------------------------|---------------------------------------------------|
|              |                            |                                       |                                              |                                                                         | ATP                                                                                   | ADP  | AMP  | Total |                                                               |                                                   |
| 1            | 1                          | 0                                     | 10                                           | 0.86                                                                    | 0.11                                                                                  | 0.55 | 1.26 | 1.91  | - 8.94                                                        | 0.46                                              |
|              | 1                          | 40                                    | 10                                           | 0.86                                                                    | 0.43                                                                                  | 0.88 | 0.82 | 2.13  | - 8.73                                                        | 0.46                                              |
| 2            | 4.7                        | 0                                     | 20                                           | 0.66                                                                    | 0.49                                                                                  | 0.63 | 0.44 | 1.56  | - 19.1                                                        | 0.54                                              |
|              | 4.7                        | 40                                    | 20                                           | 0.66                                                                    | 0.82                                                                                  | 0.45 | 0.13 | 1.40  | - 19.3                                                        | 0.53                                              |
| 3            | 20                         | 0                                     | 10                                           | 0.87                                                                    | 3.98                                                                                  | 2.52 | 0.81 | 7.31  | - 3.56                                                        | 0.51                                              |
|              | 20                         | 40                                    | 10                                           | 0.87                                                                    | 8.91                                                                                  | 1.68 | 0.20 | 10.8  | - 0.07                                                        | 0.63                                              |
|              | 20                         | 0                                     | 20                                           | 0.87                                                                    | 2.32                                                                                  | 3.58 | 3.50 | 9.40  | - 11.5                                                        | 0.63                                              |
|              | 20                         | 40                                    | 20                                           | 0.87                                                                    | 12.9                                                                                  | 2.38 | 0.27 | 15.6  | - 5.2                                                         | 0.61                                              |



and about 20% with 20 mM-nicotinamide. The addition of AMP (2.5 mM) did not affect the rate of  $\text{NAD}^+$  loss nor was this affected by increasing the  $\text{P}_i$  concentration from 5 to 20 mM.

Equilibrium in the Adenylate Kinase system: The value of the quotient  $[\text{AMP}][\text{ATP}]/[\text{ADP}]^2$ , as calculated from the data obtained in these experiments, varied between 0.45 and 0.65, apart from instances where low values for AMP introduced a large error. The quotient at equilibrium varies between 0.4 and 0.9 with the  $\text{Mg}^{2+}$  concentration (Bowen & Kerwin, 1956), while it also varies with the concentration of other complexing agents, with the total nucleotide concentration, and with the ATP/ADP ratio (Krebs, H.A., Gevers, W. & Gascoyne, T. - unpublished observations; see also Bock, 1960). Thus adenylate kinase appears to have maintained the concentrations of the reactants near the equilibrium position, suggesting that the activity of the enzyme was high compared with the rate of ATP synthesis and utilization. Assay of adenylate kinase in fresh liver homogenates and cell fractions (see METHODS) showed that

i) the average activity was 20 - 30  $\mu\text{Moles/min./g. wet wt. at } 25^\circ$  (8 birds). Since the maximum rate of  $\text{O}_2$  uptake in the presence of lactate (Section B) was about 2.5 - 3  $\mu\text{Moles/min./g. wet wt. at } 25^\circ$ , the rate of ATP synthesis was not greater than the activity of adenylate kinase.

ii) The activity was the same in livers from well-fed and starved pigeons.

iii) 55% of the activity was in the supernatant after centrifugation of a standard sucrose homogenate at 30,000g for 20 min.; the remainder



was recovered in the resuspended particulate fraction, and 90% of this was solubilized by sonication (2 treatments of 60 sec.) into the supernatant after further centrifugation at the same force.

iv) The enzyme did not exhibit "latency" in that sonication of the whole homogenate or the particles did not increase the activity in the assay. According to Chappell & Greville (1963) this implies that none of the substrates were prevented by permeability barriers from reaching the active site, supporting the view of Chappell & Crofts (1965) that most of the mitochondrial adenylate kinase lies outside the atrac-tolyside-sensitive membrane (Klingenberg & Pfaff, 1966), and that it may be concerned mainly with extra-mitochondrial metabolism. In experiments described in Chap. 2, where adenine nucleotides were added to washed mitochondria, the quotient  $[AMP][ATP] / [ADP]^2$ , where it could be accurately determined, varied between 0.10 and 4.0.

EFFECTS OF ADDED AMP ON HOMOGENATES OF LIVERS FROM STARVED PIGEONS,  
IN THE PRESENCE AND ABSENCE OF LACTATE:

Effects of the addition of AMP on the formation and disappearance of carbohydrate: The disappearance of added glucose in pigeon-liver homogenates was greatly accelerated by AMP (Table 2, cups 2 and 3). The formation of glycogen did not account for the removal of glucose. The addition of lactate plus glucose gave rise to substantial gluconeogenesis (cup 5), which was completely abolished by AMP (cup 6). In fact, in this experiment more glucose disappeared in the presence than in the absence



of lactate.

Similar effects of AMP were observed in over 20 other experiments. Final AMP concentrations of 1.25 mM were generally more effective than concentrations of 5 mM. At mM-Pi, the effect of AMP on glucose disappearance was smaller.

Effects of AMP on the concentration of intermediates of carbohydrate metabolism: The addition of AMP caused characteristic changes in the concentrations of intermediary metabolites (Table 3), which depended on the presence of lactate. When no AMP was added the concentrations of glucose 6-P and fructose 6-P increased on incubation, and these increases were approximately doubled by lactate. The addition of AMP (final concentrations 0.1 - 2 mM) led to a fall in the concentrations of both hexose phosphates and abolished the increases found on incubation with lactate.

The most striking changes were those in the concentrations of FDP and triose-P, which increased more than 100-fold on the addition of AMP in the absence of lactate. There was a relatively small increase without AMP. Lactate abolished these increases and, instead of FDP and triose-P, large quantities of  $\alpha$ -glycero-P appeared, especially when AMP was added.

The accumulation of FDP and triose-P did not occur in homogenates from starved birds to which no glucose was added; in other words, the presence of glucose or glucogen was a prerequisite of the accumulation. This suggests that the bulk of the three intermediates was derived from



Table 2. *Effects of added AMP on carbohydrate metabolism in pigeon-liver homogenate (bird starved for 48 hr.)*

The data refer to 4 ml. of liver homogenate. The medium contained  $P_i$  (20 mM) and  $NaHCO_3$  (25 mM); the gas space contained  $CO_2 + O_2$  (5:95). Glucose (5 mM) was added. AMP was added from a side arm after 15 min. and the incubation was continued for another 30 min. The tissue dry wt. was 80 mg./cup.

| Cup no. ... ..                                       | Initial | 1    | 2    | 3     | 4    | 5    | 6     |
|------------------------------------------------------|---------|------|------|-------|------|------|-------|
| Additions (other than glucose):                      |         |      |      |       |      |      |       |
| Lactate (mM) ... ..                                  |         |      |      |       | 40   | 40   | 40    |
| AMP (mM) ... ..                                      |         |      |      | 2.5   |      |      | 2.5   |
| Time of incubation (min.) ... ..                     | 0       | 15   | 45   | 45    | 15   | 45   | 45    |
| Metabolites found ( $\mu$ moles/cup)                 |         |      |      |       |      |      |       |
| Glucose                                              | 2.5     | 19.5 | 14.2 | 5.9   | 23.9 | 27.5 | 6.6   |
| Glucose + glycogen                                   | 3.9     | 21.1 | 16.8 | 8.5   | 25.5 | 30.5 | 8.4   |
| Glycogen                                             | 1.4     | 1.6  | 2.6  | 2.6   | 1.6  | 3.0  | 1.8   |
| Changes between 15 and 45 min.<br>( $\mu$ moles/cup) |         |      |      |       |      |      |       |
| Glucose                                              |         |      | -5.3 | -13.6 |      | +3.6 | -17.3 |
| Glucose + glycogen                                   |         |      | -4.3 | -12.6 |      | +5.0 | -17.1 |
| Glycogen                                             |         |      | +1.0 | +1.0  |      | +1.5 | +0.3  |

Table 3. *Effects of AMP on the concentration of intermediates of carbohydrate metabolism in pigeon-liver homogenate (starved bird)*

The data refer to the same experiment for which values for glucose and glycogen are given in Table 2.

| Cup no. ... ..                         | Initial | 1     | 2     | 3     | 4       | 5       | 6             |
|----------------------------------------|---------|-------|-------|-------|---------|---------|---------------|
| Additions other than glucose ... ..    |         | None  | None  | AMP   | Lactate | Lactate | Lactate + AMP |
| Time of incubation (min.) ... ..       | 0       | 15    | 45    | 45    | 15      | 45      | 45            |
| Intermediates found ( $\mu$ moles/cup) |         |       |       |       |         |         |               |
| Glucose 6-phosphate                    | 0.13    | 0.74  | 0.63  | 0.18  | 1.39    | 1.10    | 0.59          |
| Fructose 6-phosphate                   | 0.04    | 0.20  | 0.15  | 0.04  | 0.40    | 0.30    | 0.10          |
| Fructose 1,6-diphosphate               | 0.02    | 0.02  | 0.12  | 8.46  | 0.03    | 0.03    | 0.13          |
| Triose phosphates                      | 0.08    | 0.11  | 0.41  | 9.71  | 0.12    | 0.14    | 0.42          |
| $\alpha$ -Glycerophosphate             | 0.26    | 0.18  | 0.58  | 0.90  | 8.18    | 4.28    | 15.1          |
| 3-Phosphoglycerate                     | 0.20    | 0.16  | 0.14  | 0.08  | 1.10    | 1.24    | 0.28          |
| 2-Phosphoglycerate                     | 0.06    | 0.06  | 0.05  | 0.02  | 0.16    | 0.17    | 0.04          |
| Phosphoenolpyruvate                    | 0.12    | 0.09  | 0.09  | 0.03  | 0.55    | 0.66    | 0.10          |
| Pyruvate                               | 0.10    | 0.07  | 0.10  | 0.11  | 0.37    | 0.38    | 0.27          |
| Lactate                                | 1.47    | 0.23  | 0.80  | 0.81  | 140     | 97.5    | 89.0          |
| Changes in lactate ( $\mu$ moles/cup)  |         | -1.24 | -0.67 | -0.68 | -21.5   | -64.0   | -72.5         |



carbohydrate rather than from gluconeogenic precursors. Results similar to those in Table 3 were obtained in every experiment where (1) the AMP concentration measured at the end of incubation was 0.1 mM or higher, (2) the concentration of added  $\text{NAD}^+$  was 0.125 mM or less, (3) glucose was added and (4) the concentration of Pi was 5 mM or more. Greater amounts of 3-PGA, 2-PGA, PEP and pyruvate were found at the end of the incubation in the presence than in the absence of lactate, but AMP caused a fall in the concentrations of all four intermediates with and without lactate. This fall was greatest for PEP and least for pyruvate.

These observations are all in agreement with the assumption that some of the rate-limiting reactions, and factors controlling them in the homogenates, included the following (only activations and inhibitions relevant to the interpretation of the results are listed):

i) PFK, activated by AMP and ADP, and inhibited by ATP and citrate (Passonneau & Lowry, 1964; Underwood & Newsholme, 1965,b);

ii) Hexokinase, inhibited by glucose 6-P (Weil-Malherbe & Bone, 1951; Crane & Sols, 1954);

iii) FDPase, inhibited by AMP and by FDP above 0.1 mM (Taketa & Pogell, 1963; Underwood & Newsholme, 1965.a; see Section D);

iv) Glyceraldehyde 3-phosphate dehydrogenase, limited in the direction of glycolysis by a low concentration of  $\text{NAD}^+$ , but fully active in the reverse direction, reflecting the differences in the kinetic behaviour of the enzyme towards  $\text{NAD}^+$  and NADH (Velick & Furfine, 1963 - data for rabbit-muscle enzyme).



The three changes that occurred on the addition of AMP, namely the rise in the concentrations of FDP and triose-P, the fall in those of hexose 6-P and the increased removal of glucose, could be accounted for by the behaviour of PFK (effect 1), plus the increased activity of hexokinase (effect 2). The inhibition of FDPase by AMP and FDP (effect 3) accounted for the flow of carbohydrate metabolism in the direction of glycolysis as opposed to gluconeogenesis. That glycolysis did not proceed beyond the formation of triose-P and caused an accumulation of FDP and triose-P was due to effect 4.

The changes that occurred in the presence of lactate and absence of added AMP, namely a fall in the concentration of FDP and rise in that of hexose 6-P were accounted for by the de-inhibition of FDPase (effect 3), and possibly also by inhibition of PFK (effect 1) associated with increases in the ATP and citrate concentrations, in conjunction with a fall in both AMP and ADP. The occurrence of a net synthesis of carbohydrate shows that triose-P dehydrogenase was active in the reduction of 1,3-diPGA (effect 4), as expected on account of the measured concentration of NADH in the homogenate (6-10  $\mu$ M; Krebs & Hems, 1964).

Effects of  $\text{NAD}^+$  concentration in the absence of lactate:

Addition of AMP further accelerated the increased removal of carbohydrate caused by raising the  $\text{NAD}^+$  concentration from 0.125 mM to 0.5 mM (Table 4). At this higher  $\text{NAD}^+$  concentration the addition of AMP caused no accumulation of FDP and triose-P. The observations are accounted for by the



Table 4. *Effect of NAD<sup>+</sup> concentration on carbohydrate utilization in pigeon-liver homogenate (starved bird)*

Each cup contained 4 ml. of homogenate (dry wt., 69 mg.) prepared as described in the Methods and Materials section and incubated for 45 min. AMP was added at 15 min. The homogenate contained initially ( $\mu$ moles cup): glucose plus glycogen, 3.2; glucose, 1.8; fructose diphosphate, 0.03; triose phosphates, 0.13;  $\alpha$ -glycerophosphate, 0.37.

| Cup no.                    | 1     | 2     | 3     | 4     |
|----------------------------|-------|-------|-------|-------|
| Additions:                 |       |       |       |       |
| Glucose (mM)               | 5     | 5     | 5     | 5     |
| NAD <sup>+</sup> (mM)      |       |       | 0.5   | 0.5   |
| AMP (mM)                   |       | 2.5   |       | 2.5   |
| Changes in compounds       |       |       |       |       |
| ( $\mu$ moles cup)         |       |       |       |       |
| Glucose + glycogen         | -6.8  | -11.4 | -8.5  | -13.3 |
| Glucose                    | -9.0  | -13.2 | -0.4  | -14.7 |
| Fructose diphosphate       | +0.44 | +4.61 | +0.04 | +0.13 |
| Triose phosphates          | +1.71 | +6.87 | +0.05 | +0.16 |
| $\alpha$ -Glycerophosphate | +0.07 | +0.31 | -0.20 | -0.09 |



rate-limiting function of  $\text{NAD}^+$  in the triose-P dehydrogenase system (effect 4). At 0.5 mM- $\text{NAD}^+$  this step no longer blocked the degradation of triose-P. Hence, in the absence of lactate, more glucose was broken down to the stages of pyruvate and beyond, and this breakdown was greatly enhanced by AMP (effects 1, 2 and 3). This interpretation is confirmed by another experiment in which the yield of  $^{14}\text{CO}_2$  from  $[\text{U-}^{14}\text{C}]$ -glucose (2.5 mM) was determined during a 30 min. incubation after the addition of AMP (2.5 mM) in the absence of lactate. AMP caused a 2.5-fold increase in the number of counts in carbon dioxide.

Effects of  $\text{NAD}^+$  concentration in the presence of lactate: The synthesis of carbohydrate in the presence of lactate was not affected by the addition of  $\text{NAD}^+$  (0.5 mM) (effect 4). A detailed analysis of the effects of AMP in such homogenates to which lactate and  $[\text{U-}^{14}\text{C}]$ glucose were added, and where gluconeogenesis predominated over glycolysis, was made in a further experiment (Table 5). The synthesis of carbohydrate that occurred in the absence of AMP was associated with a progressive fall in the specific activity both of glucose and of glucose plus glycogen until, at 90 min., it was less than half the starting value. However, only about one-fifth of the total radioactivity was not recovered as glucose or glycogen, which indicates a lower rate of glucose degradation under the test conditions i.e. in the presence of lactate (see DISCUSSION). In contrast, there was a net disappearance of glucose in the early stages after the addition of AMP, where the concentration of AMP, which fell (cup 3) from 2.5 to 0.23 mM in 15 min., remained high



Table 5. *Effects of AMP on the synthesis and turnover of carbohydrate in pigeon-liver homogenate (starved bird)*

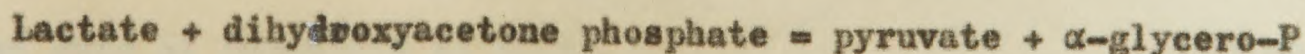
Each cup contained 4 ml. of homogenate incubated for the periods given in the Table. Each cup contained 2.5  $\mu$ C of [ $^{14}$ C]glucose, and the medium contained  $P_i$  (20 mM) and  $NAD^+$  (0.5 mM). AMP was added to the relevant cups at 15 min. Other procedures are described in detail in the text. The tissue dry wt. was 74.2 mg. cup.

| Cup no.                                   | ... | ... | ... | ... | ... | 1    | 2 <sup>a</sup> | 3    | 4    | 5    | 6    | 7    | 8    | 9    |
|-------------------------------------------|-----|-----|-----|-----|-----|------|----------------|------|------|------|------|------|------|------|
| Time (min.)                               | ... | ... | ... | ... | ... | 15   | 30             | 30   | 45   | 45   | 60   | 60   | 60   | 60   |
| Additions:                                |     |     |     |     |     |      |                |      |      |      |      |      |      |      |
| Glucose (mM)                              | ... | ... | ... | ... | ... | 2.5  | 2.5            | 2.5  | 2.5  | 2.5  | 2.5  | 2.5  | 2.5  | 2.5  |
| Lactate (mM)                              | ... | ... | ... | ... | ... | 35   | 35             | 35   | 35   | 35   | 35   | 35   | 35   | 35   |
| AMP (mM)                                  | ... | ... | ... | ... | ... | ...  | 2.5            | 2.5  | ...  | 2.5  | ...  | 2.5  | ...  | 2.5  |
| Compounds found                           |     |     |     |     |     |      |                |      |      |      |      |      |      |      |
| Glucose + glycogen:                       |     |     |     |     |     |      |                |      |      |      |      |      |      |      |
| $\mu$ moles cup                           | ... | ... | ... | ... | ... | 7.4  | 21.5           | 14.5 | 24.6 | 14.9 | 27.5 | 15.7 | 29.8 | 22.7 |
| Counts min./cup ( $\times 10^3$ )         | ... | ... | ... | ... | ... | 211  | 197            | 158  | 191  | 144  | 176  | 140  | 170  | 149  |
| Counts min./ $\mu$ mole ( $\times 10^2$ ) | ... | ... | ... | ... | ... | 121  | 92             | 109  | 78   | 97   | 64   | 89   | 57   | 65   |
| Free glucose:                             |     |     |     |     |     |      |                |      |      |      |      |      |      |      |
| $\mu$ moles cup                           | ... | ... | ... | ... | ... | 4.4  | 19.5           | 12.7 | 20.6 | 11.9 | 22.5 | 12.9 | 21.3 | 18.8 |
| Counts min./cup ( $\times 10^3$ )         | ... | ... | ... | ... | ... | 211  | 193            | 157  | 180  | 141  | 161  | 137  | 149  | 139  |
| Counts min./ $\mu$ mole ( $\times 10^2$ ) | ... | ... | ... | ... | ... | 146  | 99             | 123  | 87   | 118  | 72   | 106  | 70   | 74   |
| Glycogen:                                 |     |     |     |     |     |      |                |      |      |      |      |      |      |      |
| $\mu$ moles cup                           | ... | ... | ... | ... | ... | 3.0  | 2.0            | 1.8  | 4.0  | 3.0  | 5.0  | 2.8  | 8.5  | 3.9  |
| Counts min./cup ( $\times 10^3$ )         | ... | ... | ... | ... | ... | 0    | 4              | 1    | 11   | 3    | 15   | 3    | 21   | 10   |
| Counts min./ $\mu$ mole ( $\times 10^2$ ) | ... | ... | ... | ... | ... | 0    | 20             | 6    | 28   | 10   | 30   | 11   | 24   | 26   |
| $\alpha$ -Glycerophosphate:               |     |     |     |     |     |      |                |      |      |      |      |      |      |      |
| $\mu$ moles cup                           | ... | ... | ... | ... | ... | 0.64 | 6.79           | 12.6 | 4.08 | 13.4 | 2.56 | 12.9 | 1.61 | 6.83 |
| Counts min./cup ( $\times 10^3$ )         | ... | ... | ... | ... | ... | 0    | 6.25           | 38.8 | 6.15 | 33.2 | 3.9  | 27.1 | 1.4  | 13.5 |
| Counts min./ $\mu$ mole ( $\times 10^2$ ) | ... | ... | ... | ... | ... | 0    | 9.2            | 30.8 | 15.1 | 24.7 | 15.2 | 21.0 | 8.7  | 19.8 |
| Fructose diphosphate:                     |     |     |     |     |     |      |                |      |      |      |      |      |      |      |
| $\mu$ moles cup                           | ... | ... | ... | ... | ... | 0.04 | 0.03           | 0.03 | 0.03 | 0.03 | 0.01 | 0.03 | 0.01 | 0.01 |
| AMP:                                      | ... | ... | ... | ... | ... | ...  | ...            | ...  | ...  | ...  | ...  | ...  | ...  | ...  |
| $\mu$ moles/cup                           | ... | ... | ... | ... | ... | 0.48 | 0.37           | 0.92 | 0.32 | 0.68 | 0.29 | 0.56 | 0.33 | 0.47 |



enough to inhibit. When the concentration of AMP dropped further to 0.14 mM after 60 min., a net synthesis of glucose began. There was no fall in the specific activity of glucose until net synthesis occurred. The data for the radioactivity of glucose plus glycogen were similar to those found for free glucose; hence the fall in specific activity was not due to the formation of glucose from glycogen.

The concentrations of intermediates in these experiments were very similar to those given in Table 3, describing an experiment where the concentration of added  $\text{NAD}^+$  was 0.125 mM, except that the concentration of FDP at no stage rose above 0.01 mM (Table 5). Adding  $\text{NAD}^+$  (0.5 mM) did not abolish the extra formation of  $\alpha$ -glycero-P caused by the addition of AMP in the presence of high concentrations of lactate. The amount of radioactivity in  $\alpha$ -glycero-P increased sharply (Table 5). During the first 15 min. after AMP addition, 41900 counts/min. were lost from glucose, of which 33700 counts/min. appeared in  $\alpha$ -glycero-P; the net increase in the latter was 5.12  $\mu\text{Moles/cup}$ , which would, if derived entirely from glucose, account for 31600 counts/min. Thus AMP, on balance, caused a breakdown of carbohydrate to the level of triose-P; but, in the presence of lactate, triose-P was not dehydrogenated (effect 4) but reduced to  $\alpha$ -glycero-P in accordance with the reaction:



The concentrations of the four reactants were close to those of the equilibrium state (Table 6).

At the same time, AMP inhibited the initial stages of



gluconeogenesis from lactate to triose-P, as indicated by the fact that lactate carbon was not incorporated into glucose or  $\alpha$ -glycero-P. This inhibition was not due to decreased utilization of lactate because the data in Table 7 show that more lactate was removed when AMP was added. The conclusion must be drawn that AMP above a critical concentration caused pyruvate to undergo oxidation rather than conversion into PEP. This effect could have been exerted either by stimulation of the rate of pyruvate oxidation or by the inhibition of an enzyme concerned with the synthesis of PEP.

Evidence in favour of the first possibility was: (a) the increase in the rate of oxygen uptake, and increased oxidation of lactate to carbon dioxide, caused by AMP (see below), presumably related to the formation of ADP by the action of adenylate kinase; (b) the fall in the steady-state concentration of pyruvate to about half that found in the absence of AMP (Table 6).

Evidence in favour of the second possibility was: (a) the fall in the concentration of PEP (and in that of PGA) caused by AMP (Table 2), which could have been due to diminished activity of PEPCK or to enhanced action of PK; (b) the rise in the concentration of malate to nearly double that found when AMP was not added, in conjunction with the increased rate of formation of other intermediates of the tricarboxylic acid cycle and related amino acids (Table 7). This suggests that pyruvate carboxylase was not affected by AMP or the products of its metabolism. (See Section E). However, inhibition of PEPCK could have



Table 6. *Equilibrium between two linked dehydrogenase systems in pigeon-liver homogenate*

The values given refer to the experiment described in Table 5. Determinations were carried out as indicated in the Methods and Materials section. The value for dihydroxyacetone phosphate was calculated from that for the sum of triose phosphates, assuming that there was equilibrium in the triose phosphate-isomerase system and that  $K = [\text{dihydroxyacetone phosphate}]/[\text{glyceraldehyde 3-phosphate}] = 22$  (Baronowski, 1949).  $E'_0$  values of the lactate-pyruvate and  $\alpha$ -glycerophosphate-dihydroxyacetone phosphate couples at 25° were taken to be  $-0.185$  and  $-0.192$  v respectively (Burton, 1957), and the expected ratio  $K = [\alpha\text{-glycerophosphate}][\text{pyruvate}]/[\text{lactate}][\text{dihydroxyacetone phosphate}] = 0.58$  was derived from the relation  $\Delta E'_0 = 0.03 \log K$ .

| Cup no. | Time (min.) | Added AMP<br>( $\mu$ moles/cup) | Compounds found ( $\mu$ moles/cup) |                                    |          |                                 | $K$  |
|---------|-------------|---------------------------------|------------------------------------|------------------------------------|----------|---------------------------------|------|
|         |             |                                 | Lactate                            | Dihydroxy-<br>acetone<br>phosphate | Pyruvate | $\alpha$ -Glycero-<br>phosphate |      |
| 1       | 15          | 0                               | 124                                | 0.16                               | 1.51     | 7.53                            | 0.57 |
| 2       | 30          | 0                               | 115                                | 0.19                               | 1.81     | 6.79                            | 0.56 |
| 3       | 30          | 10                              | 101                                | 0.32                               | 1.08     | 12.6                            | 0.42 |
| 4       | 45          | 0                               | 88.9                               | 0.15                               | 1.86     | 4.08                            | 0.57 |
| 5       | 45          | 10                              | 79.0                               | 0.34                               | 0.93     | 13.4                            | 0.47 |
| 6       | 60          | 0                               | 75.3                               | 0.14                               | 1.84     | 2.56                            | 0.45 |
| 7       | 60          | 10                              | 63.1                               | 0.29                               | 0.85     | 12.9                            | 0.60 |
| 8       | 90          | 0                               | 54.0                               | 0.15                               | 0.72     | 1.61                            | 0.14 |
| 9       | 90          | 10                              | 35.6                               | 0.24                               | 0.84     | 6.83                            | 0.67 |

Table 7. *Effects of AMP on the formation of intermediates of the tricarboxylic acid cycle and related amino acids in pigeon-liver homogenates in the presence of lactate (starved bird)*

Each cup contained 4 ml. of homogenate prepared as described in the Methods and Materials section and incubation was for 60 min. The medium contained  $P_i$  (20 mM) and  $NAD^+$  (0.5 mM). AMP was present in cup 2 from zero time. The calculation of 'lactate equivalents' was made according to the principles described by Berry (1965) for the calculation of 'pyruvate equivalents' of intermediates measured. The tissue dry wt. was 80.4 mg./cup.

| Cup no.                                               | Initial | 1     | 2     |
|-------------------------------------------------------|---------|-------|-------|
| Additions:                                            |         |       |       |
| Lactate (mM)                                          |         | 40    | 40    |
| AMP (mM)                                              |         |       | 2.5   |
| Intermediates found ( $\mu$ moles/cup)                |         |       |       |
| Glutamate                                             | 0.97    | 2.11  | 2.66  |
| Glutamine                                             | 0.20    | 0.46  | 0.37  |
| Aspartate                                             | 0.71    | 0.87  | 2.09  |
| Asparagine                                            | 0.05    | 0.01  | 0.02  |
| Alanine                                               | 0.85    | 2.34  | 3.52  |
| Citrate                                               | 0.49    | 1.23  | 1.86  |
| $\alpha$ -Oxoglutarate                                | 0.14    | 3.26  | 2.94  |
| Fumarate                                              | < 0.01  | 1.10  | 1.99  |
| Malate                                                | 0.62    | 6.75  | 12.2  |
| Lactate removed ( $\mu$ moles/cup)                    |         | -57.6 | -72.2 |
| Total 'lactate equivalents' gained ( $\mu$ moles/cup) |         | +21.2 | +31.5 |



accounted for the effects of AMP. This point was further investigated (Chap. 4) since Holten & Nordlie (1965) observed an inhibition of guinea-pig liver mitochondrial PEPCK by AMP.

Effects of AMP on gas exchanges: When only glucose was added to the homogenates, a 50% increase in respiration was brought about by AMP addition (Table 8). Optimum conditions for this effect included the presence of 20 mM-Pi and 5 mM-bicarbonate or more in the medium; the effect was slight when the concentration of Pi was 1 mM, and without bicarbonate there was no stimulation by AMP. This requirement for  $\text{HCO}_3^-$  was presumably an expression of the need under these conditions to synthesize oxaloacetate from pyruvate, in order to increase the rate of oxidations in the tricarboxylic acid cycle. The increase in the  $\text{O}_2$  uptake caused by AMP in the presence of lactate was only 20 - 30%. Varying the concentration of added AMP from 1.25 mM to 5 mM made little difference. At 2.5 mM the rise in  $\text{O}_2$  uptake was progressive with time (Table 9), indicating that the process was related to formation of ADP from AMP by the action of adenylate kinase. At 5 mM initial stimulation was followed by inhibition when the concentration of Pi was 5 mM, and in the latter case extensive losses of adenine nucleotides occurred during the incubation. The inhibition was probably due to an effect of ammonia set free from AMP (see Recknagel & Potter, 1951; Berry, 1964). When  $\text{NAD}^+$  was 0.125 mM, addition of AMP did not change the respiratory quotient, despite the large increases in consumption of both  $\text{O}_2$  and carbohydrate. When  $\text{NAD}^+$  was added at 0.5 mM, the increase in  $\text{O}_2$  uptake



Table 8. *Effects of AMP on gas exchange in pigeon-liver homogenate (starved bird)*

In each experiment, 4 ml. of homogenate was incubated for 45 min.; full details of procedure are given in the Methods and Materials section. The  $P_i$  concentration was 20 mM. Experiments were carried out with and without AMP (5 mM in Expt. 1 and 2.5 mM in the others), which was added from a side arm at 15 min.  $CO_2$  buffers were used in Expt. 1 and the method of Warburg & Yabusoe (1924) in Expts. 2-5. The gas measurements refer to the periods 15-45 min. The tissue dry wt. varied from 70 to 80 mg. Expt. 1 is the same as that described in Tables 2 and 3, where values for changes in carbohydrates and intermediates are given. R.Q., Respiratory quotient.

| Expt. no. | Substrate added  | Concn. of substrate (mM) | Concn. of added $NAD^+$ (mM) | Concn. of $HCO_3^-$ (mM) | Without AMP                     |      | With AMP                        |      | Increase of $O_2$ uptake caused by AMP (%) |
|-----------|------------------|--------------------------|------------------------------|--------------------------|---------------------------------|------|---------------------------------|------|--------------------------------------------|
|           |                  |                          |                              |                          | $O_2$ uptake ( $\mu$ moles/cup) | R.Q. | $O_2$ uptake ( $\mu$ moles/cup) | R.Q. |                                            |
| 1         | Glucose          | 5                        | 0.125                        | 25                       | -39.8                           | 0.85 | -61.3                           | 0.85 | 54                                         |
|           | Glucose, lactate | 5, 40                    | 0.125                        | 25                       | -56.4                           | 0.55 | -72.0                           | 0.59 | 27                                         |
| 2         | Glucose          | 5                        | 0.125                        | 0                        | -32.0                           | 0.84 | -33.4                           | 0.85 | 4                                          |
| 3         | Glucose          | 5                        | 0.125                        | 5                        | -34.1                           | 0.91 | -50.8                           | 0.90 | 49                                         |
| 4         | Glucose          | 5                        | 0.5                          | 5                        | -33.5                           | 0.85 | -42.2                           | 0.95 | 26                                         |
| 5         | None             |                          | 0.125                        | 5                        | -25.2                           | 0.89 | -30.9                           | 0.88 | 23                                         |

Table 9. *Time-course of AMP effect on oxygen uptake by pigeon-liver homogenate*

Conditions were as described in Table 8 and Expt. 1 corresponds to Expt. 1 of that Table.  $CO_2$  buffers were used to measure the uptake of  $O_2$ . AMP was added at 15 min. The tissue dry wt. was 80 mg./cup in the first experiment and 75 mg./cup in the second.

| Expt. no. | Substrate added  | Concn. of substrate (mM) | Concn. of $P_i$ (mM) | Concn. of added AMP (mM) | $O_2$ uptake ( $\mu$ moles/cup) |            |            | Total loss of adenine nucleotides during incubation ( $\mu$ moles/cup) |
|-----------|------------------|--------------------------|----------------------|--------------------------|---------------------------------|------------|------------|------------------------------------------------------------------------|
|           |                  |                          |                      |                          | 0-15 min.                       | 15-30 min. | 30-45 min. |                                                                        |
| 1         | Glucose          | 5                        | 20                   | 0                        | -20.8                           | -20.2      | -19.6      |                                                                        |
|           | Glucose          | 5                        | 20                   | 2.5                      | -20.7                           | -27.8      | -33.5      | -3.4                                                                   |
|           | Glucose, lactate | 5, 40                    | 20                   | 0                        | -32.2                           | -30.3      | -26.1      |                                                                        |
|           | Glucose, lactate | 5, 40                    | 20                   | 2.5                      | -32.2                           | -33.5      | -38.5      | -0.1                                                                   |
| 2         | Glucose          | 5                        | 5                    | 0                        | -22.1                           | -21.5      | -21.1      |                                                                        |
|           | Glucose          | 5                        | 5                    | 5                        | -22.0                           | -27.4      | -11.2      | -18.4                                                                  |



due to AMP was smaller, but the respiratory quotient rose from 0.85 to 0.95 (Table 8), signifying that carbohydrate was being oxidised in preference to endogenous fatty acids. This correlated with the absence of a block at glyceraldehyde 3-phosphate dehydrogenase (Table 4).

In homogenates containing very small initial quantities of carbohydrate and to which no glucose was added the increase in oxygen uptake brought about by AMP was only 23% (Table 8, Expt. 5). Virtually all the preformed glucose and glycogen disappeared on incubation whether AMP was added or not.

EFFECTS OF ADDED AMP ON LIVER HOMOGENATES FROM FED PIGEONS:

Effects of AMP on the formation and degradation of carbohydrate: In homogenates of livers from well-fed pigeons, AMP caused an increase in the rate of disappearance of total carbohydrate, mainly glucose (Table 10). When lactate was added, some gluconeogenesis occurred, but the rate was low. When AMP and lactate were added together no net synthesis of carbohydrate occurred but much carbohydrate disappeared. The absolute effect of AMP when corrected for the control without AMP was about the same without lactate (cups 2 and 3: 10.3  $\mu$ Moles) and with lactate (cups 5 and 6: 11.1  $\mu$ Moles).

Effects of AMP on the concentration of intermediates: The effects of AMP on the concentrations of intermediates were similar to those found in homogenates from starved pigeons (Table 11). When  $\text{NAD}^+$  was added at 0.5 mM, FDP and triose-P did not accumulate. Iodoacetate at 0.25 mM (in the absence of lactate) and  $\text{NAD}^+$  added at 0.5 mM (Table 12)



Table 10. *Effects of added AMP on carbohydrate metabolism in pigeon-liver homogenate (well-fed bird)*

Conditions were as described in Table 2. The tissue dry wt. was 80 mg./cup.

| Cup no. ...                                           | ... | ... | Initial | 1    | 2    | 3     | 4    | 5    | 6    |
|-------------------------------------------------------|-----|-----|---------|------|------|-------|------|------|------|
| Additions:                                            |     |     |         |      |      |       |      |      |      |
| Lactate (mM) ...                                      | ... | ... |         |      |      |       | 40   | 40   | 40   |
| AMP (mM) ...                                          | ... | ... |         |      |      | 2.5   |      |      | 2.5  |
| Time of incubation (min.)                             | ... | ... | 0       | 15   | 45   | 45    | 15   | 45   | 45   |
| Metabolites found ( $\mu$ moles/cup):                 |     |     |         |      |      |       |      |      |      |
| Glucose                                               |     |     | 12.2    | 20.8 | 15.9 | 9.4   | 19.6 | 18.8 | 15.0 |
| Glucose + glycogen                                    |     |     | 71.2    | 71.5 | 70.3 | 60.0  | 70.5 | 74.8 | 63.7 |
| Glycogen                                              |     |     | 59.0    | 50.7 | 54.4 | 50.6  | 50.9 | 56.0 | 48.7 |
| Changes between 15 and 45 min.<br>( $\mu$ moles/cup): |     |     |         |      |      |       |      |      |      |
| Glucose                                               |     |     |         |      | -4.9 | -11.4 |      | -0.8 | -4.6 |
| Glucose + glycogen                                    |     |     |         |      | -1.2 | -11.5 |      | +4.3 | -6.8 |
| Glycogen                                              |     |     |         |      | +3.7 | -0.1  |      | +5.1 | -2.2 |

Table 11. *Effects of AMP on the concentration of intermediates of carbohydrate metabolism in pigeon-liver homogenate (well-fed bird)*

The values refer to the experiment described in Table 10, where conditions of incubation are given.

| Cup no. ...                            | ... | ... | Initial | 1     | 2     | 3     | 4       | 5       | 6             |
|----------------------------------------|-----|-----|---------|-------|-------|-------|---------|---------|---------------|
| Additions                              | ... | ... |         | None  | None  | AMP   | Lactate | Lactate | AMP + lactate |
| Time of incubation (min.)              | ... | ... | 0       | 15    | 45    | 45    | 15      | 45      | 45            |
| Intermediates found ( $\mu$ moles/cup) |     |     |         |       |       |       |         |         |               |
| Glucose 6-phosphate                    |     |     | 0.78    | 0.79  | 0.75  | 0.52  | 1.08    | 1.20    | 0.86          |
| Fructose 6-phosphate                   |     |     | 0.26    | 0.25  | 0.24  | 0.18  | 0.35    | 0.39    | 0.30          |
| Fructose diphosphate                   |     |     | 0.13    | 0.25  | 0.55  | 5.11  | 0.12    | 0.11    | 0.14          |
| Triose phosphates                      |     |     | 0.26    | 0.65  | 1.62  | 8.58  | 0.21    | 0.17    | 0.28          |
| $\alpha$ -Glycerophosphate             |     |     | 1.66    | 0.51  | 0.27  | 0.58  | 5.12    | 7.86    | 14.5          |
| 3-Phosphoglycerate                     |     |     | 0.32    | 0.30  | 0.30  | 0.14  | 0.45    | 0.49    | 0.21          |
| 2-Phosphoglycerate                     |     |     | 0.07    | 0.06  | 0.05  | 0.03  | 0.06    | 0.08    | 0.04          |
| Phosphoenolpyruvate                    |     |     | 0.14    | 0.13  | 0.14  | 0.06  | 0.28    | 0.26    | 0.15          |
| Pyruvate                               |     |     | 0.10    | 0.10  | 0.09  | 0.04  | 0.35    | 0.38    | 0.19          |
| Lactate                                |     |     | 2.45    | 1.10  | 0.52  | 0.71  | 149.1   | 124.0   | 119.5         |
| Change in lactate ( $\mu$ moles/cup)   |     |     |         | -1.35 | -1.93 | -1.74 | -14.8   | -39.9   | -44.4         |



prevented the removal of total carbohydrate during incubation (although breakdown of glycogen to glucose occurred), and the concentrations of intermediates did not increase significantly. When AMP was added, however, a large amount of carbohydrate disappeared; this could be accounted for quantitatively as FDP and triose-P. This confirms the conclusions reached above that the primary effects of AMP were on PFK and FDPase (effects 1 and 2).

EFFECTS OF 2,4-DINITROPHENOL: Experiments with DNP were carried out to ascertain whether interference with the maintenance of ATP synthesis would simulate the effects of AMP addition in a specific way. The ATP concentration fell slightly at 0.05 mM-DNP, and by about 60% at 0.1 mM-DNP (Table 13). There were corresponding rises in the concentrations of AMP and ADP. These changes of the adenine nucleotides were paralleled by a fall in carbohydrate synthesis. Though there was no accumulation of FDP or triose-P, the concentration of glucose 6-P was decreased and that of  $\alpha$ -glycero-P increased. It is thus feasible that *Some of* the effects of dinitrophenol on carbohydrate metabolism were due to the rise in the concentration of AMP.

EFFECTS OF ADDITION OF ADP AND ATP: The addition of ADP or ATP caused changes similar to those obtained with AMP. This may be expected, owing to the ready interconversion of the adenine nucleotides. No evidence was obtained to indicate that ATP has specific effects, except that, in homogenates of well-fed bird livers with high initial glycogen



Table 12. *Effect of iodoacetate and AMP on pigeon-liver homogenate (well-fed bird)*

Each cup contained 4 ml. of homogenate. AMP was added at 15 min. during the incubation of 45 min.; iodoacetate was present initially. The tissue dry wt. was 83 mg./cup.

| Cup. no. ... ..                        | Initial | 1    | 2    | 3    | 4    |
|----------------------------------------|---------|------|------|------|------|
| Additions:                             |         |      |      |      |      |
| NAD <sup>+</sup> (mM) ... ..           |         | 0.5  | 0.5  | 0.5  | 0.5  |
| Sodium iodoacetate (mM)...             |         |      |      | 0.25 | 0.25 |
| AMP (mM) ... ..                        |         |      | 2.5  |      | 2.5  |
| Intermediates found ( $\mu$ moles/cup) |         |      |      |      |      |
| Glucose + glycogen                     | 33.7    | 25.2 | 20.2 | 33.2 | 22.8 |
| Glucose                                | 8.45    | 5.98 | 4.23 | 13.5 | 10.0 |
| Glycogen                               | 25.2    | 19.2 | 16.0 | 19.7 | 12.8 |
| Fructose diphosphate                   | 0.06    | 0.07 | 0.10 | 0.09 | 7.80 |
| Triose phosphates                      | 0.19    | 0.26 | 0.32 | 0.47 | 10.1 |
| $\alpha$ -Glycerophosphate             | 1.39    | 0.31 | 0.54 | 1.02 | 1.19 |

Table 13. *Effects of 2,4-dinitrophenol on carbohydrate synthesis in pigeon-liver homogenate (starved bird)*

Each cup contained 4 ml. of homogenate incubated for 45 min. The medium contained lactate (40 mM) but no added glucose; the concentration of P<sub>i</sub> was 20 mM. The tissue dry wt. was 74.1 mg./cup.

| Cup. no. ... ..                              | 1     | 2     | 3     | 4     |
|----------------------------------------------|-------|-------|-------|-------|
| Dinitrophenol (mM)...                        | 0     | 0.02  | 0.05  | 0.1   |
| O <sub>2</sub> uptake ( $\mu$ moles/cup) ... | -70.4 | -72.3 | -70.5 | -68.8 |
| Metabolites found ( $\mu$ moles/cup)         |       |       |       |       |
| Glucose                                      | 13.9  | 13.2  | 7.17  | 2.68  |
| Glucose + glycogen                           | 18.1  | 15.7  | 10.1  | 5.18  |
| Glucose 6-phosphate                          | 0.88  | 0.80  | 0.59  | 0.28  |
| Fructose 6-phosphate                         | 0.22  | 0.22  | 0.14  | 0.05  |
| Fructose 1,6-diphosphate                     | 0.03  | 0.03  | 0.03  | 0.05  |
| Triose phosphates                            | 0.12  | 0.14  | 0.12  | 0.23  |
| $\alpha$ -Glycerophosphate                   | 2.78  | 4.02  | 3.63  | 4.42  |
| Pyruvate                                     | 0.77  | 0.36  | 0.29  | 0.25  |
| ATP                                          | 0.97  | 0.90  | 0.84  | 0.37  |
| ADP                                          | 0.32  | 0.38  | 0.50  | 0.71  |
| AMP                                          | 0.05  | 0.10  | 0.15  | 0.46  |



content, high concentrations (5 mM) of ATP caused rapid glycogenolysis with increases in free glucose and glucose 6-P; this was possibly due to activation of glycogen phosphorylase by 3',5'-(cyclic)-AMP formed from ATP.

#### SECTION D

FRUCTOSE DIPHOSPHATASE IN PIGEON LIVER: Underwood & Newsholme (1965,a) have reported detailed studies on the FDPase of rat liver. Their data will be used in the interpretation of the results obtained in the present work (see DISCUSSION). A limited study was undertaken to check whether the pigeon-liver enzyme was indeed similar.

When assayed in the presence of 10 mM 2-mercaptoethanol and 5 mM  $Mg^{2+}$  at pH 7.4, the activity of FDPase was 30 - 40  $\mu$ Moles/min./g. dry wt. at 25<sup>0</sup>, and did not vary during a cycle of fasting (48 hr.) and refeeding. In the absence of the thiol agent and at 1 mM  $Mg^{2+}$  (i.e. conditions resembling the standard pigeon-liver homogenates) the activity was 5 - 7  $\mu$ Moles/min./g. dry wt. at 25<sup>0</sup>; addition of 0.25 mM EDTA doubled this rate.

0.5 mM AMP inhibited the enzyme (in the full assay system with thiol agent and 5 mM  $Mg^{2+}$ ) by 98%. At 1 mM  $Mg^{2+}$ , the inhibition by 0.1 mM AMP was 85%. The optimal FDP concentration was 50 - 75  $\mu$ M; at higher concentrations there was increasing inhibition although the degree of inhibition never exceeded 70%. The apparent  $K_m$  for FDP was in the region



$2 - 5 \times 10^{-6} \text{M}$  (Lineweaver-Burk plot at low FDP concentrations). 90% of the activity was found in the supernatant fraction of a sucrose homogenate centrifuged at 30,000g for 20 min.

These results clearly indicated marked resemblance of pigeon liver FDPase to the enzyme from rat liver,

### SECTION E

#### FATE AND UTILIZATION OF $[3-^{14}\text{C}]$ -L-LACTATE IN PIGEON-LIVER

HOMOGENATES, IN THE PRESENCE AND ABSENCE OF AMP: Krebs (1964,b) outlined some of the problems involved in the interpretation of isotope incorporation data in complex systems. The utilization of  $[3-^{14}\text{C}]$ -lactate was studied in pigeon-liver homogenates specifically to answer a limited number of questions that seemed amenable to this approach:

i) Were the large amounts of malate that arose in the homogenates during lactate utilization synthesized directly from pyruvate by carboxylation, or were they formed by forward operation of the tricarboxylic acid cycle?

ii) What were the effects of AMP addition on the incorporation of  $^{14}\text{C}$  from lactate into carbohydrate and  $\alpha$ -glycero-P?

iii) Were there differences in the incorporation patterns between liver homogenates from fed and starved pigeons?

The results of experiments in which  $[3-^{14}\text{C}]$ -lactate was added to pigeon-liver homogenates are recorded in Table 14. The results of both



**Table 14.** Fate and utilization of  $[3-^{14}\text{C}]\text{-L-lactate}$  in pigeon-liver homogenates (starved and fed birds).

Standard homogenates were prepared, using a liver from a starved bird (Expt. A) and one from a fed bird (Expt. B). They were incubated for 60 min. as described in METHODS. L-lactate (150  $\mu\text{Moles}$ , specific activity 8000 - 8100 counts/min./ $\mu\text{Mole}$ ) was added to each cup. Added  $\text{NAD}^+$  was 0.25 mM, and Pi was 20 mM. The measurement of intermediates and their radioactivity was as described in METHODS. Tissue dry wt. was 80 mg. in Expt. A and 77 mg. in Expt. B.

| Expt. No.                           |                                  | A       |      |       | B       |       |
|-------------------------------------|----------------------------------|---------|------|-------|---------|-------|
| Cup No.                             |                                  | Initial | 1    | 2     | Initial | 1     |
| Added AMP ( $\mu\text{Moles/cup}$ ) |                                  |         |      | 10    |         |       |
| Compounds found:                    |                                  |         |      |       |         |       |
| Lactate                             | $\mu\text{Moles/cup}$            | 150     | 92.6 | 77.5  | 153     | 102   |
|                                     | Counts/min./cup( $\times 10^2$ ) | 12170   | 7500 | 6280  | 12170   | 8160  |
|                                     | Counts/min./ $\mu\text{Atom C}$  | 2700    | 2700 | 2700  | 2660    | 2660  |
| Malate                              | $\mu\text{Moles/cup}$            | 0.62    | 6.75 | 12.2  | 0.88    | 10.45 |
|                                     | Counts/min./cup( $\times 10^2$ ) | -       | 720  | 1250  | -       | 1040  |
|                                     | Counts/min./ $\mu\text{Atom C}$  | -       | 2670 | 2565  | -       | 2490  |
| $\alpha$ -Oxo-glutarate             | $\mu\text{Moles/cup}$            | 0.14    | 3.26 | 2.94  | 0.11    | 4.55  |
|                                     | Counts/min./cup( $\times 10^2$ ) | -       | 510  | 447   | -       | 665   |
|                                     | Counts/min./ $\mu\text{Atom C}$  | -       | 3130 | 3040  | -       | 2880  |
| Glutamate                           | $\mu\text{Moles/cup}$            | 0.97    | 2.11 | 2.66  | 0.68    | 1.85  |
|                                     | Counts/min./cup( $\times 10^2$ ) | -       | 267  | 318   | -       | 257   |
|                                     | Counts/min./ $\mu\text{Atom C}$  | -       | 2530 | 2390  | -       | 2860  |
| $\alpha$ -Glycero-P                 | $\mu\text{Moles/cup}$            | 0.53    | 3.64 | 11.75 | 1.36    | 11.05 |
|                                     | Counts/min./cup( $\times 10^2$ ) | -       | 301  | 520   | -       | 510   |
|                                     | Counts/min./ $\mu\text{Atom C}$  | -       | 2760 | 1475  | -       | 1540  |
| Glucose                             | $\mu\text{Moles/cup}$            | 4.26    | 12.7 | 5.52  | 7.65    | 8.02  |
|                                     | Counts/min./cup( $\times 10^2$ ) | -       | 1275 | 646   | -       | 270   |
|                                     | Counts/min./ $\mu\text{Atom C}$  | -       | 1672 | 1952  | -       | 562   |
| Glycogen                            | $\mu\text{Moles/cup}$            | 2.46    | 4.20 | 1.15  | 66.5    | 60.4  |
|                                     | Counts/min./cup( $\times 10^2$ ) | -       | 196  | 40    | -       | 311   |
|                                     | Counts/min./ $\mu\text{Atom C}$  | -       | 775  | 442   | -       | 86    |
| $\text{CO}_2$                       | Counts/min./cup( $\times 10^2$ ) | -       | 708  | 1314  | -       | 612   |



experiments showed that accumulation of malate from lactate occurred almost exclusively by carboxylation of pyruvate, and not by a combination of carboxylation and decarboxylation followed by forward operation of the tricarboxylic acid cycle. This conclusion is based on the specific activity of malate (counts/min./ $\mu$ Atom C), which was either less than, or equal to, the specific activity of the lactate added. In the event of a sequence of reactions in which lactate was the precursor of both oxaloacetate and acetyl CoA, which then condensed to form citrate and were oxidised as such, the malate formed would have had a specific activity one and a half times that of lactate. This is a maximum value and would be less if endogenous precursors of acetyl CoA were present. However, the data for glutamate and  $\alpha$ -oxo-glutarate show that utilization of endogenous substrates (e.g. fatty acids) was low in the presence of added lactate; the specific activities of these compounds were those expected if they were formed by forward operation of the tricarboxylic acid cycle following synthesis of citrate, most molecules of which contained 2 labelled atoms. Since there was little incorporation of such extra counts into malate, the stages of the cycle between  $\alpha$ -oxo-glutarate and malate were not active under these conditions, independent of the nutritional state of the birds.

AMP addition inhibited incorporation of [3-<sup>14</sup>C]-lactate into carbohydrate (by 50%); incorporation into  $\alpha$ -glycero-P was increased, in contrast with the results recorded in Table 5, but the bulk of the net increase in this compound on AMP addition was due to carbohydrate



degradation, as in the other experiment. The difference between the two experiments may have resided in the fact that no time course was followed in the present instance; changes in AMP concentration subsequent to its addition may have been accompanied by secondary changes in the metabolic pattern. AMP increased the oxidation of  $[3-^{14}\text{C}]$ -lactate to  $\text{CO}_2$ , as well as the carboxylation of pyruvate to malate; there was some dilution of label in the products of pyruvate metabolism, presumably due to formation of unlabelled pyruvate from carbohydrate (see Section C).

Comparing the results of  $[3-^{14}\text{C}]$ -lactate incorporation in the two nutritional states, differences are apparent in the diminished entry of  $^{14}\text{C}$  into carbohydrate in the homogenates from fed birds, as well as the smaller contribution made by lactate to the  $\alpha$ -glycero-P formed in the absence of AMP.

## SECTION F

### EFFECTS OF LACTATE CONCENTRATION ON INTERMEDIATES OF CARBOHYDRATE

METABOLISM IN PIGEON-LIVER HOMOGENATES: Krebs et al., (1964) found that high concentrations of lactate were required for optimal rates of gluconeogenesis in pigeon-liver homogenates, and attributed this to the rapid removal of lactate (of which 25% was converted into carbohydrate) and to kinetic properties of lactate dehydrogenase, such as low affinity for lactate (Nisselbaum & Bodansky, 1963) and inhibition by pyruvate (Zewe & Fromm, 1962). A study was undertaken of the changes occurring



in the rate of carbohydrate synthesis and lactate removal, and in the concentrations of intermediates of gluconeogenesis, at various lactate concentrations during short-term incubations. These experiments were designed to answer the question of how lactate addition, besides making available precursor molecules, favoured high rates of glucose formation. Key regulatory steps might thus be identified.

Standard pigeon-liver homogenates were incubated with added lactate in the range 2 - 40 mM, for 15 min.; some of the cups were incubated for 30 min. (Table 15). Pyruvate concentrations rose as lactate was increased, although the pyruvate/lactate ratio progressively declined. The level of oxaloacetate was so low as to be unmeasurable, but the malate concentration rose roughly in parallel with pyruvate. Citrate followed a similar course. The amounts of PEP, 2- and 3-PGA fell as the lactate concentration was raised, excepting that they increased sharply at very low levels of added lactate. Triose-P increased slightly above lactate concentrations of 0.25 mM, as did FDP. Hexose 6-P was increased at low levels of lactate but there was no further change when more than 4 mM substrate was present. The amounts of total carbohydrates were similar in all the cups except that the increase due to lactate was smaller at concentrations of the latter less than 4 mM. The other principal product of lactate metabolism via the gluconeogenic pathway,  $\alpha$ -glycero-P, increased in parallel with lactate up to the highest concentrations employed (40 mM). The cups incubated for longer periods confirmed that the rates of carbohydrate synthesis were constant



**Table 15.** Effects of lactate concentration on intermediates of carbohydrate metabolism in pigeon-liver homogenate (starved bird).

Standard homogenates were prepared as described in METHODS.  $P_i$  was 20 mM and added  $NAD^+$  was 0.25 mM. Each cup contained 2.5 mM added glucose. Incubations were terminated by rapid freezing and acidification. The dry wt. was 57 mg/cup. Zero-time samples contained the following ( $\mu$ Moles/cup): lactate, 1.22; glucose + glycogen, 3.60; free glucose, 2.01.

| No                                          | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      |
|---------------------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Added lactate (mM)                          | 40     | 160    | 0      | 2      | 3.5    | 6.5    | 10     | 20     | 40     |
| Time of incubation (min.)                   | 30     | 30     | 15     | 15     | 15     | 15     | 15     | 15     | 15     |
| Metabolites found<br>(all $\mu$ Moles/cup): |        |        |        |        |        |        |        |        |        |
| Lactate                                     | 4.33   | 108    | 0.10   | 0.38   | 1.09   | 6.23   | 15.9   | 54.5   | 136.2  |
| Pyruvate                                    | 0.17   | 0.34   | 0.04   | 0.06   | 0.09   | 0.21   | 0.25   | 0.31   | 0.36   |
| Oxaloacetate                                | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 |
| Citrate                                     | 1.51   | 2.24   | 0.14   | 0.28   | 0.71   | 0.95   | 1.50   | 2.01   | 2.24   |
| Malate                                      | 2.46   | 4.84   | 0.12   | 0.56   | 1.26   | 2.32   | 2.72   | 2.84   | 2.99   |
| PEP                                         | 0.87   | 0.10   | 0.06   | 0.37   | 0.54   | 0.52   | 0.36   | 0.18   | 0.11   |
| 2-PGA                                       | 0.20   | 0.03   | 0.04   | 0.10   | 0.15   | 0.12   | 0.09   | 0.04   | 0.02   |
| 3-PGA                                       | 1.57   | 0.19   | 0.13   | 0.79   | 1.00   | 0.89   | 0.63   | 0.26   | 0.16   |
| Triosephosphates                            | 0.11   | 0.16   | 0.08   | 0.10   | 0.10   | 0.11   | 0.13   | 0.13   | 0.11   |
| $\alpha$ -Glycero-P                         | 0.56   | 7.98   | 0.09   | 0.18   | 0.38   | 0.91   | 1.99   | 4.46   | 6.86   |
| FDP                                         | 0.04   | 0.05   | 0.02   | 0.02   | 0.02   | 0.02   | 0.03   | 0.02   | 0.02   |
| Fructose 6-P                                | 0.21   | 0.31   | 0.12   | 0.16   | 0.24   | 0.29   | 0.32   | 0.32   | 0.31   |
| Glucose 6-P                                 | 0.60   | 0.89   | 0.38   | 0.47   | 0.70   | 0.85   | 0.89   | 0.93   | 0.90   |
| Glucose                                     | 19.2   | 20.2   | 12.3   | 13.0   | 14.1   | 15.5   | 15.8   | 15.8   | 15.8   |
| Glucose + glycogen                          | 23.2   | 23.0   | 14.1   | 14.7   | 16.6   | 17.4   | 17.4   | 17.6   | 17.6   |
| ATP                                         | 1.21   | 1.18   | 0.95   | 0.97   | 1.09   | 1.19   | 1.13   | 1.11   | 1.10   |
| AMP                                         | 0.07   | 0.07   | 0.15   | 0.13   | 0.12   | 0.08   | 0.08   | 0.09   | 0.08   |
| Changes ( $\mu$ Moles/cup):                 |        |        |        |        |        |        |        |        |        |
| Lactate                                     | -36.9  | -52.4  | -1.1   | -8.8   | -14.1  | -21.0  | -25.3  | -26.7  | -25.0  |
| Glucose + glycogen                          | +9.6   | +9.4   | +0.5   | +1.1   | +3.0   | +3.8   | +3.8   | +4.0   | +4.0   |



above 3 - 4 mM lactate, while the amount of lactate removed varied with substrate concentration only below 10 mM added lactate. The changes in adenine nucleotides were in agreement with the data of Table 1, showing that lactate oxidation increased ATP and decreased AMP levels.

These changes may be interpreted as follows: Without added lactate, the mitochondria, which were suspended in a "cytoplasm" diluted 15 times during homogenization, were short of oxidizable substrates other than the products of aerobic glycolysis and endogenous non-carbohydrates (State 2 of Chance & Williams, 1955). At low lactate concentrations (<4 mM), pyruvate was formed in sufficient quantity to stimulate both oxidative and decarboxylative pathways, since the  $O_2$  uptake was increased (see Section B), and the citrate and malate concentrations rose. At the same time PEP formation was enhanced (possible mechanisms for this will be discussed in Chap. 4), while hexose 6-P was increased, indicating that FDPase activity was stimulated, either directly or in conjunction with inhibition of PFK. All these changes led to glucose synthesis.

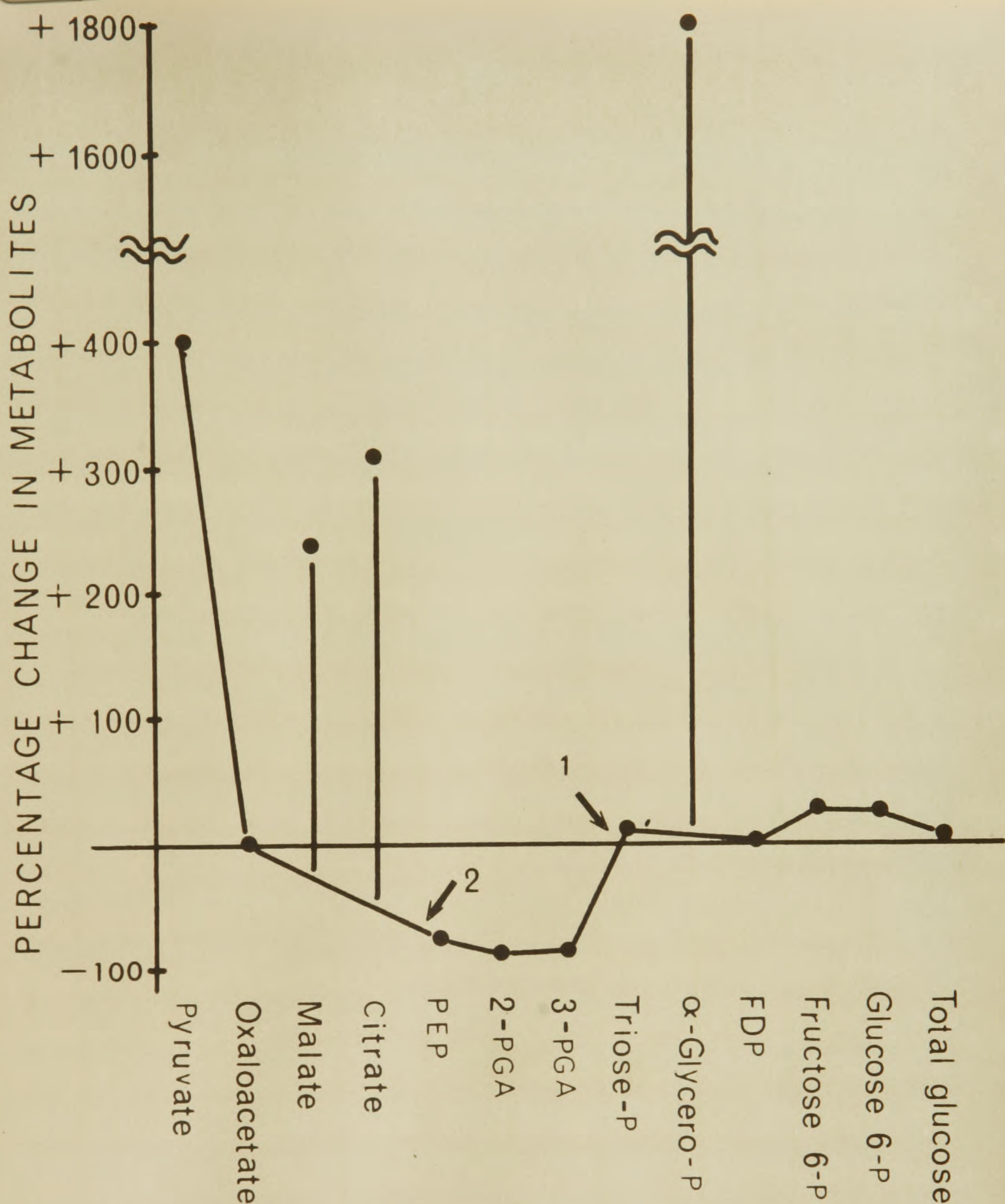
At higher lactate concentrations, the rate of glucose formation did not increase, and there was no further change in the concentrations of hexose 6-P or FDP. The increase in  $\alpha$ -glycero-P which was still occurring, despite the progressive fall in PEP and PGA, suggests that flow of lactate carbon from dicarboxylic acid to triose-P was still increasing. The low pyruvate/lactate ratio was presumably responsible for these metabolite changes, due to the effects of the raised  $NADH/NAD^+$



ratio on the triose-P dehydrogenase equilibrium, as well as on the reaction catalysed by  $\alpha$ -glycero-P dehydrogenase. The FDPase - PFK step may have been rate-limiting for carbohydrate synthesis at lactate concentrations above 4 mM, but an alternative explanation cannot be excluded, viz. that reduction of triose-P to  $\alpha$ -glycero-P limited the supply of FDP available for hydrolysis by FDPase. The effect of lactate in stimulating the conversion of FDP into hexose 6-P may have been due to 2 factors: The rise in citrate and ATP, and the fall in AMP.

It is difficult on the basis of these results to assign regulatory importance to the reducing step of gluconeogenesis, despite the "cross-over point" there obtained (Fig. 6, No. 1 - see Chance & Williams, 1956). This is in view of the need for caution in interpreting changes in intermediates according to this theory, when complex metabolic pathways are considered. These may contain steps or sequences of steps maintained by highly-active enzymes near to their equilibrium positions, a fact especially significant when such reactions involve multiple substrates including coenzymes. In general terms, reactions at near-equilibrium (readily-reversible) are unlikely to be rate-limiting steps unless marked dis-equilibrium is established. Thus, when extra-mitochondrial NADH is low, as during metabolism of high concentrations of pyruvate in pigeon-liver homogenates or slices, the reduction of 1,3-diPGA may in fact limit the rate of gluconeogenesis (Krebs & Hems, 1964) (see also Section J). No other control points in the pathway can be identified by these data, since the oxaloacetate concentrations were too low to be





**Fig. 6.** Percentage changes in concentrations of metabolites at 2 different lactate concentrations in pigeon-liver homogenate.

The data refer to cups 5 and 9 of Table 15, and are plotted as the percentage increase or decrease in each metabolite when the lactate concentration changed from 0.27 to 34 mM. Intermediates not presumed to be on the direct pathway of gluconeogenesis from lactate to glucose are represented by vertical lines abutting on the continuous line joining the other points. The "cross-over points" referred to in the text are indicated with arrows.



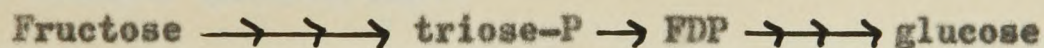
determined. The reversed "cross-over point" at PEPCK (Fig. 6, No. 2) appears in this case to have been associated with increased activity of the enzyme.

Experiments on similar lines with liver homogenates from fed birds gave essentially the same results; no differences in control points were evident.

### SECTION G

#### GLUCONEOGENESIS FROM D-FRUCTOSE IN PIGEON-LIVER HOMOGENATES:

D-Fructose was a good precursor of glucose in the homogenates both in the absence, and in the presence of added glucose (Table 16). Since phosphorylation of fructose at the 6'-position by hexokinase would be strongly inhibited in the presence of 5 mM glucose (Walker, 1963) it was assumed that the synthetic pathway under such conditions included the FDPase reaction:



(see Hers, 1957; Muntz & Vanko, 1962; Rauschenbach & Lamprecht, 1964).

Glucose formation from lactate + fructose was less than the sum of that from either substrate alone, while AMP completely inhibited gluconeogenesis from fructose, indicating that FDPase was probably involved.

Iodoacetate (0.25 mM) inhibited glucose formation from fructose by less than 50% but at the same time, the production of lactate (which occurred more rapidly from added fructose than from glucose - see cups 1 and 3)



**Table 16.** Synthesis of glucose from D-fructose in pigeon-liver homogenate (starved bird).

4 ml. of pigeon-liver homogenate, prepared as described in METHODS were incubated in each cup for 45 min. at 40°. Pi was 20 mM and added NAD<sup>+</sup> was 0.25 mM. The tissue dry wt. was 82 mg./cup.

| No.                             | Initial | 1    | 2    | 3     | 4     | 5    | 6    |
|---------------------------------|---------|------|------|-------|-------|------|------|
| Additions (mM):                 |         |      |      |       |       |      |      |
| Glucose                         |         | 5    | 5    | 5     | 5     | 5    | 5    |
| Lactate                         |         |      | 25   |       | 25    |      |      |
| Fructose                        |         |      |      | 15    | 15    | 15   | 15   |
| AMP                             |         |      |      |       |       | 2.5  |      |
| Iodoacetate                     |         |      |      |       |       |      | 0.25 |
| Compounds found (μMoles/cup):   |         |      |      |       |       |      |      |
| Glucose + glycogen              | 3.9     | 18.6 | 29.8 | 34.1  | 38.0  | 21.0 | 29.5 |
| Glucose                         | 2.82    | 16.4 | 27.2 | 31.4  | 35.4  | 19.9 | 28.6 |
| Lactate                         | 1.22    | 0.64 | 68.1 | 6.85  | 75.2  | 3.9  | 0.51 |
| α-Glycero-P                     | 0.41    | 0.52 | 2.50 | 0.68  | 2.68  | 1.22 | 1.98 |
| Metabolic changes (μMoles/cup): |         |      |      |       |       |      |      |
| Glucose + glycogen              | -       | -5.3 | +5.9 | +10.2 | +14.1 | -2.9 | +5.6 |



was almost completely stopped. These observations suggest that the pathway of gluconeogenesis from fructose did not involve triose-P dehydrogenase, but that other effects of the inhibitor were responsible for the fall in glucose synthesis.

An implication of these results is that the rate-limiting step of carbohydrate synthesis from lactate was at a stage earlier than FDPase, since fructose addition increased this synthesis from lactate by a near-additive amount. On the other hand, it is conceivable that FDPase may have limited glucose formation from fructose, and that lactate addition may have brought about changes in the control of the FDP-fructose 6-P inter-conversion to increase the rate of glucose formation from fructose. Since part of such a mechanism could be an increase in the concentration of citrate (see Section F), the effect of citrate addition on the conversion of [U- $^{14}\text{C}$ ]-fructose to glucose was tested (Table 17). In this experiment, 11 mM  $\text{Mg}^{2+}$  was added to all the cups to ensure that free  $\text{Mg}^{2+}$  was still present despite the addition of 10 mM citrate to some of the cups. The incorporation of  $^{14}\text{C}$  into glucose was enhanced some 4 times in the presence of citrate, and net synthesis of glucose was greater than the sum of that in the presence of either substrate alone. Effects similar to those of Table 16 were observed with lactate addition in cups 4 and 5; here the incorporation of  $^{14}\text{C}$  from fructose into glucose was increased by 75% when lactate was present. The concentrations of AMP were lower in the presence of citrate and lactate, while the concentrations of hexose 6-P were increased, and those of FDP and triose-P



**Table 17.** Effects of citrate and lactate on the incorporation of  $^{14}\text{C}$  from  $[\text{U-}^{14}\text{C}]\text{-D-fructose}$  into glucose in pigeon-liver homogenate.

4 ml. of homogenate were prepared and incubated for 45 min. at  $40^{\circ}$  as described in METHODS. Each cup contained 11 mM  $\text{Mg}^{2+}$ , 20 mM Pi and 0.25 mM  $\text{NAD}^{+}$ .  $[\text{U-}^{14}\text{C}]\text{-D-fructose}$  was added to some of the cups at a specific activity of 12,250 counts/min./ $\mu\text{Mole}$ . Tissue dry wt. was 85 mg./cup. The procedures for chromatography and measurement of radioactivity were as described in METHODS.

| No.                                                    | Initial | 1    | 2    | 3     | 4     | 5     |
|--------------------------------------------------------|---------|------|------|-------|-------|-------|
| Additions (mM):                                        |         |      |      |       |       |       |
| Glucose                                                |         | 2.5  | 2.5  | 2.5   | 2.5   | 2.5   |
| $[\text{U-}^{14}\text{C}]\text{-fructose}$             |         | 3.75 |      | 3.75  |       | 3.75  |
| Citrate                                                |         |      | 10   | 10    |       |       |
| Lactate                                                |         |      |      |       | 40    | 40    |
| Compounds found ( $\mu\text{Moles/cup}$ ):             |         |      |      |       |       |       |
| Glucose + glycogen                                     | 4.70    | 22.2 | 19.4 | 31.6  | 27.3  | 31.0  |
| Hexose 6-P                                             | 0.11    | 0.98 | 1.35 | 1.86  | 1.22  | 1.24  |
| FDP + triose-P                                         | 0.25    | 0.17 | 0.10 | 0.10  | 0.08  | 0.13  |
| $\alpha\text{-Glycero-P}$                              | 0.58    | 0.25 | 0.23 | 0.40  | 4.85  | 5.86  |
| AMP                                                    | 0.04    | 0.14 | 0.09 | 0.08  | 0.06  | 0.07  |
| Metabolic changes ( $\mu\text{Moles/cup}$ ):           |         |      |      |       |       |       |
| Glucose + glycogen                                     | -       | +7.5 | +4.7 | +16.9 | +12.6 | +16.3 |
| Radioactivity (counts/min./<br>cup $\times 10^{-2}$ ): |         |      |      |       |       |       |
| Glucose + glycogen                                     | -       | 391  | -    | 1591  | -     | 676   |



slightly decreased.

Both citrate and lactate addition thus stimulated glucose formation from fructose, and the changes in intermediates suggest that facilitation of FDP conversion into fructose 6-P was the factor responsible. This may have been due to the effects of citrate on PFK, and of AMP on FDPase (see DISCUSSION).

## SECTION H

EFFECTS OF CITRATE ON GLUCOSE SYNTHESIS FROM LACTATE: Citrate was a poor precursor of carbohydrate in pigeon-liver homogenates (Table 18). In this experiment, 6 mM  $Mg^{2+}$  was added to compensate for chelation by the substrate, and citrate was added twice from side-arms during the incubation (3.75 mM on each occasion) to ensure that a raised citrate concentration was continuously present. No glucose was utilized in the presence of citrate, and carbohydrate formation from lactate was increased by 25%. Synthesis in the presence of both substrates was thus not more than additive of that with either substrate alone. There was an increase in the concentration of hexose 6-P and it is likely that an effect on PFK was being exerted; however, the addition of citrate failed to stimulate some other rate-limiting step (see Sections F and G) and carbohydrate synthesis was accordingly not enhanced to a significant extent. This contrasted with the effect of citrate on glucose synthesis from fructose (see Section G).



**Table 18.** Effect of recurrent citrate addition on carbohydrate synthesis from lactate in pigeon-liver homogenate (starved bird).

4 ml. of liver homogenate were prepared and incubated for 45 min. as described in METHODS. 15  $\mu$ Moles of citrate were present initially where indicated; at 15 and 30 min. similar amounts were added from side-arms. Tissue dry wt. was 75 mg./cup.

| No.                                   | Initial | 1     | 2     | 3    | 4     |
|---------------------------------------|---------|-------|-------|------|-------|
| Additions (mM):                       |         |       |       |      |       |
| Lactate                               |         |       |       | 140  | 140   |
| Citrate                               |         |       | 3.75  |      | 3.75  |
| Glucose                               |         | 2.5   | 2.5   | 2.5  | 2.5   |
| Compounds found ( $\mu$ Moles/cup):   |         |       |       |      |       |
| Glucose + glycogen                    | 7.77    | 11.2  | 17.6  | 25.0 | 28.6  |
| Glucose                               | 5.57    | 8.30  | 14.6  | 21.2 | 24.6  |
| Hexose 6-P                            | 0.17    | 0.44  | 0.86  | 0.95 | 1.21  |
| $\alpha$ -Glycero-P                   | 0.99    | 0.21  | 0.01  | 4.58 | 2.18  |
| Lactate                               | 2.77    | 0.58  | 3.02  | 69.8 | 88.0  |
| Metabolic changes ( $\mu$ Moles/cup): |         |       |       |      |       |
| Glucose + glycogen                    | -       | -6.6  | -0.2  | +7.2 | +10.8 |
| Lactate                               | -       | -2.19 | +0.25 | -73  | -53   |



## SECTION I

### EFFECT OF GLUCOSE CONCENTRATION ON THE RATE OF CARBOHYDRATE

SYNTHESIS FROM LACTATE: The rate of net synthesis of carbohydrate from lactate was independent of the concentration of added glucose (Table 19). The glucose 6-P concentration varied little over the range of 0.5 - 10 mM added glucose. This suggests that glucose did not exert control in this in vitro system on its own synthesis from lactate, and that the glucose 6-P concentration was regulated by independent mechanisms.

## SECTION J

### METABOLISM OF L-MALATE IN PIGEON-LIVER HOMOGENATE: Krebs et al.,

(1964) showed that pigeon-liver homogenates were able to synthesize carbohydrate from malate or fumarate. In the present work the rate of synthesis from malate was always rather less than that from lactate (Table 20, cups 3 and 4).  $\text{NAD}^+$  (0.5 mM) was required for maximal activity (cup 2). In the presence of  $\text{NAD}^+$ , carbohydrate formation from malate + lactate (cup 5) was about equal to the sum of that which occurred from either substrate alone. Incubation with malate, in the presence and absence of  $\text{NAD}^+$ , was associated with a large increase in PEP and 2- and 3-PGA (when  $\text{P}_i$  was 20 mM), as well as with increases in pyruvate, lactate and oxaloacetate. Very little  $\alpha$ -glycero-P was formed. These findings suggested that shortage of NADH for the reduction of 1,3-diPGA limited



**Table 19.** Effect of added glucose concentration on synthesis of carbohydrate from lactate in pigeon-liver homogenate.

4 ml. of homogenate were prepared and incubated at 40° for 45 min. according to METHODS. All the cups contained 40 mM lactate, 0.125 mM NAD<sup>+</sup> and 5 mM Pi. Tissue dry wt. was 72 mg./cup.

| No.                                     | 1     | 2     | 3     | 4     | 5     |
|-----------------------------------------|-------|-------|-------|-------|-------|
| Added glucose (mM):                     | 0     | 1.1   | 2.2   | 4.4   | 8.8   |
| Metabolic changes (μMoles/cup):         |       |       |       |       |       |
| Glucose + glycogen                      | +15.0 | +15.6 | +14.9 | +14.8 | +14.6 |
| Lactate                                 | -105  | -106  | -115  | -108  | -109  |
| Glucose 6-P concentration (μMoles/cup): | 0.62  | 0.64  | 0.78  | 0.72  | 0.71  |



**Table 20.** Carbohydrate synthesis from L-malate in pigeon-liver homogenate (starved bird).

4 ml. of standard homogenate were prepared and incubated as in METHODS. The time was 45 min. There were 66 mg. dry wt. of tissue/cup.

| No.                                                   | Initial | 1    | 2     | 3     | 4     | 5     | 6     |
|-------------------------------------------------------|---------|------|-------|-------|-------|-------|-------|
| <b>Additions (mM):</b>                                |         |      |       |       |       |       |       |
| Pi                                                    |         | 5    | 20    | 20    | 20    | 20    | 20    |
| Malate                                                |         | 30   | 30    |       | 30    | 30    | 30    |
| Lactate                                               |         |      |       | 30    |       | 30    |       |
| DNP                                                   |         |      |       |       |       |       | 0.075 |
| NAD <sup>+</sup>                                      |         | 0.5  |       | 0.5   | 0.5   | 0.5   | 0.5   |
| <b>Compounds found (<math>\mu</math>Moles/cup):</b>   |         |      |       |       |       |       |       |
| Glucose +glycogen                                     | 5.90    | 11.2 | 7.25  | 19.7  | 11.5  | 24.4  | 6.28  |
| Glucose                                               | 4.92    | 10.4 | 6.52  | 19.2  | 11.1  | 23.7  | 5.55  |
| Malate                                                | 0.65    | 47.0 | 52.6  | 4.95  | 38.2  | 56.6  | 36.2  |
| Oxaloacetate                                          | 0.01    | 1.60 | 1.21  | 0.06  | 2.36  | 0.66  | 1.39  |
| Lactate                                               | 1.30    | 2.01 | 5.35  | 50.5  | 3.72  | 69.2  | 1.33  |
| Pyruvate                                              | 0.03    | 0.40 | 4.19  | 2.36  | 1.23  | 3.40  | 0.25  |
| PEP                                                   | 0.04    | 0.96 | 13.6  | 1.20  | 13.0  | 5.17  | 2.26  |
| 2-PGA                                                 | 0.01    | 0.28 | 2.29  | 0.25  | 2.15  | 0.90  | 0.42  |
| 3-PGA                                                 | 0.08    | 2.14 | 23.1  | 2.06  | 27.1  | 8.03  | 4.21  |
| $\alpha$ -Glycero-P                                   | 0.33    | 0.25 | 0.03  | 3.65  | 0.23  | 3.62  | 0.01  |
| <b>Metabolic changes (<math>\mu</math>Moles/cup):</b> |         |      |       |       |       |       |       |
| Glucose + glycogen                                    | -       | +5.3 | +1.35 | +13.8 | +5.6  | +18.5 | +0.4  |
| Malate                                                | -       | -73  | -68   | +4.3  | -82   | -64   | -84   |
| PEP + 2- and 3-PGA                                    | -       | +3.3 | +38.9 | +3.4  | +37.1 | +14.0 | +6.8  |



the rate of gluconeogenesis from malate, despite the high rate of PEP formation. When lactate was also added, the concentrations of the phosphorylated intermediates fell by 60% and oxaloacetate was decreased. At 5 mM Pi carbohydrate synthesis was equal to that at 20 mM Pi, but there was no significant accumulation of intermediates (cup 1). In the presence of  $0.75 \times 10^{-4}$  M DNP, both carbohydrate synthesis and the accumulation of PEP and PGA were greatly diminished (cup 6), although malate removal was unchanged.

Shrago & Lardy (1966) have shown that PEP is synthesized rapidly from malate in rat-liver "cytosol" (high speed supernatant). In the present experiments it is evident that in pigeon-liver homogenates this conversion, though very rapid, was not associated with the maintenance of high concentrations of NADH in the extra-mitochondrial compartment, as was found for example on incubation with lactate. Evidence for this was the absence of  $\alpha$ -glycero-P formation and the accumulation of 3-PGA, 2-PGA and PEP. This may imply that malate was oxidized primarily within the mitochondria, or that some PEP synthesis proceeded over the steps Malate  $\rightarrow$  pyruvate  $\rightarrow$  oxaloacetate  $\rightarrow$  PEP, catalysed by malate enzyme, pyruvate carboxylase and PEPCK respectively. This sequence of reactions would supply NADPH but not NADH (see Seubert & Huth, 1965).

The role of Pi in these experiments is not clear. Although the concentration of Pi might be expected to play an important part in determining the equilibrium disposition of the reactants in the complex reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase, such that



high Pi concentrations would be associated with an increase in the ratio of 1,3-diPGA/glyceraldehyde 3-phosphate (other reactants being unchanged), this may not be the whole explanation of the metabolite accumulations, since net carbohydrate synthesis was the same at both high and low Pi concentrations. Judging from the ratios of pyruvate/lactate and triose-P/ $\alpha$ -glycero-P, there appears to have been no change in the extramitochondrial redox status with the change in Pi concentration; thus flux over the reducing step of gluconeogenesis in both situations was limited by the supply of NADH. The Pi concentration must be assumed to be less important in the regulation of the rate of this reaction. However, an effect of Pi on the formation or removal of PEP (see Chap. 4) may have been partly responsible for these findings. DNP likewise may have affected the supply of PEP for conversion into 1,3-diPGA (Chap. 4).

The fact that carbohydrate synthesis from malate + lactate was additive is difficult to explain in terms of rate-limiting steps in the formation of glucose from either substrate. The following factors may have been concerned in this complex case:

i) Inhibition of pyruvate carboxylase at high malate concentrations (Freedman & Kohn, 1964);

ii) Chelation of essential metal ions by phosphorylated compounds accumulating in the presence of malate and 20 mM Pi, e.g.  $Mn^{2+}$  or  $Mg^{2+}$  by PEP and 2-PGA (Wold & Ballou, 1957). This is likely to affect reactions requiring these ions such as FDPase (Underwood & Newsholme, 1965,a) and PK (Mildvan & Cohn, 1966). The experiments nevertheless



clearly emphasize the limiting role of pyruvate carboxylase in PEP synthesis from lactate in whole homogenates, and the requirement for a supply of extra-mitochondrial NADH in order to permit rapid reduction of 1,3-diPGA (see Section F).



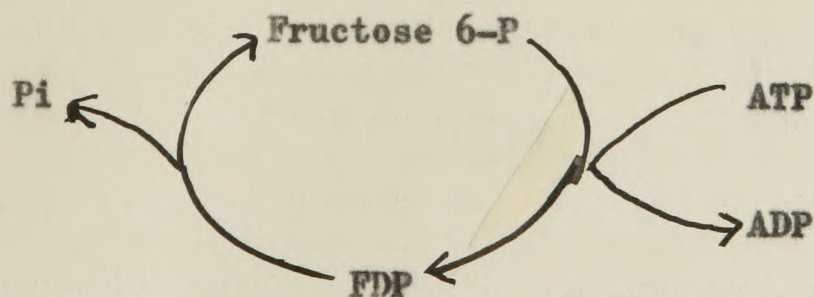
### DISCUSSION

The results obtained with pigeon-liver homogenates (especially the stimulation of glucose degradation and the inhibition of glucose synthesis by AMP, and the evidence indicating that points of action of AMP are PFK and FDPase) support the concept that the reactions catalysed by PFK and FDPase are among these which limit the rate of glycolysis and gluconeogenesis respectively, and that the concentrations of intracellular modifiers, in particular AMP and ATP, regulate the activity of these enzymes. The fact that AMP at low concentrations (0.2 mM) is a powerful inhibitor of FDPase, as well as a powerful activator of PFK, suggests that AMP is in the present system one of the most important single modifiers among the various cell constituents which can influence the activities of the two enzymes, though ADP has similar effects on PFK, the quantitative significance of which cannot yet be assessed. The concentration of AMP is a particularly apt 'signal' for regulating glycolysis and gluconeogenesis, as an energy-consuming process, because it is directly linked to the energy status of the cytoplasmic space i.e. to the concentration of ATP. The concentrations of AMP and ATP are governed by the equilibrium constant  $K = [\text{AMP}][\text{ATP}] / [\text{ADP}]^2$  of the adenylate-kinase reaction. As the activity of this enzyme is sufficiently high to maintain the concentrations of the three reactants near equilibrium, a fall in the concentration of ATP will cause a rise in the concentration of AMP, and this, by activating PFK, will tend to increase the rate of ATP synthesis. On the other hand, if the adenine



nucleotides of the cell are present predominantly as ATP, the AMP concentration will be low, PFK blocked and the pathway of gluconeogenesis open.

The kinetics of the AMP effect on PFK are very intricate because they depend not only on the concentration of AMP but also on those of  $Mg^{2+}$  and of ATP (see Table 21) and, as the concentrations of cell constituents may differ from compartment to compartment within tissue homogenates, it is not possible to predict the degree of inhibition of activation from information on the overall concentration of AMP alone. The kinetics of the AMP inhibition of FDPase appear to be less involved, though they depend on the concentration of  $Mg^{2+}$  (Table 21) as expected because of the formation of chelates between AMP and  $Mg^{2+}$ . Rather lower concentrations of AMP (below 0.1 mM) are needed for an almost complete inhibition of FDPase than for the activation of PFK (above 0.1 mM). These characteristics, according to available data, bear on the co-ordinated control of the two enzymes: at very low concentrations of AMP when FDPase is active PFK is inactive, being inhibited by ATP; as the concentration of AMP rises FDPase becomes inactivated before PFK is activated. This avoids a wastage of energy through the occurrence of the futile cycle:



It is not yet clear whether a physiological significance can be



Table 21 *Effects of various 'modifiers' on fructose diphosphatase and phosphofructokinase of liver*

The values illustrate that inhibitions depend on the presence of various substances in the medium. They are selected from data of Underwood & Newsholme (1965a,b), obtained with partially purified rat-liver enzymes. The substrate for fructose diphosphatase was fructose diphosphate and that for phosphofructokinase glucose 6-phosphate, phosphohexose isomerase being present. It is emphasized that the values indicate only the order of magnitude of the effects involved; there are complicating factors, such as the effects of enzyme dilution, that make precise statements on inhibitions difficult.

| Metabolite                  | Concn. of<br>substrate<br>(mM) | Concn.<br>of $Mg^{2+}$<br>(mM) | Inhibition (%)                 |                               |
|-----------------------------|--------------------------------|--------------------------------|--------------------------------|-------------------------------|
|                             |                                |                                | Fructose<br>diphos-<br>phatase | Phospho-<br>fructo-<br>kinase |
| 0.1 mM-AMP                  | 0.1                            | 5                              | 95                             |                               |
| 0.05 mM-AMP                 | 0.1                            | 0.5                            | 98                             |                               |
| —                           | 0.1                            | 5                              | 20                             |                               |
| —                           | 0.25                           | 0.5                            | 15                             |                               |
| 0.16 mM-ATP                 | 3.3                            | 5                              |                                | 100                           |
| 0.16 mM-ATP, 0.3 mM-AMP     | 3.3                            | 5                              |                                | 18                            |
| 1.2 mM-ATP, 0.6 mM-AMP      | 3.3                            | 5                              |                                | 90                            |
| 0.01 mM-ATP                 | 3.3                            | 5                              |                                | 0                             |
| 0.01 mM-ATP, 0.5 mM-citrate | 3.3                            | 5                              |                                | 50                            |



attributed to the inhibition of FDPase by FDP. Underwood & Newsholme (1965,b) have considered the possibility that the FDP concentration (by its positive feedback action on PFK and its inhibitory effect on FDPase) could act as an "amplifier" when small changes in the enzyme activities occur due to the primary agency of other modifiers. So far concentrations of FDP (estimated as the overall tissue content) inhibitory to FDPase have not yet been demonstrated in intact liver preparations or in vivo, nor have significant changes been observed under different nutritional conditions (see Soling et al., 1966; Underwood, 1965). Lowry & Passonneau, (1964) have proposed that part of the intracellular FDP is compartmented, to explain some of their results in brain preparations.

The overall concentration of ATP in homogenates was, in some experiments, high enough, considering the concentration of the de-inhibitors fructose 6-P, FDP, Pi and AMP, to cause a virtually complete inhibition of PFK (Underwood & Newsholme, 1965,b). In fact PFK was not inactivated. This indicates that certain quantitative characteristics of the enzyme change on purification and dilution, as suggested by the observations of Underwood & Newsholme (1965,b), or, more likely, that the concentration of ATP at the site of the enzyme was substantially below the average concentration in the homogenate. Two factors could be responsible for this. One is the uneven distribution of adenine nucleotides between the mitochondria and the cytoplasm or medium (see Chap. 4 - DISCUSSION). The second factor is chelation of ATP with  $Mg^{2+}$  and  $Ca^{2+}$ .

It follows from these considerations that the movement of adenine



nucleotides into and out of the mitochondria and other organelles could be a major factor in the regulation of metabolic processes. This is also true for other solutes that can be actively accumulated by mitochondria and that can affect the activity of pace-maker enzymes. Active transport into the mitochondria has been shown, for example, for  $\text{Ca}^{2+}$  (see Ernster & Lee, 1964; Drahota et al., 1965; Carafoli et al., 1965; Chance, 1965),  $\text{Mg}^{2+}$  (Carafoli et al., 1964), Pi (Greenawalt et al., 1964; Rossi & Lehninger, 1964; Vasington & Greenawalt, 1964), citrate (Schneider et al., 1956; Gamble, 1965) and ATP and ADP (Carafoli et al., 1965). Though much attention has been paid to the mechanism of the active transport of these solutes into the mitochondria, the physiological significance of these energy-consuming movements has not yet been analysed. It may well be the regulation of enzyme activities. Traffic in one direction of substances that cannot be metabolized, like  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , would make no biological sense. Thus there must be mechanisms that bring about the release of these substances from the mitochondria into the cytoplasm. This might be entirely passive as Chance (1965) suggests, in which case it occurs continuously according to the concentration gradient and is counteracted by active transport in the opposite direction.

It is well established that the concentration of ADP can regulate the rate of the aerobic degradation of substrates generally and this raises the question whether the stimulating effect of adenine nucleotides is, at least in part, due to the formation of ADP and its role as phosphate acceptor. It is possible that this was a component in stimulating the



conversion of glucose into pyruvate in the pigeon-liver homogenates, but the following facts show that in this system the main stimulating effects of the adenine nucleotides were those on PFK: (1) The stimulation of glucose degradation to pyruvate caused by AMP in the presence of adequate concentrations of  $\text{NAD}^+$  was accompanied by a rise in the concentrations of FDP and triose-P, and by a fall in those of 3-PGA and hexose 6-P (Tables 3, 4 and 11). (2) The increase in the rate of glucose removal when AMP was added was the same when sufficient  $\text{NAD}^+$  was present to saturate the triose-P dehydrogenase system and when the rate of this step was limited by lack of  $\text{NAD}^+$  or by iodoacetate. In the first case, there were small increases in the concentrations of FDP and triose-P; in the second, the large accumulation of these intermediates accounted quantitatively for the carbohydrate removed (see Tables 3, 4 and 12).

Wenner et al., (1953) noted that the addition of phosphate acceptor did not affect the rate of formation of  $^{14}\text{CO}_2$  from  $[^{14}\text{C}]$ glucose in rat-liver homogenates while  $\text{NAD}^+$  (2 mM), as in the present experiments, was an obligatory requirement for optimum rates of oxidation. Tiedemann & Born (1963), who studied the effects of adenine nucleotides on the glycolysis of homogenates from ascites-tumour cells, found that phosphate acceptors did not limit the rates and that adenine nucleotides had effects similar to those found with pigeon-liver homogenates. The similarity, of course, is restricted to the control of glucose degradation, as gluconeogenesis does not occur in ascites cells.

The control of the inter-conversion of FDP and fructose 6-P



illustrates an intrinsic mechanism of liver cells in which anabolic activity is directly related to the energy status ("steady-state control"), and indirectly therefore, to the supply of respiratory fuels. This mechanism might be expected to come into operation when the cells are anoxic or during intoxications. For example, Frunder et al., (1961) demonstrated that egress of ATP from the mitochondria was impaired in  $\text{CCl}_4$ -damaged livers. Chance et al., (1965) showed that the AMP content of rat liver rose markedly during anoxia. This control by AMP constitutes a "safety factor". Whether the potent effects of adenine nucleotides have a wider physiological significance is not yet clear (see Chap. 4 - DISCUSSION). It is possible that post-ethanolic hypoglycaemia in fasting humans (Field et al., 1963) and the related stimulation of glucose uptake by ethanol in perfused livers from starved rats (Forsander et al., 1965) may be due to a rise in the cytoplasmic AMP concentration. Under these conditions, a rapid increase in the rate of acetate activation by the reaction



might be expected, since acetate is the principal end-product of hepatic ethanol metabolism (see Forsander & R  ih  , 1960). In pigeon-liver homogenates ethanol (40 mM) or acetate (10 mM) addition did not depress gluconeogenesis from lactate to a significant degree (unpublished work), although this may have been due to the low activity of diluted cytoplasmic enzymes. Underwood (1965) was unable to find differences in the tissue content of adenine nucleotides during various nutritional treatments of



rats, such as to suggest that a significant role might be played in the regulation of glycolysis and gluconeogenesis. At the present time, technical problems prevent the direct measurement of metabolite concentrations in the cytoplasmic space.

It is an interesting reflection that the stimulatory effect of AMP on hepatic PFK is a "safety factor" for the individual cell rather than the whole animal, since it serves to decrease the amount of glucose liberated into the circulation by the action of glucose 6-Pase.

The effects of citrate addition on glucose synthesis from fructose (Section G) introduce a new dimension into the regulation of metabolism at the level of the hexose phosphates. Although the results are not as unambiguous as those obtained by Newsholme & Underwood (1966) in rat kidney-cortex slices, in that small changes in the adenine nucleotide concentrations accompanied the addition of citrate to pigeon-liver homogenates, they do suggest that stimulation of the net conversion of FDP into fructose 6-P can result from inhibition solely of PFK, since no effects of citrate on FDPase have yet been demonstrated (Underwood & Newsholme, 1965,a). This implies that in the standard homogenates metabolizing added glucose and fructose in the absence of citrate, a considerable amount of "cycling" occurred, catalysed by PFK and FDPase. The stimulation of net glucose synthesis by citrate was presumably due to inhibition of the "cycle"; it also follows from the substantial effect of citrate that the amount of "cycling" in the absence of citrate was extensive, i.e. about half of the molecules of FDP formed were



rephosphorylated by PFK.

In the experiment (Section C) described in Table 5, a certain amount of  $^{14}\text{C}$  was lost continuously from the total glucose pool (including glycogen) during the incubation with high concentrations of lactate, despite the net synthesis of carbohydrate which occurred. This indicates that a limited amount of "cycling" took place under these conditions, since less than a fifth of the original radioactivity was lost during 90 min. of incubation. In the presence of high concentrations of AMP, "cycling" was abolished, but restored when the AMP concentration fell to levels compatible with the initiation of gluconeogenesis; as the rate of glucose synthesis increased, the extent of "cycling" diminished. Although an effect of lactate (or the fall in AMP) is theoretically not excluded as the explanation of these events, it is most likely that one of the factors inhibiting the "cycle" was the raised concentration of citrate.

In biological terms, such a cycle is wasteful of energy, although there are a number of well-established examples of similar ATP-consuming "cycles", e.g. the hydrolysis of triglycerides and continual re-esterification in adipose tissue (Vaughan, 1961), especially in brown adipose tissue, where it may serve as a mechanism of heat production (Steiner & Cahill, 1963). Cahill et al., (1959) have discussed the possible operation of such a "cycle" catalysed by glucokinase activities and glucose 6-Pase, and have stressed the advantages of fine control of both rate and direction of metabolic pathways afforded by such an arrangement. The possibility that rat-liver FDPase may exist in two forms (see Chap. 1)



suggests that a further kind of control could be exerted by hormones to influence the operation of such "cycles". Another factor could be compartmentation of multi-enzyme systems in the cytoplasm of the liver cells on the lines proposed for the glycolytic system of red cells by Green et al., (1965). The microsomal location of glucose 6-Pase is a pointer in this direction.

Mendicino & Utter (1962) showed that FDPase limited the rate of glucose 6-P formation in a reconstituted system consisting of pure enzymes and washed mitochondria. Exton & Park (1965) concluded from measurements of hexose- and triose-phosphates that FDPase was not a limiting stage in gluconeogenesis (at high concentrations of lactate) in perfused livers from starved rats, and that the formation of triose-P from pyruvate was the rate-limiting process. This concept is supported by the previous findings of Krebs et al., (1963) on the rates of formation of glucose from substrates (at high concentrations) entering the "common pathway" of gluconeogenesis above and below the stage of triose-P dehydrogenase, in kidney cortex slices. More recently, Exton & Park (1966) have demonstrated a possible effect of adrenaline on the conversion of FDP into fructose 6-P in perfused rat livers metabolizing lactate, based on changes in the relevant metabolites during increased flux ("cross-over point"); a similar effect of glucagon had earlier been shown by Schimassek & Mitzkat (1963), and has also been demonstrated to occur when lactate is added to the perfusion medium of rat livers (Hems, Ross, Berry & Krebs, in the press). Although such effects do not prove that FDPase is rate-limiting for the



entire sequence of reactions from lactate to glucose, they do imply that it may be involved in a co-ordinate system of controls. It is also reasonably clear that FDPase limits the rate of glucose formation from triose-P (see Section G; also Newsholme & Underwood, 1966).

Another factor that indicates a regulatory role for FDPase in the liver is the apparent or real change in the tissue enzyme activity which results from administration to rabbits of high protein or high fructose diets (low in glucose) (Mokrasch et al., 1956), or from alloxan diabetes and glucocorticoid treatment in rats (see Weber et al., 1965, a for review). These changes are co-ordinate with changes in other key gluconeogenic enzymes like glucose 6-Pase and PEPCK. Mokrasch et al., (1956) considered that the early increase in the enzyme activity, which now was less susceptible to proteolytic activation, might not be due to de novo protein synthesis, but to changes analogous to those occurring during papain treatment. It is now established that the early glycogenic effect of glucocorticoid administration does not require the induction of enzymes such as FDPase or PEPCK, since it occurs long before the changes can be elicited (Segal & Lopex, 1963), and is not prevented when protein synthesis is totally inhibited by ethionine (Kvam & Parks, 1960). The possibility that FDPase in various physiological situations, is present predominantly in different forms and at different concentrations, may indicate that it is rate-limiting only at certain times, and that it may not always be subject to "allosteric" control to the same extent.

It was first observed many years ago that the rate of lactate



formation in liver homogenates was greater when FDP was added than when glucose or glucose 6-P were the substrates (see Olson, 1951). Cahill et al., (1959) suggested that PFK was rate-limiting for hepatic glycolysis because in their experiments great variations in the rate of glucose 6-P formation from glucose or glycogen occurred without effects on the rate of glycolysis. Studies in the metabolism of fructose, which is thought to enter the glycolytic pathway beyond the PFK step, showed that  $\alpha$ -glycero-P and lactate formation proceeded more rapidly from fructose than from glucose (see Section G; Kornacker & Lowenstein, 1965). Wu (1963) has demonstrated directly the rate-limiting function of PFK in kidney-cortex slices ("cross-over point" in anoxia) and a regulatory role for PFK in this tissue was confirmed by Newsholme & Underwood (1966). Lea & Walker (1965) have concluded from studies on rat-liver cytoplasmic extracts that PFK and hexokinase are co-ordinately involved in the control of glycolysis.

The part played by citrate in the control of the FDP-fructose 6-P inter-conversion has important implications for the effects of fatty acid oxidation on carbohydrate metabolism in the liver and kidney. Although technical difficulties have frustrated numerous attempts to obtain reliable values for the liver citrate content under various conditions, recent (unpublished) work by Miss C. Start in this laboratory has shown that the content of citrate is significantly increased under conditions where fatty acid oxidation might be expected to be increased. (See also Tubbs & Garland, 1964). The mechanism of this increase may be similar to that proposed by Garland & Randle (1964); this depends on an assumption



of equilibrium conditions in the citrate synthase reaction, such that an increase in the acetyl CoA/CoA ratio, with constant oxaloacetate concentration, would be associated with an increase in the level of citrate. Another possible factor is the inhibition of citrate oxidation in the tricarboxylic acid cycle during fatty acid oxidation (Wieland et al., 1961). The effects of adenine nucleotides on iso-citrate dehydrogenase (Hathaway & Atkinson, 1963) and citrate synthase (Hathaway & Atkinson, 1965) have been proposed to be the basis of a mechanism to control both the metabolic fate of acetyl CoA and the citrate concentration in *E. coli*, and possibly in mammalian systems.

(Note: The important role of citrate in lipogenesis (see Srere, 1965) is paradoxical in this context, and underlines the need for caution in interpreting results.)

Struck et al., (1965) reported that oleate stimulated glucose synthesis from lactate in perfused rat livers; a similar effect of glucagon was ascribed to enhanced lipolysis, giving rise to increased fatty acid oxidation. Haynes (1965,b) claimed that octanoate increased the rate of carbohydrate synthesis from alanine in rat-liver slices. A recent report (Williamson, 1966) appears to confirm the stimulatory effect of oleate addition on gluconeogenesis in the perfused liver, and brings evidence for a "cross-over point" at triose-P dehydrogenase. The interpretation of the data to suggest that gluconeogenesis is limited in the intact liver by an unfavourable ratio of NADH to NAD<sup>+</sup>, is not supported by a number of other studies, e.g. Soling et al., (1966), who



found no changes in the reduction state of the cytoplasmic  $\text{NAD}^+$ -NADH system when livers were perfused with caproate, Hohorst et al., (1964) who also failed to show a change, and Hems, Ross, Berry & Krebs (in the press) who have not found such an effect in perfusions of rat livers with oleate. Most authors do, however, agree that the cytoplasmic  $\text{NAD}^+$ -NADH system is more reduced in alloxan-diabetic rat livers, and it is possible that in this case an effect might be exerted on triose-P dehydrogenase. (See Sections F and J). It is certainly feasible that the liver may behave similarly to kidney-cortex with respect to co-ordinate control of gluconeogenesis at the levels of PEP synthesis from pyruvate (see Chap. 4 - DISCUSSION) and the conversion of FDP into fructose 6-P.



CHAPTER 4

THE REGULATION OF PHOSPHOENOLPYRUVATE SYNTHESIS IN PIGEON LIVER

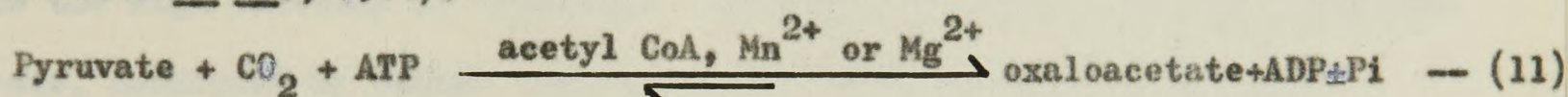
Krebs (1954) systematically reviewed the thermodynamic considerations underlying the metabolic synthesis of carbohydrate from lactate and other precursors. He discussed the role of the specific hydrolytic enzymes of gluconeogenic tissues, glucose 6-Pase and FDPase, in the circumvention of energy barriers set up by unfavourable free energy changes in the reversal of the glycolytic pathway. The remaining energy barrier among the individual steps was considered to be the formation of PEP from pyruvate and ATP. The mechanism of this overall reaction has been the subject of considerable research because it appears to involve not one special enzyme but a sequence of reactions. These are catalysed by enzymes which are not all confined in their distribution to gluconeogenic tissues, and which in some cases do not even share a common intracellular localization.

The mechanism of PEP synthesis in pigeon liver has been investigated in the work reported in this chapter. First, some of the enzymes thought to be involved in PEP formation will be described (Sections A, B, C, D and E) followed by an examination of the regulation of their activity in situ in isolated mitochondria (Section F). The results will be correlated as far as possible with past and current work in this field.



# SECTION A

PYRUVATE CARBOXYLASE IN PIGEON LIVER: Pyruvate carboxylase was characterized by Utter & Keech (1963) in chicken-liver mitochondria. It catalyses the ATP-dependent carboxylation of pyruvate (reaction 11) in the presence of  $Mn^{2+}$  or  $Mg^{2+}$ , and has an obligatory requirement for acetyl CoA or related compounds (for review and references to detailed publications, see Utter *et al.*, 1964).



3 groups of procedures have been described for the assay of pyruvate carboxylase:

i) A spectrophotometric method, suitable for the accurate direct measurement of activity in highly purified preparations (Utter & Keech, 1963; Seubert & Remberger, 1961), is subject to interference from side-reactions when the enzymes catalysing these are present in large amounts, e.g. lactate dehydrogenase.

ii) Methods based on the incorporation of  $^{14}\text{C-HCO}_3$  into oxaloacetate, with subsequent reduction of the latter to malate (Utter & Keech, 1963; Wagle, 1964), suffer from lack of specificity and the occurrence of "exchange" or "back-reactions" (see below).

iii) A method which depends on incorporation of  $^{14}\text{C}$  from  $[1-^{14}\text{C}]$ -pyruvate via oxaloacetate into citrate, in the presence of an excess of citrate synthase and acetyl CoA (Henning & Seubert, 1964), is also complicated by side-reactions of oxaloacetate, e.g. malate formation and



citrate cleavage.

The use of the method (belonging to group ii), described under "Assays of Enzyme Activity" in the METHODS section, was investigated in pigeon liver, both as an assay of pyruvate carboxylase and in the hope that aspects of mitochondrial metabolism associated with  $\text{CO}_2$  fixation might be clarified. The results suggest that radiochemical assay of pyruvate carboxylase, using  $^{14}\text{C-HCO}_3$ , is not suitable for this tissue due to the high activity of "exchange" reactions between  $\text{H}^{14}\text{CO}_3$  and oxaloacetate, catalysed by PEPCK. The use of  $[1-^{14}\text{C}]$ -pyruvate in the same procedure was found to be satisfactory.

1. Incorporation of  $\text{H}^{14}\text{CO}_3$  in pigeon liver. When a pigeon-liver homogenate was incubated according to the basic procedure, (see METHODS) the rate of  $\text{H}^{14}\text{CO}_3$  incorporation was not linear (Fig. 7). The initial rate was 18  $\mu\text{Moles/min./g. wet wt.}$  at  $25^\circ$ , a value about 25 times that obtained in rat-liver homogenates similarly incubated. The rate over short periods was proportional to extract concentration in the range 25 - 100 mg. wet wt. of tissue/cup. The "blank" rates of  $\text{H}^{14}\text{CO}_3$  incorporation viz. those occurring in the absence of added pyruvate and malate or in the presence of either compound alone, are also given in Fig. 7. In the absence of pyruvate (the  $\text{H}^{14}\text{CO}_3$  acceptor) the net incorporation was nearly as high as that in the full system containing 20  $\mu\text{Moles}$  of pyruvate, while omission of malate decreased the fixation by 66%. Two-dimensional paper chromatography of the extracts (in the solvent systems



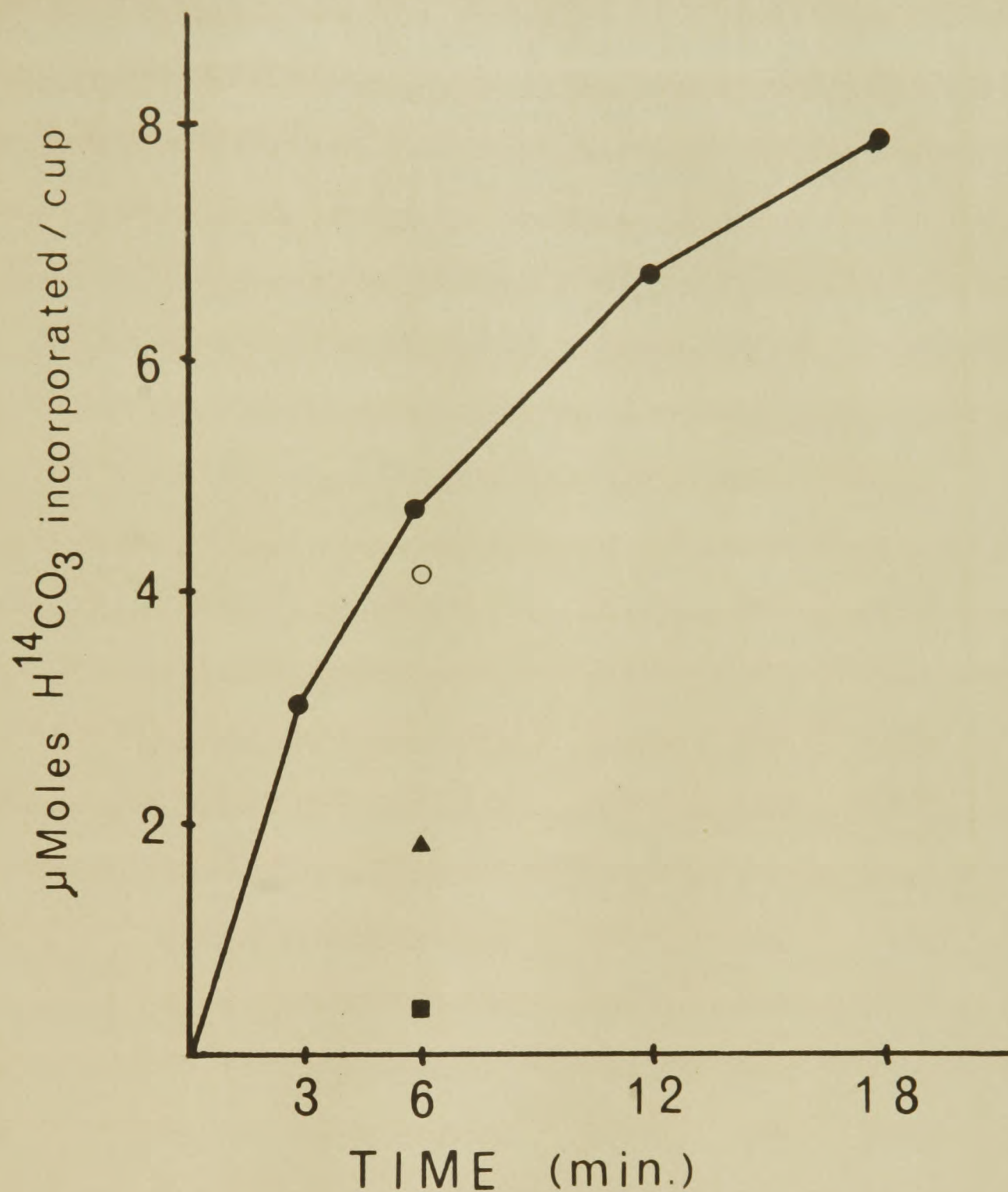


Fig. 7. Time course of  $H^{14}CO_3$  incorporation in pigeon-liver homogenate.

The procedure was that described in METHODS under "Assays of Enzyme Activity". 17.5 mg. dry wt. of tissue were added to each cup. Incubation was at  $23^{\circ}$ . ■ : No pyruvate or malate added; ▲ : pyruvate added; ○ : malate added; ● : malate + pyruvate added.



previously described), followed by autoradiography, revealed that in all 4 cases about 90 - 95% of the total counts on the paper were in areas identifiable as malate and fumarate. Although pyruvate is not very stable to these procedures, the amounts remaining were sufficient to justify the conclusion that there was little radioactivity in pyruvate. After 15 min. counts appeared in citrate.

In a similar experiment, the total incorporation after 9 min. was about 17% of all the counts added. By simple calculation, assuming that each incorporated  $\text{H}^{14}\text{CO}_3$  molecule represented the net synthesis of a molecule of dicarboxylic acid, 5.7  $\mu\text{Moles}$  of acceptor had been carboxylated at this time. The specific activity of the 20  $\mu\text{Moles}$  of malate theoretically now present would still be less than  $1/3$  that of the  $\text{CO}_2$  system. In Table 22, the concentrations of metabolites determined in these extracts are given. In the full system, 2.3  $\mu\text{Moles}$  of pyruvate (+ lactate) had been removed during 9 min., while 2.2  $\mu\text{Moles}$  of malate (+fumarate) had disappeared. The increases in citrate,  $\alpha$ -oxo-glutarate, glutamate, aspartate and PEP did not account fully for these losses. Oxaloacetate was present in such low amounts that accurate measurements were not possible. The theoretical gain of 5.7  $\mu\text{Moles}$  of dicarboxylic acid at the expense of pyruvate was thus not confirmed. Further, in the "blank" system without added pyruvate, the high incorporation rate had occurred in the presence of a very low concentration of pyruvate (0.08 mM) formed from endogenous sources or by "back-reactions".

Further additions of ATP, CoA, acetyl CoA or  $\text{NAD}^+$  to the full



**Table 22.** Metabolite changes during fixation of  $\text{H}^{14}\text{CO}_3$  by a pigeon-liver homogenate.

The standard incubation system as described under "Assays of Enzyme Activity" in METHODS, was employed. The liver was from a starved pigeon. Incubation was for 9 min. at  $25^\circ$ .

| No.                                                                                          | 1    | 2     | 3     | 4    |
|----------------------------------------------------------------------------------------------|------|-------|-------|------|
| Substrates added ( $\mu\text{Moles}$ ):                                                      |      |       |       |      |
| Pyruvate                                                                                     |      | 15.0  |       | 15.0 |
| Malate                                                                                       |      |       | 13.6  | 13.6 |
| $\mu\text{Moles H}^{14}\text{CO}_3$ incorporated<br>(using simple calculation -<br>see text) | 0.45 | 1.49  | 5.20  | 5.72 |
| Metabolites found ( $\mu\text{Moles/cup}$ ):                                                 |      |       |       |      |
| Pyruvate                                                                                     | 0.01 | 10.9  | 0.08  | 11.0 |
| Lactate                                                                                      | 0.04 | 1.70  | 0.12  | 1.73 |
| Malate                                                                                       | 0.02 | 0.85  | 9.00  | 9.55 |
| Fumarate                                                                                     | 0.01 | 0.20  | 1.75  | 1.55 |
| PEP                                                                                          | 0.01 | 0.15  | 0.30  | 0.38 |
| Citrate                                                                                      | 0.04 | 0.19  | 0.24  | 0.65 |
| $\alpha$ -Oxo-glutarate                                                                      | 0.01 | 0.28  | 0.32  | 0.58 |
| Glutamate                                                                                    | 0.10 | 0.41  | 0.40  | 0.78 |
| Oxaloacetate                                                                                 | 0.01 | 0.01  | 0.01  | 0.01 |
| Aspartate                                                                                    | 0.01 | 0.20  | 0.22  | 0.12 |
| Net changes in metabolites<br>( $\mu\text{Moles/cup}$ ):                                     |      |       |       |      |
| Pyruvate + lactate                                                                           |      | -2.4  |       | -2.3 |
| Malate + fumarate                                                                            |      | +1.05 | -2.85 | -2.2 |



system failed to increase the net incorporation, while addition of  $Mn^{2+}$ ,  $Mg^{2+}$ , ATP and  $NAD^+$  (or NADH) in the amounts employed was required for maximal activity.  $Ca^{2+}$  inhibited, while inhibition by EDTA could be reversed by a slight excess of  $Mn^{2+}$ .

An important requisite of the method is that the rate of reduction of oxaloacetate by malate dehydrogenase and NADH should not limit the overall rate of  $H^{14}CO_3$  fixation. Addition of succinate (20 mM) might be expected to increase intra-mitochondrial NADH (Krebs et al., 1961); in fact, no increase (or slight inhibition) of  $H^{14}CO_3$  incorporation was observed, although the concentrations of malate and fumarate were increased by 40% relative to controls. Butyrate (5 mM) was added to test whether the concentration of intra-mitochondrial acetyl CoA under the standard assay conditions was sufficient maximally to activate pyruvate carboxylase - according to Dr. M.C. Scrutton (personal communication) pigeon-liver mitochondrial pyruvate carboxylase is highly sensitive to activation by this metabolite. There was no effect of butyrate addition on  $H^{14}CO_3$  incorporation, and malate and pyruvate removal were unchanged.

The rates were the same in livers from fed and starved pigeons.

Taken together, the data from experiments with whole homogenates suggest that if the fixation of  $H^{14}CO_3$  were catalysed primarily by pyruvate carboxylase, then the rapid incorporation in the absence of added pyruvate must have been associated with "back-reactions" synthesizing pyruvate from malate or oxaloacetate at a rate sufficient to allow



near-maximal activity of pyruvate carboxylase. Pyruvate could also have arisen from carbohydrates or amino acids. However:

1. The  $K_m$  of pyruvate carboxylase for pyruvate (0.4 mM - Keech & Utter, 1963) is about 5 times the measured pyruvate concentration in the relevant extracts.

2. The absence of counts in pyruvate in the full system implies that under these conditions, where 15  $\mu$ Moles of pyruvate were added, few or no "back-reactions" occurred.

3. 20 mM fluoride did not inhibit the "blank" incorporation in the absence of added pyruvate, eliminating aerobic glycolysis as a source of endogenous pyruvate.

4. Exclusion of PK and malate enzyme from the reaction system by using washed mitochondria (see Sections B and D for distribution studies on these enzymes) did not prevent the occurrence of the high "blank" activity found also in this preparation.

Thus it appears likely that a large part of the fixation of  $H^{14}CO_3$ , especially in the absence of added pyruvate, was not due to pyruvate carboxylase. It was also not due to malate enzyme, which has a  $K_m$  for pyruvate of 1.2 mM (Utter, 1963) and which was removed from the system without effect when washed mitochondria were used. 87% of the  $H^{14}CO_3$ -fixing activity of a fresh homogenate was located in the twice-washed "cyclophorase" preparation (representing the sediment of a standard sucrose homogenate of the same liver centrifuged at 2500g for 10 min.). Washed pure mitochondria had a specific activity 4 times that of the whole



homogenate from which they were prepared, although they contained only  $1/6$  of the dry wt. These findings suggest that the bulk of the activity was mitochondrial.

The ability of PEPCK of pigeon liver to incorporate  $^{14}\text{CO}_2$  into oxaloacetate in the absence of PEP has been known for many years and has formed a basis for an assay of this enzyme (Utter, 1951; Utter & Kurahashi, 1954; Kurahashi et al., 1957). The rate of fixation was greatly stimulated by ATP addition, although later studies with pure nucleotides and purified proteins showed that GTP or ITP were the direct reactants in the "exchange reaction". Lane et al., (1966) have described the occurrence of an  $\text{H}^{14}\text{CO}_3$ -oxaloacetate exchange catalysed by purified pig-liver mitochondrial PEPCK at a rate greatly in excess of the overall carboxylation or decarboxylation reaction. Pyruvate carboxylase does not carry out a rapid "exchange" of this type (Scrutton & Utter, 1965).

It is likely that PEPCK, present in high activity in pigeon-liver mitochondria (see Section C) carried out a large part of the  $\text{H}^{14}\text{CO}_3$  fixation observed in these experiments, by virtue of an exchange between  $\text{H}^{14}\text{CO}_3$  and oxaloacetate independent of added PEP (although some PEP was in fact formed).

The method with  $\text{H}^{14}\text{CO}_3$  is not suitable for use as an assay of pyruvate carboxylase in this tissue.

2. Incorporation of  $[1-^{14}\text{C}]$ -pyruvate in pigeon liver. Since the incorporation of  $\text{H}^{14}\text{CO}_3$  could not be used to give an indication of pyruvate carboxylase activity in pigeon liver preparations, the method of



Henning & Seubert (1964) was adapted to give a more exact assay.  $[1-^{14}\text{C}]$ -pyruvate was now used as the labelled precursor but the basic principle and practice of the method was retained. Since the  $^{14}\text{C}$ -oxaloacetate was trapped in a large pool of unlabelled malate, there was a low probability that  $^{14}\text{C}$  would be lost from oxaloacetate in the exchange reaction. This follows from the fact that the exchange involved the other carboxyl group of oxaloacetate; randomization of label in the two carboxyls was prevented by the presence of the large pool of added malate. Radioactivity in citrate + malate was measured after paper chromatography of the extracts. Knowledge of the specific activity of added pyruvate allowed the calculation of the  $\mu\text{Moles}$  of  $^{14}\text{C}$  incorporated.

The addition of  $\text{Mn}^{2+}$  and  $\text{Mg}^{2+}$  ions was required for maximal activity, as was the addition of  $\text{NAD}^+$ . Omission of ATP left only 20% of the original incorporation. The other components of the assay mixture were optimal. The assay was linear for 10 min. and was proportional to extract concentration. When compared with the rate of  $\text{H}^{14}\text{CO}_3$  incorporation, the rate was lower by more than two-thirds. 75% was present in twice-washed mitochondria prepared at low temperature (see Wagle, 1964). Succinate addition (20 mM) did not affect the assay, showing that the rate of oxaloacetate reduction did not limit the overall rate of  $^{14}\text{C}$  incorporation. Since the enzyme was being assayed in situ in mitochondria, it was not possible to test for avidin sensitivity.

Values for pyruvate carboxylase obtained by the above method are given in Table 23. The small number of observations precludes a valid



**Table 23.** Assay of pyruvate carboxylase in pigeon liver by the incorporation of  $[1-^{14}\text{C}]$ -pyruvate into malate and citrate.

The assays were conducted on fresh homogenates as described in METHODS and in Section A. The values refer to  $\mu\text{Moles/min./g. dry wt.}$  at 25°.

| No. | Nutritional state | Activity |
|-----|-------------------|----------|
| 1.  | Well-fed          | 11.9     |
| 2.  | Well-fed          | 9.65     |
| 3.  | Starved 48 hr.    | 12.1     |
| 4.  | Starved 48 hr.    | 11.2     |



statistical analysis, but the data suggest that the activity did not change on starvation for 48 hr., which is supported by the findings (Section F) that isolated mitochondria from fed and starved birds did not differ in their capacity to form PEP from pyruvate.

Henning et al., (1966) have reported that in rat liver and kidney cortex about half of the pyruvate carboxylase activity is non-particulate, if steps are taken gently to dissociate the enzyme from the particles by changes in the tonicity of the medium. They also found that both particulate and soluble enzyme activities were increased after 12 hr. starvation, in alloxan diabetes and after cortisol administration. Changes in pyruvate carboxylase in diabetic rats were also shown by Wagle (1964), Freedman & Kohn (1964) and Prinz & Seubert (1964). Shrago & Lardy (1966) and Krebs (1965) have found no changes in hepatic pyruvate carboxylase activity in rats given glucocorticoids or made diabetic, respectively. These discrepancies may be due to the use of different assay procedures. Scrutton (1965) stated that "starvation for 24 - 36 hr. caused a  $1\frac{1}{2}$  - 2fold increase in the specific activity of the enzyme in the mitochondrial extract" when purifying pyruvate carboxylase from chicken liver. The above data suggest that no such changes occur in pigeon liver.

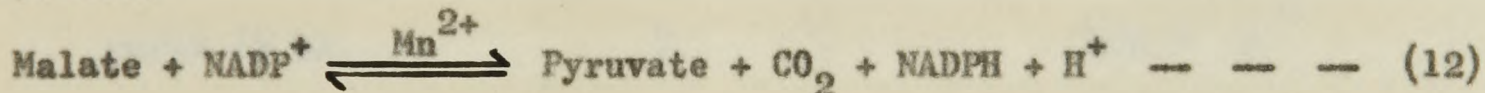
Of the key gluconeogenic enzymes tested, pyruvate carboxylase had the lowest activity, which was not greatly in excess of the maximum rates of PEP synthesis from pyruvate in isolated mitochondria, or of carbohydrate synthesis from lactate in homogenates. This supports the



conclusion (see Section F) that the enzyme is rate-limiting for gluconeogenesis from pyruvate and its precursors in pigeon-liver homogenates, and probably also in the intact liver.

## SECTION B

MALATE ENZYME IN PIGEON LIVER. This enzyme, which catalyses reaction (12), has been studied in partially-purified form by Rutter & Lardy (1958) and Stickland (1959,a,b). In agreement with the former, malate enzyme was found to occur almost exclusively (>90%) in the supernatant fraction of a sucrose homogenate centrifuged at 30,000g for 30 min.



The activity of malate enzyme in avian liver is particularly high in comparison with other species (Ochoa et al., 1950); this was borne out in the present work, where the average activity (60 - 70  $\mu\text{Moles/min./g. wet wt. at } 25^\circ$ ) exceeded that found by Pande et al., (1964) in livers of fed rats, by some 40 times.

Washed mitochondria (Section F) were almost entirely free of malate enzyme activity.

Variations in the activity during fasting and refeeding are shown in Table 24. The data agree with Wise & Ball (1964) and Young et al., (1964) relating to changes in rat liver malate enzyme in such nutritional states. It appears certain from the work of these authors that malate



**Table 24.** Malate enzyme activity in pigeon liver during a cycle of fasting and refeeding (with standard diet).

Assays were carried out as described in METHODS. The values refer to the mean activity in  $\mu\text{Moles/min./g}$ , dry wt. at  $25^{\circ}$ , with standard deviations.

| Nutritional state    | No. of birds | Activity      |
|----------------------|--------------|---------------|
| Well-fed             | 4            | $201 \pm 16$  |
| Starved 24 hr.       | 3            | $123 \pm 6^*$ |
| Starved 48 hr.       | 4            | $57 \pm 21^*$ |
| Starved 48 hr., then |              |               |
| refed 6 hr.          | 3            | $93 \pm 16^*$ |
| refed 18 hr.         | 3            | $131 \pm 8^*$ |
| refed 40 hr.         | 3            | $217 \pm 20$  |

\* = significantly different from values for well-fed birds ( $p < 0.001$ )



enzyme is intimately concerned with lipogenesis in adipose tissue and mammary gland, and in the liver. Being  $\text{NADP}^+$ -linked, it acts as a transhydrogenase (in conjunction with  $\text{NAD}^+$ -linked malate dehydrogenase) and - more generally - as a generator of NADPH for reductive syntheses. In the context of gluconeogenesis, however, it is possible that the marked changes in malate enzyme during hormonal and nutritional treatments could divert dicarboxylic acids to pyruvate instead of PEP, in competition with malate dehydrogenase and PEPCK.

### SECTION C

PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) IN PIGEON LIVER. There is little doubt that PEPCK is concerned in gluconeogenesis from oxaloacetate and its precursors in liver and kidney tissue. (See Section F). A special interest in the enzyme resides in its intracellular location in different species (Nordlie & Lardy, 1963,b). In avian liver (as in the livers of certain other species, such as the guinea-pig (Nordlie & Lardy, 1963,b) lamb and rabbit (Bandurski & Lipmann, 1956)), PEPCK has been found in the mitochondria (Kurahashi et al., 1957; Stickland, 1959,c).

Some properties of pigeon-liver PEPCK were investigated, with particular reference to phenomena that could play a part in the regulation of the activity in situ.

The enzyme could not be solubilized from particulate fractions without their disruption, sonication being the best method. (See METHODS).



This "latency", in conjunction with the evidence presented in Section F concerning the effects of atractyloside on PEP synthesis in isolated mitochondria, strongly suggests that PEPCK is located in the "matrix space" (Klingenberg & Pfaff, 1966).

The intracellular distribution of PEPCK was studied by centrifugation of a fresh standard sucrose homogenate. The results (Table 25) show that the bulk of PEPCK was in the particulate fraction, and that more than 80% of this was solubilized by sonication. In another experiment, 65% of the PEPCK activity of the original homogenate was present in the pure washed mitochondrial fraction, 20% in the fraction containing intact cells, nuclei and debris, and 10% in the two supernatants obtained during the washing procedures. It is evident that there is very little PEPCK in the "cytosol" in pigeon liver, contrasting with rat liver. The intracellular distribution was the same in well-fed and starved pigeons.

There was no significant variation in PEPCK activity in the livers of pigeons during a cycle of fasting and refeeding (Table 26). In addition, the activity of washed mitochondria was 70 - 85  $\mu$ Moles/min./g. dry wt. at 25<sup>0</sup>, irrespective of the nutritional state of the birds. This correlates with the finding of Nordlie et al., (1965) that mitochondrial PEPCK in rats and guinea-pigs did not change during various hormonal and dietary treatments, whereas the soluble enzyme was subject to wide variations. PEPCK was twice as active as pyruvate carboxylase in pigeon liver.

No detailed kinetic studies have yet been carried out on PEPCK,



Table 25. Intracellular distribution of PEPCK in pigeon liver.

A starved pigeon was used. A standard sucrose homogenate (see METHODS) was centrifuged for 20 min. at 30,000g at 0°. Samples were sonicated for 2 periods of 60 sec. each, and the sonicates were centrifuged as before. Assays were carried out in the direction of oxaloacetate formation (see METHODS).

| No. | Fraction                                            | Activity<br>( $\mu$ Moles/fraction/min. at 25°) |
|-----|-----------------------------------------------------|-------------------------------------------------|
| 1.  | Whole homogenate                                    | 10.2                                            |
| 2.  | Supernatant                                         | 3.18                                            |
| 3.  | Resuspended particles                               | 8.85                                            |
| 4.  | Sonicated whole homogenate                          | 75.0                                            |
| 5.  | Sonicated supernatant                               | 4.10                                            |
| 6.  | Sonicated resuspended particles                     | 75.0                                            |
| 7.  | Supernatant of (6) after centrifugation             | 58.5                                            |
| 8.  | Resuspended sediment of (6) after<br>centrifugation | 11.6                                            |



**Table 26.** Changes in PEPCK activity in pigeon liver during fasting and refeeding (with standard diet).

Whole homogenates were prepared and assayed as in METHODS. The mean values are given with the standard deviations.

| Nutritional state    | No. of birds | Activity                              |
|----------------------|--------------|---------------------------------------|
|                      |              | ( $\mu$ Moles/min./g. dry wt. at 25°) |
| Well-fed             | 5            | 31.2 $\pm$ 5.2                        |
| Starved 24 hr.       | 3            | 27.6 $\pm$ 3.0                        |
| Starved 48 hr.       | 6            | 33.1 $\pm$ 5.7                        |
| Starved 48 hr., then |              |                                       |
| refed 6 hr.          | 3            | 27.7 $\pm$ 2.9                        |
| refed 18 hr.         | 3            | 32.1 $\pm$ 3.5                        |
| refed 40 hr.         | 3            | 29.8 $\pm$ 4.8                        |

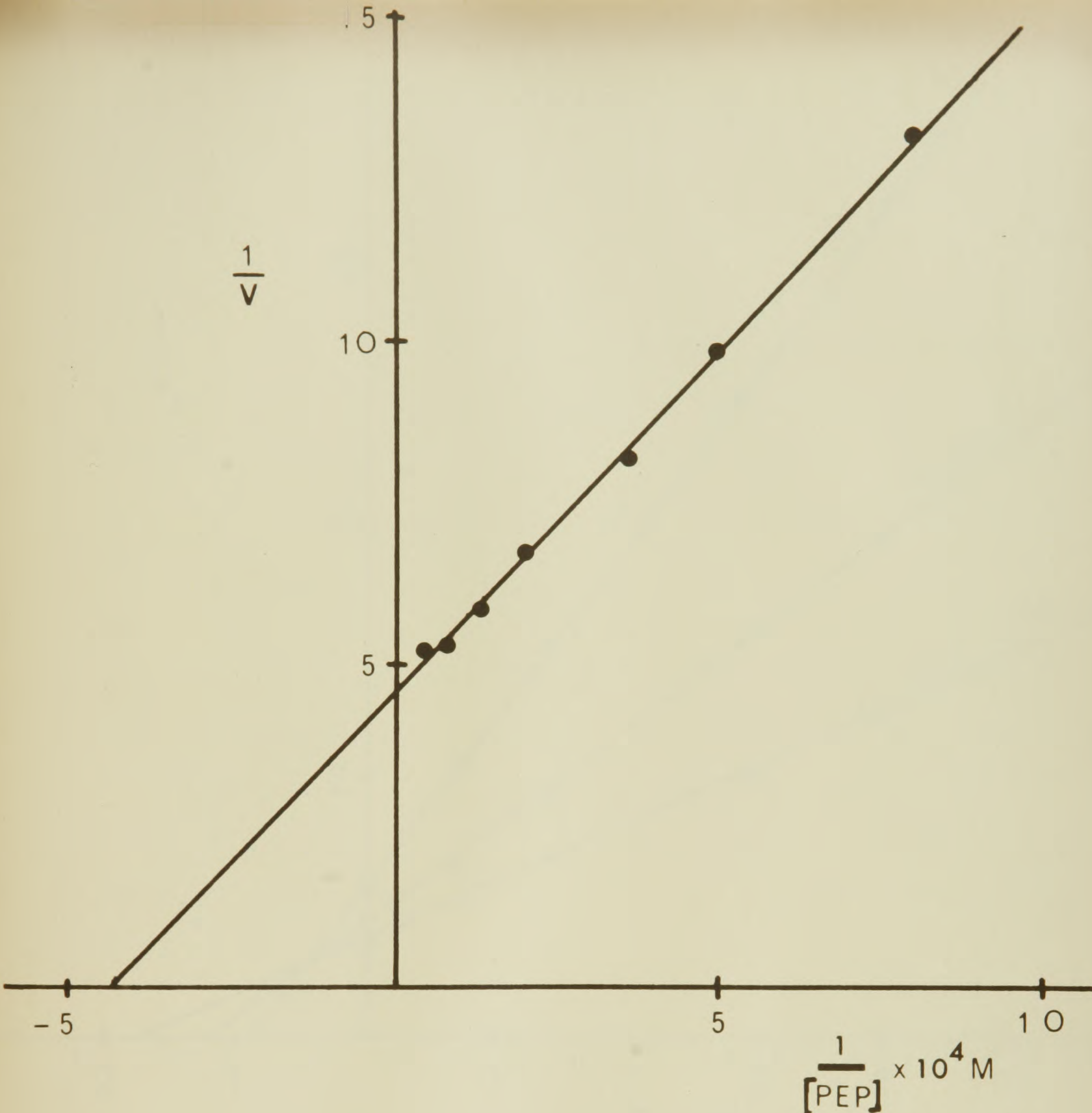


since high degrees of purification have until recently not been achieved. The apparent  $K_m$  for PEP (IDP saturating; IDP/ $Mn^{2+}$  ratio 1.3) was  $23 \mu M$  (Fig. 8). The effects of PEP concentration on the initial velocity differed from those described by Stickland (1959,c) in that a "normal" hyperbolic relationship appeared to exist. The result was similar when 2-mercaptoethanol was omitted from the assay procedure. The apparent  $K_m$  for IDP was  $30 \mu M$ . According to Stickland (1959,c) the  $K_m$  for  $HCO_3^-$  is about  $25 mM$ .

AMP and ADP (both  $0.8 mM$ ) inhibited by less than 5% in the standard assay with  $1 mM Mn^{2+}$ ; there was no inhibition when the  $Mn^{2+}$  concentration was raised to  $3 mM$ . <sup>5 mM</sup> ATP inhibited by 80% in the standard system; again the effect was reversible when excess  $Mn^{2+}$  was present. These negative findings are in contrast with those of Utter & Kurahashi, (1954). Cyclic 3',5'-AMP ( $0.1 mM$ ) had no effect. CoA ( $0.4 mM$ ) was without effect; acetyl CoA ( $0.33 mM$ ) inhibited by 20% - this was not reversed by extra  $Mn^{2+}$  ions. L- $\alpha$ -glycero-P ( $1 mM$ ), glucose 6-P ( $1 mM$ ), glucose ( $5 mM$ ), FDP ( $1 mM$ ), 3-PGA ( $1 mM$ ), L-malate ( $3 mM$ ) and Pi ( $6 mM$ ) did not influence the enzyme activity. Citrate ( $0.5 mM$ ) inhibited by 10%, which was reversed by adding  $Mn^{2+}$  in excess.

The maximal velocity of the same preparation of PEPCK, when assayed in the direction of oxaloacetate decarboxylation, was similar to that obtained in the other assay. The apparent  $K_m$  for ITP was  $1.6 mM$ , when oxaloacetate was  $3.3 mM$  and  $Mg^{2+}$   $13 mM$  (Fig. 9), in agreement with Nordlie & Lardy (1963,b). According to these authors, the oxaloacetate





**Fig. 8.** Effect of PEP concentration on the initial velocity of PEPCK (direction of carboxylation).

An extract consisting of fresh sonicated washed mitochondria was used and assays were carried out as described in METHODS. The  $\text{Mn}^{2+}$  concentration was 1 mM and that of IDP 1.66 mM. Tests showed that substrate inhibition due to excess IDP did not occur. The apparent  $K_m$  for PEP was 23  $\mu\text{M}$ . A similar value was obtained when  $\text{Mn}^{2+}$  was added at 3 mM. [The initial velocity was expressed as  $-E/3\text{min.}$  at  $25^\circ$  for a constant amount of added enzyme.]



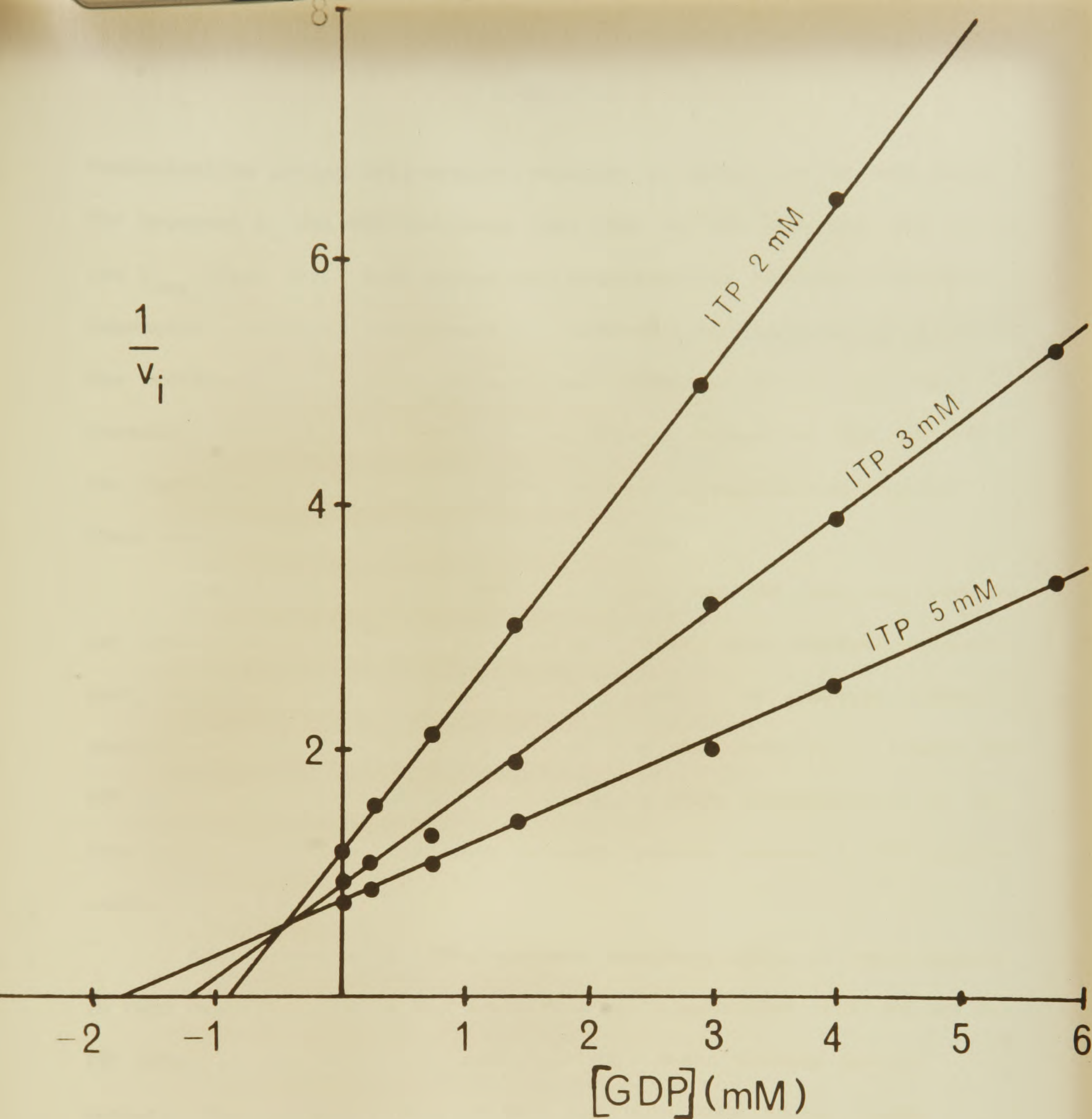


Fig. 9. Inhibition of PEPCK by GDP, and determination of apparent  $K_m$  for ITP (direction of decarboxylation).

Fresh sonicated washed mitochondria were used; the assays were carried out with ITP as nucleotide substrate as described in METHODS. The velocities were expressed as  $\mu$ Moles of PEP formed/10 min. at 25° by a constant amount of enzyme. The results were plotted according to the method of Dixon (1953); this allowed calculation of the  $K_i$  for GDP (0.4 mM) and of the apparent  $K_m$  for ITP (1.6 mM).



concentration giving half-maximal velocity is about 1 mM in this assay. The apparent  $K_m$  for GTP was lower than that for ITP (0.4 mM), but so was the  $V_{max}$  (Fig. 10). Both nucleoside triphosphates exhibited hyperbolic substrate - velocity relationships. According to Kurahashi et al., (1957) the "affinity" of purified chicken-liver PEPCK for GTP is very high; recently, Lane et al., (1966) have reported  $K_S$  values for GTP and PEP for the highly-purified pig-liver PEPCK obtained by equilibrium dialysis - these are 10 times lower than the above values.

Heldt & Klingenberg (1965) have shown that ITP does not occur in rat liver mitochondria, and Heldt et al., (1964)<sup>b</sup> have obtained evidence that endogenous GDP is the physiological acceptor for substrate-level phosphorylation in the same preparation. On this basis it is likely that GTP is the usual substrate for mitochondrial PEPCK; determination of the true kinetic constants will have to await greater success in the purification of the enzyme.

GDP inhibited the ITP-dependent decarboxylation of oxaloacetate in this assay (Fig. 9). The inhibition was competitive with respect to ITP (Fig. 11) and the  $K_i$  was about 0.4 mM. Metal binding was not a factor. Part of this effect of GDP could have been due to "blank" activity of PK, since GDP is 2 - 3 times more effective than IDP in that reaction (see METHODS). All possible steps were taken (see METHODS) to reduce interference from this enzyme; further, in the carefully-timed procedure for the determination of PEP accumulated during the assay, there appeared to be no difference in the amount of pyruvate present in the



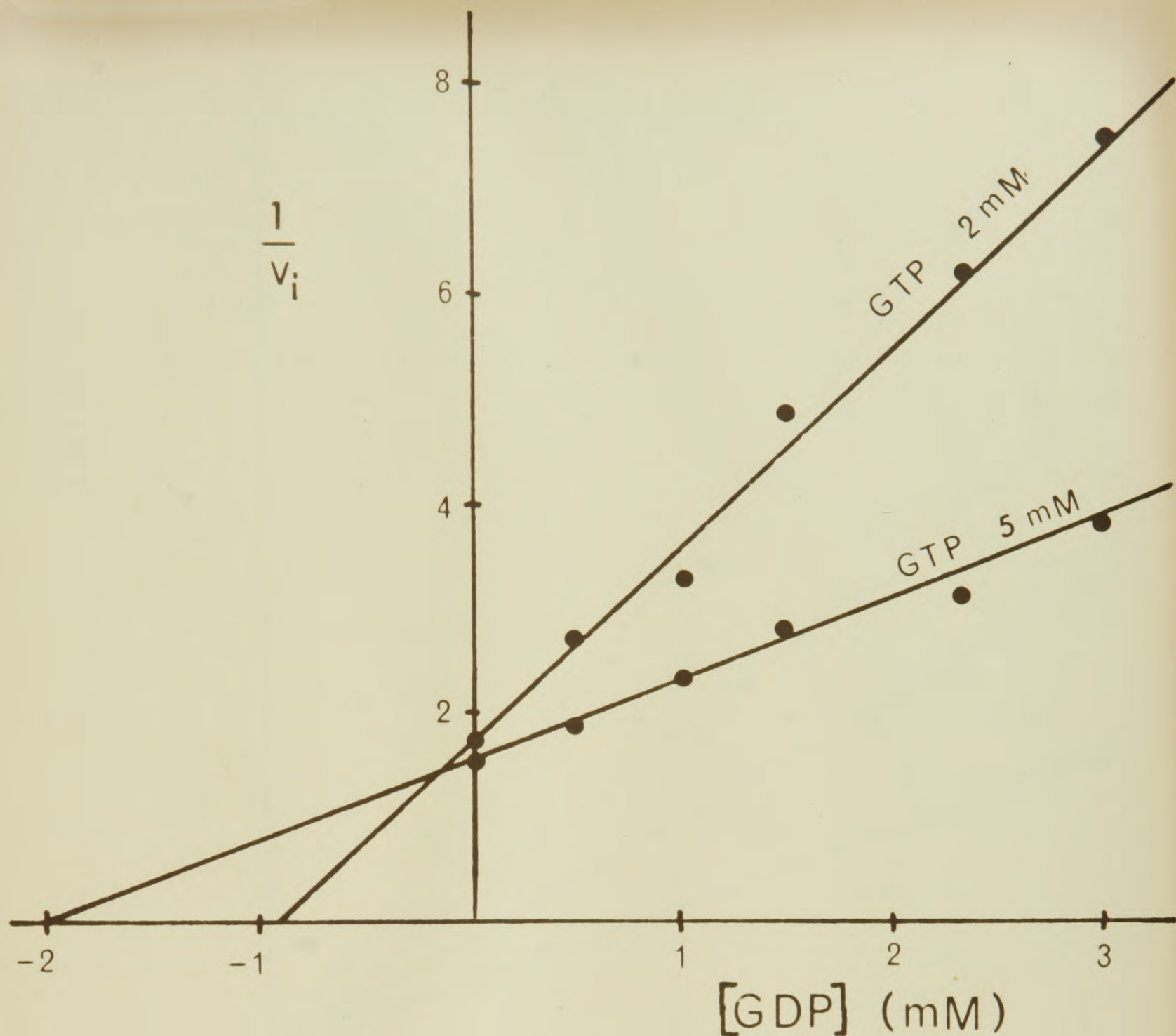


Fig. 10. Inhibition of PEPCK by GDP and determination of the apparent  $K_m$  for GTP (direction of decarboxylation).

The method was as described for Fig. 9, except that GTP replaced ITP as substrate. The  $K_i$  for GDP was about 0.15 mM, and the apparent  $K_m$  for GTP was 0.4 mM. The  $V_{max}$  with GTP was about 50% of that with ITP (see Fig. 9).



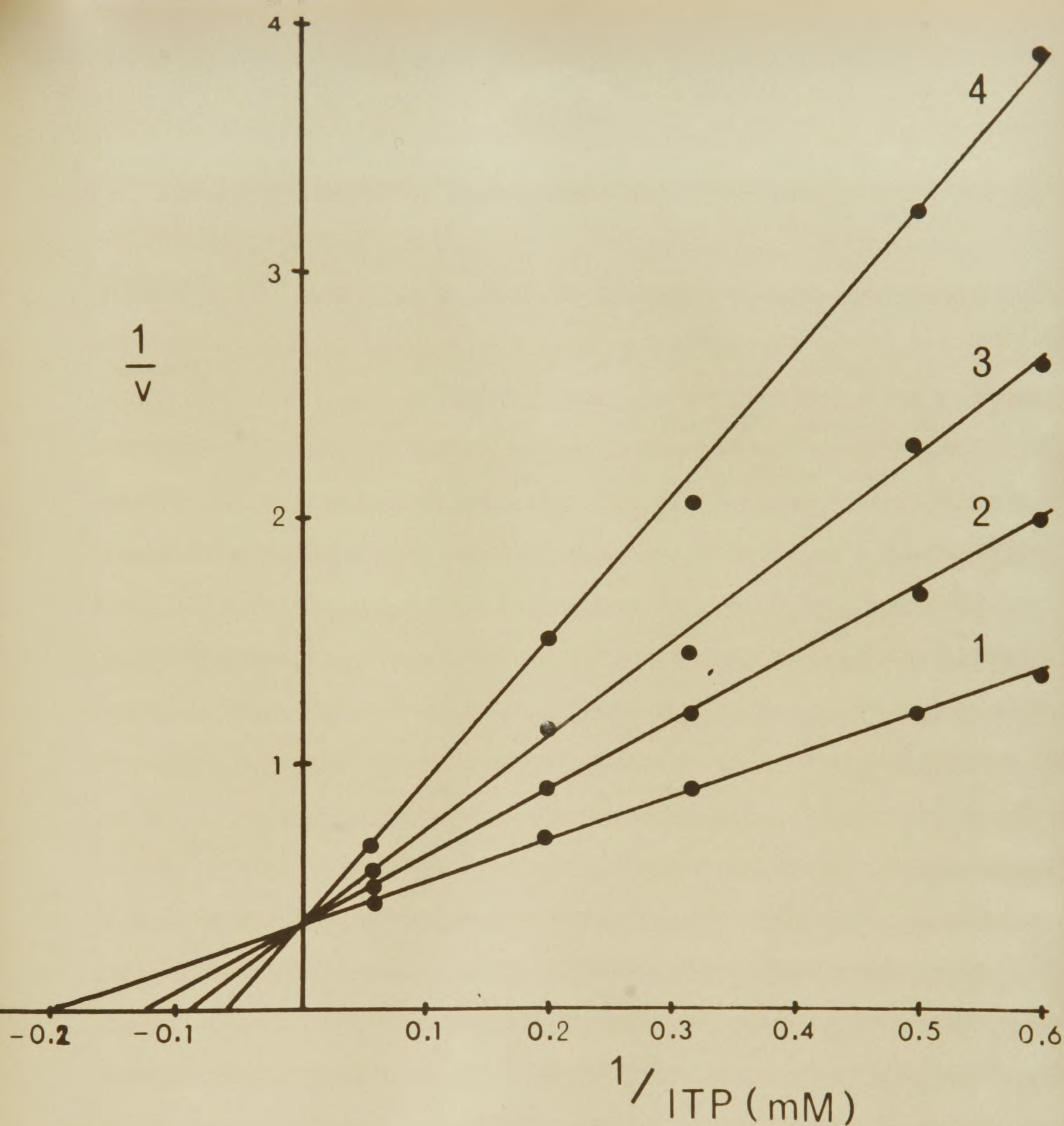


Fig. 11. Competitive inhibition of PEPCK by GDP (direction of decarboxylation).

Assays were conducted as described for Fig. 9, on which some of the points appear. The inhibition by GDP was competitive with ITP. 1. GDP 0; 2. GDP 0.5 mM; 3. GDP 0.75 mM; 4. GDP 1.45 mM.



samples with and without GDP, suggesting that all the pyruvate was formed by decarboxylation of oxaloacetate. The fact that the inhibition by GDP was competitive with ITP also indicates that the effects were not due to changes in the rate of PEP removal. Finally, when GDP was used as acceptor in the carboxylation reaction, the increase in the blank rate of NADH oxidation was only 10 - 20%, at the highest GDP concentrations used.

Similar results were obtained in the GTP-linked assay, except that the  $K_i$  was 0.1 mM (Fig. 10). The lower  $V_{max}$  in the presence of GTP may have been due to contamination of GTP samples with GDP, which varied between 10 - 25%, despite all precautions taken. Although the accuracy of these kinetic data is decreased by the enforced use of a sampling method, with consequent difficulty in determining initial rates, it is reasonably certain that GDP is a strong competitive inhibitor of PEPCK with respect to the nucleoside triphosphate substrate, and that the GDP/GTP ratio will be an important determinant of the overall rate of conversion of oxaloacetate into PEP.

Adenine nucleotides did not inhibit the rate of oxaloacetate decarboxylation (within the limits of accuracy imposed by high blanks) at concentrations of 3 mM (ATP), and 1 mM (AMP and ADP). The following substances did not affect the rate in this direction: acetyl CoA (0.3 mM), CoA (0.1 mM), citrate (0.5 mM), malate (2 mM), Pi (6 mM), 3-PGA (0.8 mM), glucose 6-P (1 mM) and palmityl CoA (0.1 mM).

The above studies did not suggest that "co-operative effects" exist in the kinetic behaviour of PEPCK with respect to 3 of its substrates



(PEP, ITP, and GTP) or an inhibitor (GDP). No evidence of "allosteric" or second-site regulation was found.

It is apparent that pigeon-liver PEPCK differs greatly from the soluble enzyme of rat liver and kidney cortex. The list of species studied from the point of view of intracellular PEPCK distribution is too short to allow general correlations to be made, e.g. from the nature of diets or of special functional physiology. For example, ketosis in both rats and cattle has been ascribed to enhanced PEPCK activity in the presence of unchanged capacity to form oxaloacetate from pyruvate (Krebs, 1965). According to Miss P. Lund (personal communication) PEPCK in bovine liver is at least partly mitochondrial (rat liver mitochondria contain almost no PEPCK). Pigeons also are liable to ketosis following pancreatectomy (Hazelwood, 1965). The studies of Exton & Park (1965) have suggested an action of glucagon and adrenal hormones on PEPCK, based on changes in intermediates in perfused rat livers. Foster et al., (1966) have described a paradoxical effect of L-tryptophan administration on PEPCK of rat liver, consisting of a marked and early induction of PEPCK in intact and adrenalectomized rats, but a concomitant inhibition of the glycogenic effect of hydrocortisone, or of glycogenesis from pyruvate, malate and aspartate. This work followed on the earlier studies of Ray et al., (1964) showing that the increased PEPCK activity following on hydrocortisone administration to rats was not directly due to protein synthesis, but involved activation phenomena. These results point to complex regulatory mechanisms governing the activity of PEPCK in rat liver;



pigeon liver has not yet been studied from the point of view of hormonal effects.

#### SECTION D

PYRUVATE KINASE (PK) AND ENOLASE IN PIGEON LIVER. Krebs & Eggleston (1965) pointed out the important role that PK activity might play in the regulation of gluconeogenesis in the liver at the level of PEP metabolism. They demonstrated a fall of 66% in the hepatic PK activity of rats starved for 48 hr. or fed a low carbohydrate diet, while an increase of more than 300% occurred on a sucrose-casein (70 : 30) diet for 2 - 3 days. No such changes occurred in the kidney cortices of the same animals. Tanaka et al., (1965) obtained evidence for 2 forms of PK in rat liver, using immuno-precipitation techniques and differential fractionation. They also reported changes in the "L" -form of the enzyme in liver during dietary and hormonal treatments. Weber et al., (1965,b) have described changes in rat liver PK consonant with the above, and have emphasized the requirement for insulin to permit induction. No changes of a similar nature have been reported for liver enolase.

PK and enolase activities in pigeon livers during a cycle of fasting and refeeding are shown in Table 27. No significant variations occurred. The bulk of both enzymes was found in the soluble fraction (Table 28). Besides an obligatory requirement for  $K^+$  ions, PK required  $Mg^{2+}$  or  $Mn^{2+}$  for activity in this assay. The apparent  $K_m$  for ADP (at an optimal PEP concentration of 1.1 mM) was 0.6 mM; that for PEP was 0.5 mM.



**Table 27.** Activities of pyruvate kinase (PK) and enolase in pigeon liver during a cycle of fasting and refeeding (with standard diet).

The assays were carried out on whole homogenates as described in METHODS. Mean activities are expressed as  $\mu\text{Moles/min./g. dry wt. at } 25^{\circ}$ , with standard deviations.

| Nutritional state     | No. of birds | PK activity | Enolase activity |
|-----------------------|--------------|-------------|------------------|
| Well-fed              | 4            | $56 \pm 4$  | $202 \pm 17$     |
| Starved 24 hr.        | 3            | $69 \pm 5$  | $175 \pm 29$     |
| Starved 48 hr.        | 4            | $58 \pm 9$  | $162 \pm 12$     |
| Starved 48 hr., then: |              |             |                  |
| refed 6 hr.           | 3            | $60 \pm 4$  | $174 \pm 26$     |
| refed 18 hr.          | 3            | $51 \pm 3$  | $149 \pm 22$     |
| refed 40 hr.          | 3            | $49 \pm 7$  | $192 \pm 31$     |



**Table 28.** Intracellular distribution of pyruvate kinase (PK) and enolase in pigeon liver.

A fresh sucrose homogenate was prepared (1 : 4, w/v - see METHODS) using the liver of a starved pigeon. 2 sets of particles were prepared; the first was the unwashed sediment of a 20 min. centrifugation at 30,000g at 0°, the other consisted of pure twice-washed mitochondria. Both pellets were resuspended in homogenizing medium to the original volume of homogenate from which they were prepared - percentage yields could then be derived for each fraction.

| Fraction            | Activity<br>( $\mu$ Moles/min./fraction at 25°) |         | Percentage of<br>original activity |         |
|---------------------|-------------------------------------------------|---------|------------------------------------|---------|
|                     | PK                                              | Enolase | PK                                 | Enolase |
| Whole homogenate    | 379                                             | 640     | 100                                | 100     |
| 30,000g supernatant | 285                                             | 562     | 75                                 | 88      |
| 30,000g sediment    | 104                                             | 86      | 27                                 | 13      |
| Washed mitochondria | 8                                               | 3       | 2                                  | 1       |



Inhibition was observed when the concentration of PEP was raised above 2 mM. This occurred in the presence of an excess of  $Mg^{2+}$  and may have had a kinetic basis on the lines of that proposed by Ferdinand (1966) for certain 2-substrate reactions.

There was no inhibition by pyruvate below 3 mM. ATP inhibited at relatively high concentrations (3 mM ATP gave 50% inhibition when  $Mg^{2+}$  was 10 mM and the pH 7.4). This was similar to the behaviour of muscle PK (Reynard et al., 1961). No detailed studies were done since the kinetics appeared to be complex and are under study in various laboratories (Tanaka et al., 1965; Mildvan & Cohn, 1966). The same comments apply to the effects of  $Ca^{2+}$  ions; inhibition occurred in competition with  $Mg^{2+}$  (see Kachmar & Boyer, 1953; Mildvan & Cohn, 1966).

Glucose, glucose 6-P, FDP,  $\alpha$ -glycero-P and 3-PGA (all at 1 mM) did not affect the enzyme. Malate, glutamate, aspartate and  $\alpha$ -oxo-glutarate (all at 8 mM) had no effect. Citrate was tested because of the close functional correlation between PFK and PK in glycolysis - there was no inhibition at 3 mM citrate, in the presence of a 3-fold excess of  $Mg^{2+}$  ions. The inhibition by ATP was not potentiated.  $NH_4^+$  and Pi ions (6 mM) activated by 10 - 20%. AMP and cyclic 3',5'-AMP had no effect (1 mM and 0.2 mM, respectively). CoA (0.2 mM) and acetyl CoA (0.4 mM) inhibited by less than 10%. Palmityl CoA was without effect (0.1 mM).

PK of pigeon liver appears thus not to undergo variations in activity during starvation or refeeding, and is significantly sensitive, amongst a variety of metabolites tested, only to inhibition by ATP and

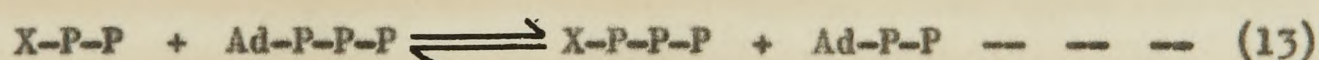


$\text{Ca}^{2+}$ , and to "activation" by its substrates (ADP and PEP) and metal ion cofactors ( $\text{Mg}^{2+}$  and  $\text{K}^+$ ).

The high activity of enolase, and its failure to change during starvation and refeeding, are in agreement with the view that it is a "bifunctional" enzyme of both glycolysis and gluconeogenesis (Weber et al., 1965,a).

## SECTION E

NUCLEOSIDE DIPHOSPHOKINASE (NuDiKi) IN PIGEON LIVER. This enzyme probably plays a key role in the intracellular metabolism of nucleotides (see Weaver, 1962) but relatively little is known of its kinetic properties or intracellular distribution, or even whether one enzyme entity catalyses the numerous reactions between ADP or ATP and non-adenosine polyphosphates, according to reaction (13):



Since PEP synthesis in liver mitochondria is thought to involve the participation of guanosine nucleotides (Section C), and because the kinetic studies in that Section suggested that the ratio of GDP/GTP might be important in the regulation of PEPCK activity, the distribution, activity and kinetic properties of NuDiKi were examined in pigeon liver.

The intracellular distribution of NuDiKi (Table 29) was principally particulate, although 12% was found in the cytoplasm. Sonication solubilized about  $\frac{2}{3}$  of the particulate activity. In another experiment



**Table 29.** Intracellular distribution of nucleoside diphosphokinase (NuDiKi) in pigeon liver.

Exactly the same sonicated fractions were assayed as those described in Table 25. The assays were carried out as described in METHODS.

| No. | Fraction                                                     | Activity<br>( $\mu$ Moles/min./fraction at 25°) |
|-----|--------------------------------------------------------------|-------------------------------------------------|
| 1.  | Sonicated whole homogenate                                   | 4600                                            |
| 2.  | Sonicated supernatant                                        | 480                                             |
| 3.  | Sonicated resuspended particles                              | 3870                                            |
| 4.  | Supernatant after recentrifugation<br>of sonicated particles | 2610                                            |
| 5.  | Resuspended sediment of centrifuga-<br>tion mentioned in (4) | 1246                                            |



66% of all the activity in an homogenate was recovered in the pure washed mitochondrial fraction. Since both the 450g sediment and the two "washing" supernatants contained mitochondria, it is a reasonable conclusion that 75% of the total NuDiKi in pigeon liver is mitochondrial. This applies also to fed pigeons.

The tissue activity of NuDiKi was very high (400 - 600  $\mu$ Moles/min./g. dry wt. at 25<sup>0</sup>), in comparison with PEPCK or pyruvate carboxylase. There was no apparent difference between starved and fed birds.

The "latency" of NuDiKi (i.e. the requirement for particle disruption for full activity, best achieved by sonication - see METHODS) and the effects of atractyloside on isolated mitochondria reported in Section F, suggest that the enzyme is located in the mitochondrial "matrix space" within the "adenine nucleotide translocase" barrier (Klingenberg & Pfaff, 1966). Heldt & Schwalbach ( 1966 ) said rat-liver NuDiKi appears to be mainly extra-mitochondrial, like PEPCK. However, according to Klingenberg & Pfaff (1966) NuDiKi in rat liver is almost exclusively mitochondrial.

Kinetic studies were carried out using as enzyme preparation a high-speed supernatant of sonicated washed mitochondria. The  $K'_S$  values for GTP and ADP were determined by the method of Florini & Vestling (1957), based on the initial velocities at pH 8. A family of parallel lines was obtained in double-reciprocal plots where ADP was the variable substrate and GTP was the fixed-changing substrate (Fig. 12), except that substrate inhibition occurred at ADP concentrations higher than 0.1 mM; this was



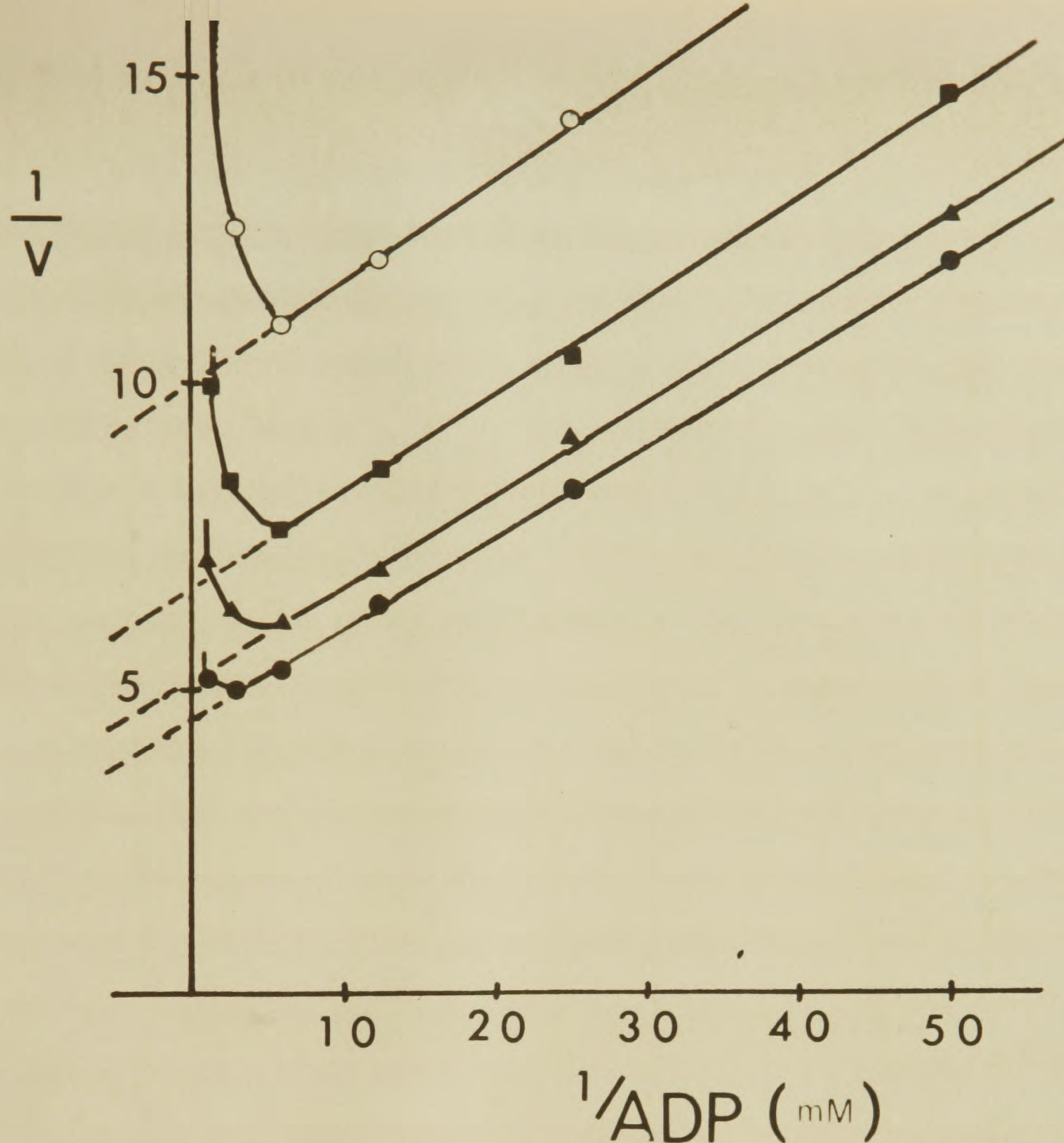


Fig. 12. Effect of ADP concentration on the initial velocity of nucleoside diphosphokinase (NuDiKi) at various concentrations of GTP.

The assays were conducted as in METHODS, using the 30,000g supernatant of freshly-prepared and sonicated mitochondria. The values for the (extrapolated) intercepts on the ordinate were used to construct a Florini & Vestling (1957) plot to determine the  $K'_S$  for GTP. The initial velocity was expressed as  $+ E/4\text{min.}$  at  $25^\circ$  for a constant amount of enzyme. ●—● GTP  $1.2 \times 10^{-3}\text{M}$ ; ▲—▲ GTP  $3.3 \times 10^{-4}\text{M}$ ; ■—■ GTP  $1.5 \times 10^{-4}\text{M}$ ; ○—○ GTP  $6 \times 10^{-5}\text{M}$ .



competitive with GTP. The inhibition could not be overcome by adding more hexokinase or glucose 6-P dehydrogenase, or by addition of  $Mg^{2+}$ . These tests were done because it was suspected that high concentrations of ADP might increasingly inhibit hexokinase (Crane & Sols, 1954). This did not appear to be the case. When GTP was the variable substrate and ADP was the fixed-changing substrate, parallel lines were also observed at low ADP concentrations. The Florini-Vestling plots were linear for both substrates and gave  $K'_S$  values of  $8 - 9 \times 10^{-5} M$  for GTP and  $4 - 5 \times 10^{-5} M$  for ADP. Mourad & Parks (1966) in their studies on erythrocyte NADKi obtained similar results, although they used rather small amounts of auxiliary enzymes in their assays. It may therefore be assumed that their finding that one protein entity catalyses the reactions with several species of nucleotide substrates can be extrapolated to the pigeon liver enzyme. The mechanism of the reaction from both sets of data appears to be of the "double displacement" type, and the inhibition at high concentrations may be due to the formation of an "abortive complex" as suggested by Mourad & Parks (1966). The values given by these authors for  $K'_S$  of ATP and GDP were  $4 - 8 \times 10^{-4} M$  and  $3 - 6 \times 10^{-4} M$ , respectively.

The equilibrium constant for the reaction:  $GTP + ADP \rightleftharpoons GDP + ATP$ , is not known. Thermodynamic considerations, and the value derived by Berg & Joklik (1953) for the reaction:  $ITP + ADP \rightleftharpoons IDP + ATP$ , suggest that it would be near to unity. The concentrations of metal ions may be expected to have little effect on the equilibrium position, since the association constants for  $Mg^{2+}$  of GTP and ATP are very similar.



The following factors may be of importance in an analysis of the possible regulatory role of NuDiKi in PEP synthesis from oxaloacetate:

i) The high activity of the enzyme, and the reversibility of the overall reaction, indicate that metabolic control might not occur at this point. On the other hand, it is likely that the ratio GDP/GTP will at all times be closely "geared" to that of ADP/ATP, and therefore to the "phosphorylation potential" of the mitochondrial matrix space.

ii) The inhibition of NuDiKi activity at ADP concentrations such as might reasonably occur in mitochondria (0.1 mM), is competitive with GTP and may thus be even more marked at low concentrations of GTP. According to Heldt & Klingenberg (1965), the ratio of guanosine to adenosine nucleotides in rat liver mitochondria is about 1 : 100. The high activity of the enzyme would require very severe inhibition if GTP formation were to be regulated efficiently by this mechanism.

iii) Competition for adenine nucleotides between various species of non-adenosine nucleotides may be important in intact cells.

## SECTION F

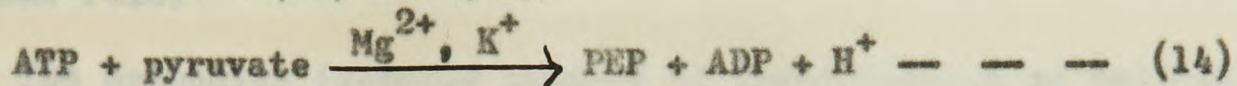
### SYNTHESIS OF PEP IN PIGEON-LIVER MITOCHONDRIA.

#### 1. Introduction

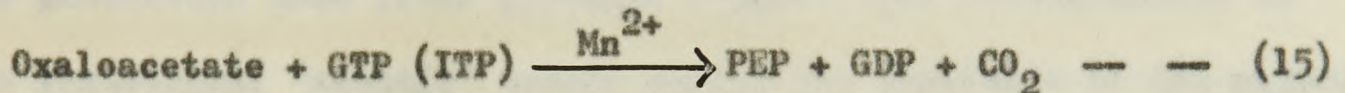
It is now almost certain that hepatic or renal synthesis of carbohydrate from pyruvate or oxaloacetate (and all precursors of these key intermediates) involves the formation of PEP. This is followed by the



reversal of the glycolytic pathway in terms of intermediates, but not in terms of all the participating enzymes (see Krebs, 1964,a). Only 2 enzymes are likely to catalyse formation of PEP, namely PK and PEPCK, by the following reactions (14) and (15):



and



There is considerable evidence that PK is not concerned in PEP synthesis in vivo, viz.

i) The reaction is highly endergonic ( $\Delta G = +6.1$  Kcal. - Burton, 1957) and can only occur in the presence of very high ATP/ADP ratios. Krinsky (1959) has shown that intact liver mitochondria can maintain sufficiently high ratios to permit PEP synthesis definitely catalysed by PK in vitro.

ii) The apparent  $K_m$  of PK from muscle for pyruvate is about 10 mM (McQuate & Utter, 1959). The  $K_m$  of the liver enzyme is not known.

iii) The maximal velocity is very slow in this direction (about  $1/200$  th of the reverse reaction - McQuate & Utter, 1959). If this applies to the pigeon-liver enzyme, rates of PEP formation of  $0.3 \mu\text{Moles/min./g. dry wt. at } 25^{\circ}$  might be possible (see Table 27). This is much less than observed rates of PEP formation during carbohydrate synthesis in liver homogenates.

ix) In certain tissues, e.g. rat liver, the PK activity varies inversely with the glycogenic capacity. This does not apply to pigeon liver or rat kidney cortex. (See Section D).



v) PK is present in many tissues, and is particularly active in those tissues where no gluconeogenesis occurs. The discovery by Tanaka et al., (1965) of a separate "L"-form of PK in liver may imply a different functional role.

vi) The extensive isotopic studies of incorporation of selectively labelled precursors into liver glycogen suggest that randomization of  $^{14}\text{C}$  by a symmetrical "shuttle" occurs during the synthesis of the majority of new hexose molecules (see Weinman et al., 1957). However, these data do not exclude the possibility that net synthesis of PEP occurs via PK, and that the labelling is due to an "exchange shuttle" between pyruvate and fumarate.

vii) Washed mitochondria from certain glycogenic tissues carry out rapid synthesis of PEP from a variety of precursors (see below). Most of these preparations contain very small amounts of PK.

There is much evidence supporting a role of PEPCK in PEP synthesis in the liver or kidney:

i) The enzyme has been found primarily in gluconeogenic tissues, although it occurs also in certain white muscles and in brain (L.H. Opie and L.V. Eggleston, personal communication), and it may also be present in adipose tissue (Dougherty et al., 1966).

ii) The potential capacities of PEPCK in liver and kidney exceed the maximum rates of carbohydrate synthesis measured in these organs.

iii) The kinetic constants for the substrates are such that the enzyme might be expected to be appreciably active in vivo.



iv) The changes in the activity of "soluble" PEPCK in rat and guinea-pig livers during hormonal and dietary treatments (see Section C for references) are such as to suggest a close relationship to gluconeogenesis, in terms of the "increased through-put" theory of Fitch & Chaikoff (1960). No such changes occur in mitochondrial PEPCK, even in pigeon liver where no "soluble" PEPCK appears to exist.

v) PEP synthesis from dicarboxylic acids in isolated mitochondria occurs only in those species where PEPCK is largely mitochondrial (Nordlie & Lardy, 1963,b).

Starting from the conclusions reached above concerning the roles of PK and PEPCK in PEP synthesis in the liver, studies have been carried out to examine the regulation of PEP formation in isolated pigeon-liver mitochondria, from the point of view both of substrate supply and of the integrated control of mitochondrial metabolism related to it.

## 2. Previous studies of PEP synthesis in mitochondria.

Kalckar (1939) first observed the formation of PEP from malate and fumarate in crude extracts of kidneys from cats and rabbits, and suggested that it might be important in carbohydrate synthesis from dicarboxylic or amino acids. Leloir & Munoz (1944) demonstrated that PEP was formed from citrate, glutamate, succinate, fumarate and malate in a preparation of lysed liver particles from guinea-pigs, and showed a requirement for added Pi, AMP and cytochrome c. The oxidation of butyrate inhibited the formation of PEP (Munoz & Leloir, 1943). Stanbury & Mudge (1954) described the synthesis of PEP in rabbit-liver mitochondria in the



presence and absence of DNP; only citrate,  $\alpha$ -oxo-glutarate, glutamate and proline were effective as precursors. In each case a biphasic stimulation of  $O_2$  uptake and PEP formation occurred when progressively more DNP was added, with a minimum at  $5 \times 10^{-5} M$  DNP. The effects were only observed (after a time lag) in the presence of  $O_2$ , high substrate concentrations and Pi.

Bartley (1954,a,b), Bartley & Avi-Dor (1955) and Bartley (1955) reported extensive studies on the mechanism of PEP synthesis in washed sheep-kidney particles, and in particles from rat and pigeon liver. The conclusions were:

i) PEP, as well as 2- and 3-PGA, was formed during short incubations in the presence of high concentrations of oxaloacetate, malate, fumarate, succinate,  $\alpha$ -oxo-glutarate, citrate and pyruvate (the last only in the presence of  $7.5 \text{ mM HCO}_3^-$ ). The formation was not due to glycolysis.

ii) Rapid "turnover" of PEP occurred, as shown by net chemical changes and by isotope incorporation data.

iii) Kinetic studies of the specific activities of added  $^{32}P$ i, ATP and PEP during synthesis of PEP from  $\alpha$ -oxo-glutarate showed that PEP was not directly formed from Pi and suggested that ATP might be the donor.

iv) Pigeon-liver "cyclophorase" or mitochondria were very much more active than rat-liver preparations.

v) Anaerobically, only oxaloacetate gave rise to PEP in the presence of added ATP and fluoride, during short incubations.

vi) Under certain conditions the formation of PEP was reversible.



vii) PEP synthesis from malate and oxaloacetate occurred in the absence of added ATP or ITP, even when the particles were washed to decrease endogenous nucleotides. Added ATP or ITP were rapidly hydrolysed and inhibited PEP accumulation above rather low concentrations. The kinetics of  $^{32}\text{P}_i$  incorporation into phosphorylated compounds in the presence of these substrates suggested that PEP was formed without the intervention of ATP or ITP, but possibly via GTP. Bartley concluded that the transfer of  $\sim\text{P}$  (from its origin during substrate oxidation) to PEP might proceed over different pathways depending on the concentration of oxaloacetate, with the implication that ADP and oxaloacetate competed for  $\sim\text{P}$  of GTP formed during oxidative phosphorylation. Although the question of the nucleotide specificity of oxidative phosphorylation is still not settled (see Klingenberg & Pfaff, 1966) it is generally believed that GTP can be synthesized in intact mitochondria only by the NuDiKi reaction and by substrate-level phosphorylation. Some aspects of these relationships have been studied (see below).

Bandurski & Lipmann (1956) found that guinea-pig liver mitochondria synthesized PEP from citrate, pyruvate and  $\alpha$ -oxo-glutarate; fumarate, succinate and malate were less effective. They confirmed that only oxaloacetate gave rise to PEP in the absence of  $\text{O}_2$ . Mendicino & Utter (1962) studied the formation of PEP from malate in chicken-liver mitochondria. This was not linear with time, was independent of the particle concentration within certain limits, and was stimulated by low concentrations of ATP. "Turnover" due to contaminant PK occurred.



Nordlie & Lardy (1963,a) observed that PEP synthesis in guinea-pig liver mitochondria was related to the rate of  $\alpha$ -oxo-glutarate oxidation, such that addition of DNP or of a glucose-hexokinase ATP-trap increased  $O_2$  uptake, Pi uptake and PEP formation. Malonate addition was inhibitory, while PEP synthesis was enhanced by malate. The conclusion was reached that the biphasic effects of DNP were related to the interplay of various effects of the uncoupler on mitochondrial metabolism, including the stimulation of respiration without loss of  $\sim P$  at low concentrations, and increasing stimulation of ATPase at higher concentrations. Variations in the oxaloacetate concentration were also considered to be important. The data appeared to support the earlier postulate of Azzone & Ernster (1961) that compartmentation of  $\sim P$  produced by substrate-level phosphorylation rendered it unavailable to the DNP-stimulated ATPase or to the exogenous glucose-hexokinase trap.

This problem was re-examined by Scholte & Tager (1965) in rat-liver mitochondria oxidizing glutamate or  $\alpha$ -oxo-glutarate in the absence of added Pi and in the presence and absence of DNP. It was shown that the depletion of endogenous Pi which gradually limits the rate of respiration in this system, was due to the formation of PEP. From the ratio of PEP molecules formed to the substrate molecules removed, they concluded that only 1 in 10 of the GTP molecules formed reacted with oxaloacetate, that the rest reacted with ADP to form ATP which was then hydrolysed, and that there was no real evidence for compartmentation of the  $\sim P$  derived from substrate-level phosphorylation. The weakness in this argument lay in the



fact that no attempt was made to assess the activity of PK in the system (see below), which was a potentially serious cause of error in the computation.

Papa et al., (1965) considered a possible role of PEPCK in the control of succinate oxidation in rabbit-kidney mitochondria. Their data indicate that the effect of ATP in relieving succinate oxidation inhibited by high intra-mitochondrial oxaloacetate concentrations, might be due to the removal of the inhibitor to form PEP. The re-activation was insensitive to oligomycin and sensitive to atractyloside.

Lardy et al., (1965) and Shrago & Lardy (1966) investigated the mechanisms of PEP synthesis in rat liver, using both washed mitochondria and high-speed supernatant fractions. Evidence was obtained to support the concept of a complex path of carbon from intra-mitochondrial pyruvate to extra-mitochondrial PEP, involving the transfer of oxaloacetate units out of the particles as the more readily-diffusible malate and aspartate. "Cytosol" enzymes were able to convert these metabolites rapidly to PEP, and their activity was increased in alloxan diabetes. Haynes (1965) obtained evidence for a similar scheme in studies on  $^{14}\text{CO}_2$  incorporation into rat-liver mitochondria. Henning et al., (1966) have claimed that this complex transfer might be unnecessary in view of their finding of pyruvate carboxylase in the cytoplasm, in both rat liver and kidney cortex. It is evident that in pigeon liver the problem is not one of oxaloacetate translocation into the cytoplasm, but of the movement of PEP. Diffusion of malate could still be involved in lipogenesis from pyruvate and amino



acids as part of a trans-hydrogenation mechanism (Section B).

Since the discovery of pyruvate carboxylase by Utter & Keech (1963) a number of studies have appeared dealing with the regulation of the activity of this enzyme in intact tissues or mitochondria. An activation due to increased concentrations of acetyl CoA appeared to be involved in the stimulation of glucose synthesis from lactate in rat kidney cortex slices in the presence of added acetoacetate (Krebs et al., 1965). No such evidence has yet been obtained in liver preparations. According to Struck et al., (1965) the stimulation of gluconeogenesis from lactate caused by oleate in perfused rat liver was not associated with changes in the activity of pyruvate carboxylase on direct assay of tissue samples; this does not exclude the possibility that activation by acetyl CoA had occurred reversibly in situ. Berry (1965) investigated the role of adenine nucleotides in the regulation of pyruvate carboxylase activity in intact rat liver cells and mitochondria. He showed that the reaction was sensitive to low ATP/ADP ratios, and that it was important in the maintenance of oxaloacetate concentrations sufficiently high to prevent the formation of ketone bodies. Freedman & Kohn (1964) demonstrated "feed-back" inhibition of pyruvate carboxylase by malate and other intermediates of the tricarboxylic acid cycle.

### 3. General properties of pigeon-liver mitochondria.

Pure washed mitochondria were used in most experiments for the following reasons:

- i) Liver nuclei contain a number of glycolytic enzymes, including



enolase and aldolase (Dounce, 1950), which would interfere with accurate measurements of PEP formation.

ii) It was desirable to exclude the ATPase thought to be present in nuclei (Potter et al., 1951).

iii) According to the data of Section C, there seemed to be little or no PEPCK in nuclei, which meant that only mitochondria were involved in PEP synthesis and its regulation.

The basic conditions for the incubation of mitochondria were established as follows: It was initially decided to use 2 media, the first corresponding roughly to that of Bartley (1954) and the second to that of Stanbury & Mudge (1954), including Pi and 'tris' as the principal buffers, respectively. When particles were incubated with pyruvate or malate and with added ATP, the yield of PEP was greater at higher Pi concentrations. (Table 30). This was not due to inhibition by 'tris'. Malate formation from pyruvate, substrate removal in either case, and the ATP/ADP ratios, were higher as the Pi concentration was increased.

When  $Mg^{2+}$  ions were omitted, net accumulation of PEP (and a small amount of 2- and 3-PGA) occurred, and was linear over 40 min. (Fig. 13). In this experiment, 10  $\mu$ Moles of PEP were added at the start; in the presence of 2.5 mM  $Mg^{2+}$ , there was a progressive loss of PEP, while there was no change during the incubation in the presence of 2.5 mM  $Ca^{2+}$ . More 3-PGA was formed when  $Mg^{2+}$  was present, as would be expected on account of the requirement of enolase for divalent metal ions. (Note: In all the studies reported here, 2- and 3-PGA were always measured in conjunction



**Table 30.** Effect of Pi concentration on PEP formation from pyruvate and malate in pigeon-liver mitochondria.

Fresh washed mitochondria (21 mg. dry wt./cup) were prepared as in METHODS and incubated at 30° for 30 min. Each cup, excepting no. 11, contained 40 mM 'tris' buffer, pH 7.4 and 1 mM ATP. Pi was added as the neutral potassium salt.

| No.                                | 1    | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    |
|------------------------------------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Additions (mM):                    |      |       |       |       |       |       |       |       |       |       |       |
| Pyruvate                           | 10   | 10    | 10    | 10    | 10    |       |       |       |       |       |       |
| Malate                             |      |       |       |       |       | 15    | 15    | 15    | 15    | 15    | 15    |
| HCO <sub>3</sub>                   | 4    | 4     | 4     | 4     | 4     |       |       |       |       |       |       |
| Pi                                 | 0    | 0.5   | 1     | 3     | 10    | 0     | 2.5   | 5     | 10    | 20    | 20    |
| Metabolic changes<br>(μMoles/cup): |      |       |       |       |       |       |       |       |       |       |       |
| Pyruvate                           | -6.2 | -12.3 | -18.9 | -26.5 | -28.5 |       |       |       |       |       |       |
| Malate                             | +0.4 | +1.3  | +4.7  | +5.8  | +5.9  | -13.6 | -21.8 | -27.2 | -29.3 | -31.7 | -30.1 |
| Metabolites found<br>(μMoles/cup): |      |       |       |       |       |       |       |       |       |       |       |
| PEP + PGA                          | 0.01 | 0.01  | 0.45  | 4.98  | 7.46  | 1.81  | 8.16  | 8.38  | 9.44  | 11.2  | 11.4  |
| ATP                                | 0.80 | 2.57  | 3.04  | 3.34  | 3.48  | 1.30  | 2.70  | 3.40  | 3.34  | 3.41  | 3.29  |
| ADP                                | 2.58 | 1.12  | 0.78  | 0.66  | 0.48  | 2.05  | 0.85  | 0.33  | 0.37  | 0.34  | 0.40  |



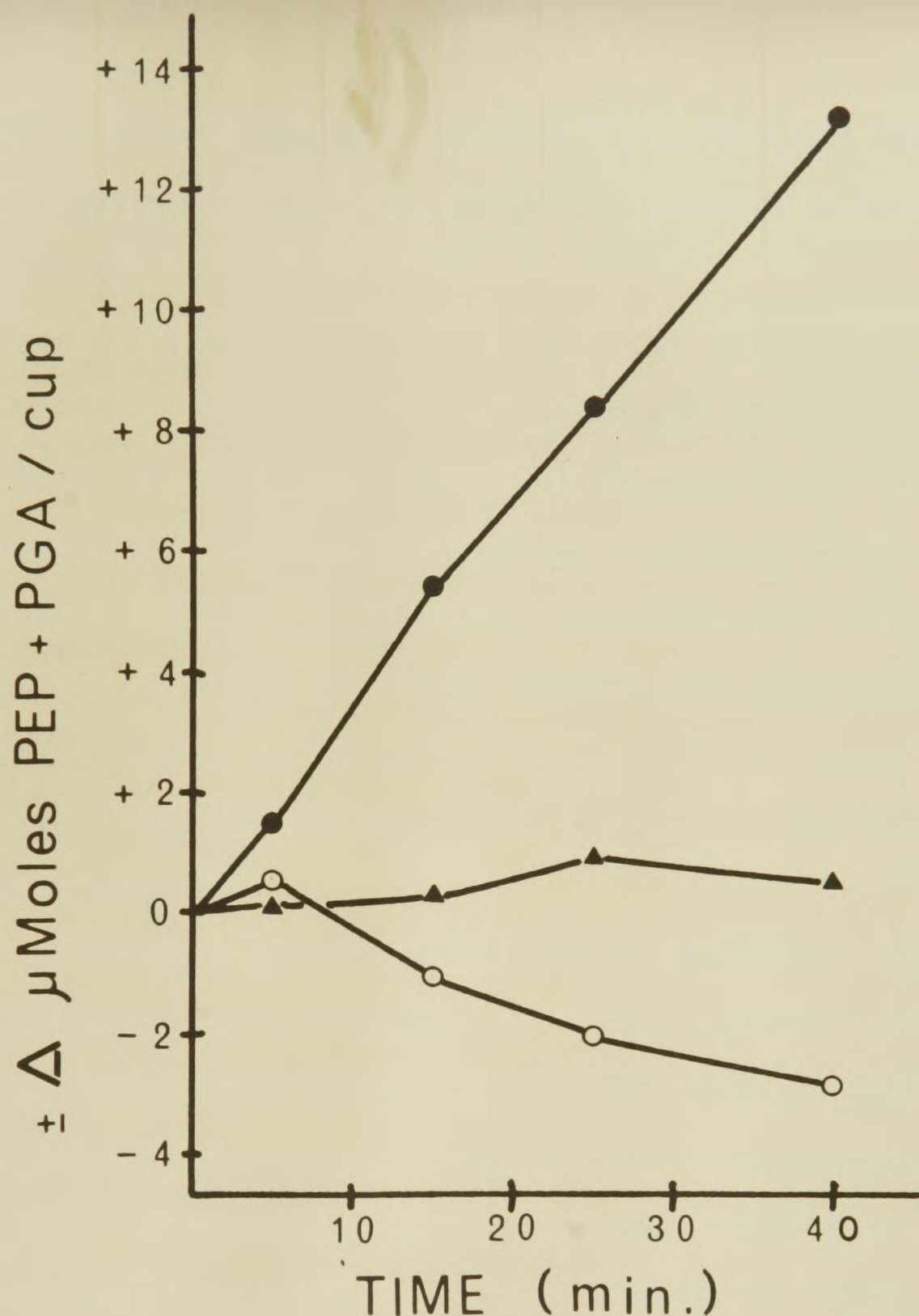


Fig. 13. Effect of  $Mg^{2+}$  and  $Ca^{2+}$  addition on PEP accumulation from malate in pigeon-liver mitochondria.

Mitochondria were prepared as described in METHODS. They were incubated (20 mg. dry wt./cup) at  $30^{\circ}$  in a medium containing 15 mM Pi (pH 7.4), 2 mM ADP, 2.5 mM PEP and isotonic KCl, with additions as indicated. At the times indicated, incubations were terminated and the phosphorylated compounds measured in the neutral extracts. ● No added divalent ions; ▲ + 2.5 mM  $Ca^{2+}$ ; ○ + 2.5 mM  $Mg^{2+}$ .



with PEP). It was tentatively concluded that PK contamination (Table 18) was responsible for the failure of PEP to accumulate in the presence of  $Mg^{2+}$  and ADP.

Added adenine nucleotides (ATP or ADP) were required for maximal accumulation of PEP from all substrates tested. The roles of added  $Mg^{2+}$ ,  $Ca^{2+}$ , Pi and adenine nucleotides were examined in detail (see below). The standard incubation medium for synthesis of PEP from malate, oxaloacetate, fumarate, succinate,  $\alpha$ -oxo-glutarate, glutamate and citrate contained 15 mM Pi, 1 - 2 mM ADP, 10 - 15 mM substrate and isotonic KCl, all at pH 7.4; in the case of all substrates excepting malate and oxaloacetate, 0.625 mM  $Mg^{2+}$  was included to increase the rate of oxidation, which was otherwise very slow.

As found by Bartley (1954,b) PEP was formed from pyruvate only when  $HCO_3^-$  ions were also added; 4 - 8 mM were optimal, since at 25 mM  $HCO_3^-$  the formation of PEP was depressed, although the rate of oxidation was increased (Table 31).  $HCO_3^-$  had no effect on the synthesis of PEP from malate.  $K^+$  ions were required for maximal activity,  $Na^+$  did not appear to be essential but was usually present. The formation of PEP from malate was proportional to the concentration of mitochondria in the range 10 - 40 mg./cup (Fig. 14). This finding is in contrast to that of Mendicino & Utter (1962) who included 5 mM  $Mg^{2+}$  in their incubation medium. Results with mitochondria from livers of fed and starved pigeons were qualitatively and quantitatively similar.

A number of functional properties of the mitochondria were



**Table 31.** Effect of bicarbonate concentration on the formation of PEP from pyruvate in pigeon-liver mitochondria.

Mitochondria (15 mg. dry wt./cup) were prepared and incubated for 30 min. at 30°. The medium contained 15 mM Pi, pH 7.4, 1 mM ATP and 10 mM pyruvate. In all the cups excepting No. 4, NaOH was present in the centre wells.

| No.                                  | 1     | 2     | 3     | 4     | 5     |
|--------------------------------------|-------|-------|-------|-------|-------|
| HCO <sub>3</sub> concentration (mM): | 0     | 1     | 4     | 8     | 25    |
| Metabolic changes<br>(μMoles/cup):   |       |       |       |       |       |
| PEP + PGA                            | +2.15 | +3.75 | +5.26 | +5.45 | +3.26 |
| Pyruvate                             | -6.92 | -8.40 | -11.4 | -14.7 | -13.8 |



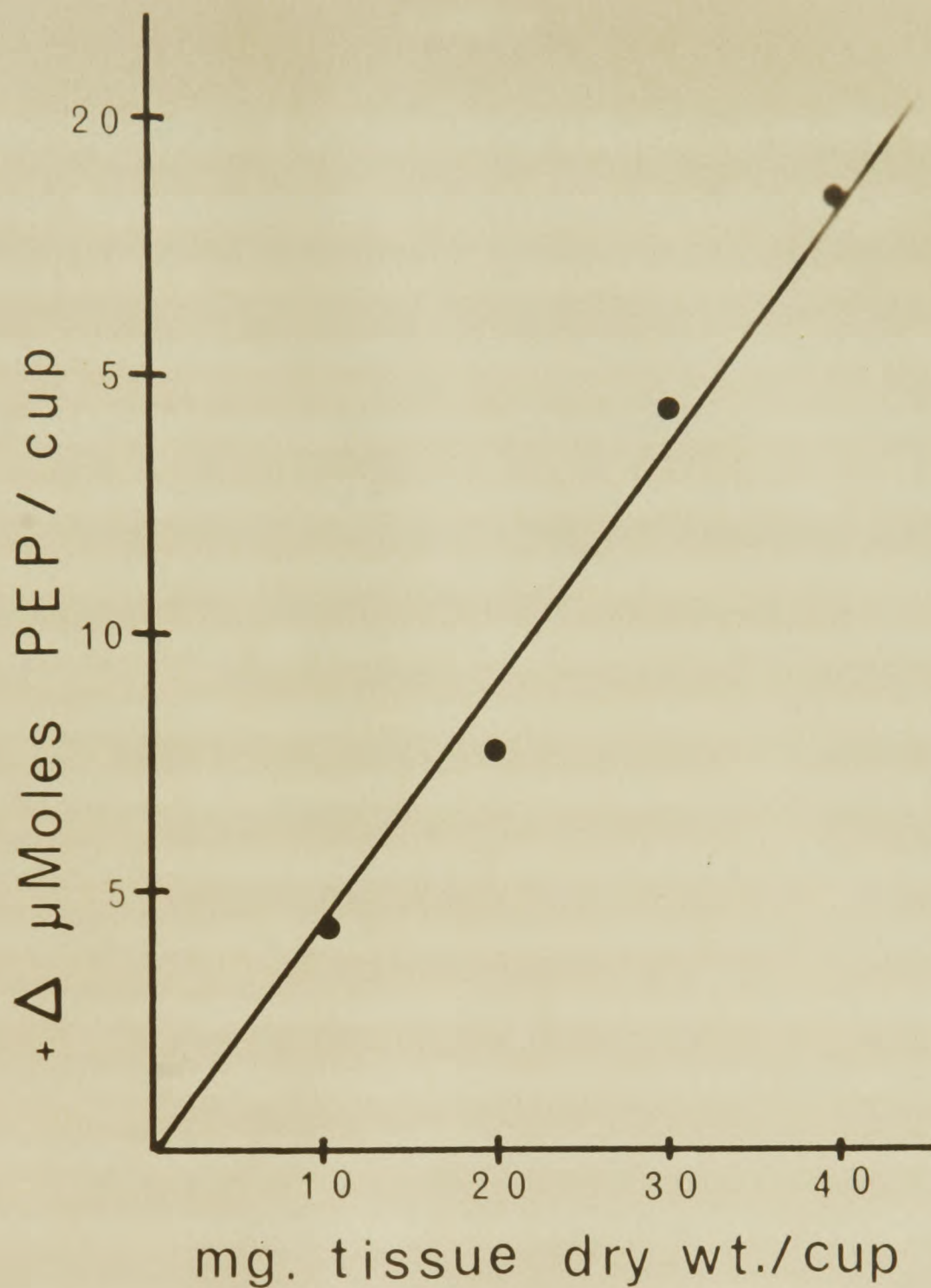


Fig. 14. Effect of mitochondrial concentration on PEP synthesis from pyruvate.

Conditions were exactly as described for Fig. 13 (no  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  ions were added). Incubation was for 25 min. at  $30^{\circ}$ .



investigated in order to assess whether significant damage to membranes or multi-enzyme systems had occurred during preparation or incubation in the above media:

i) Phase contrast microscopy showed that the particles had the spherical appearance characteristic of mitochondria isolated in 0.25M sucrose (see Whittaker, 1966). In this sense they were swollen mitochondria.

ii) The respiratory control ratio (Chance & Williams, 1956) was determined in the high phosphate medium as above, but containing 1.25 mM  $Mg^{2+}$  and no added ADP or ATP. The ratio of the rate of  $O_2$  uptake before and after addition of 1 mM ADP was 5.6 in the case of citrate, 4.5 (glutamate), 3.3 ( $\alpha$ -oxo-glutarate), 3.3 (pyruvate +  $HCO_3^-$ ) and 2.5 (malate). All substrates were at 10 mM. The same results were obtained if the ADP was added after 20 min. of incubation, or if  $Mg^{2+}$  was omitted.

iii) Addition of DNP (an uncoupler of oxidative phosphorylation - Lardy & Wellman, 1952) at  $5 \times 10^{-5}M$  doubled the rate of respiration in the presence of pyruvate, malate or  $\alpha$ -oxo-glutarate, and absence of ADP (see below).

iv) Oligomycin (an inhibitor of respiration coupled to oxidative phosphorylation - Lardy et al., 1958; Huijing & Slater, 1961) at low concentrations inhibited both respiration and substrate removal (see below).

v) Evidence was obtained for an intact "attractyloside-sensitive barrier" in the mitochondria (see below).

vi) The "latency" of the mitochondrial enzymes, such as PEPCK and NuDiKi (see Sections C, E) suggests that the mitochondrial membranes were



not damaged during the fractionation and washing procedures.

From these studies it was concluded that the mitochondria contained intact and well-coupled electron transport and oxidative phosphorylation systems. Since the formation of PEP is an "ATPase" activity, the lower respiratory control ratio in the presence of malate is not unexpected, since this was the best precursor of PEP of the substrates tested (Table 32). Much the same effects were obtained at  $P_i$  concentrations of 5 or 10 mM, in the presence of 'tris' buffer. Addition of EDTA (0.1 - 0.5 mM) as a protective agent against swelling damage (Slater & Cleland, 1952) or of 0.5% bovine serum albumen (Wojtczak & Lehninger, 1961) did not affect the metabolic activity; at high concentrations (2 mM), EDTA inhibited all parameters.

The distribution of malate and PEP between medium and particles was tested by the simplest procedure available, viz. rapid centrifugation in the cold (2500g for 5 min.) at the end of an incubation with pyruvate as substrate. Both metabolites appeared to be quite evenly distributed, although the hypoxic period may have caused permeability barriers to break down. The absence of enolase from pure washed mitochondria suggests that PEP must be the metabolite transferred during gluconeogenesis from the matrix space to the cytosol.

#### 4. PEP synthesis from malate and oxaloacetate.

These two substrates gave the highest rates of PEP accumulation under optimal conditions of all substrates tested (Table 32). In the absence of added  $Mg^{2+}$ , but presence of added ADP or ATP, between 60 and



**Table 32.** Formation of PEP from various substrates in pigeon-liver mitochondria under optimal conditions.

Mitochondria were prepared as in METHODS and incubated for 30 min. at 30° in a medium containing 10 mM substrate as indicated, 15 mM Pi, pH 7.4, 1 mM ADP and (in the case of all substrates except malate and oxaloacetate), 0.625 mM  $Mg^{2+}$ . Tissue dry wt. was 26 mg./cup.

| No. | Substrate               | PEP formed ( $\mu$ Moles/cup) |
|-----|-------------------------|-------------------------------|
| 1.  | Oxaloacetate            | 20.2                          |
| 2.  | Malate                  | 20.7                          |
| 3.  | Fumarate                | 16.7                          |
| 4.  | Succinate               | 7.69                          |
| 5.  | $\alpha$ -Oxo-glutarate | 6.95                          |
| 6.  | Citrate                 | 5.71                          |
| 7.  | Pyruvate                | 7.38                          |
| 8.  | Pyruvate + 4 mM $HCO_3$ | 12.8                          |



80% of the substrate removed was converted into PEP. This was about a quarter of the maximal velocity of PEPCK when assayed after sonication in the same particles (direction of PEP synthesis). The  $O_2$  uptake declined progressively during the incubation, but in the case of malate it was at all times sufficient for the conversion of malate into oxaloacetate before phosphorylation. The ATP/ADP ratio was high (about 5) even when large quantities of ADP were added. Small amounts of citrate and  $\alpha$ -oxo-glutarate were formed. The amounts of endogenous non-adenosine nucleotides were so small that no accurate measurements could be made. Omission of added adenine nucleotides decreased PEP formation by 33 - 50%; maximal rates were already obtained at 1 - 2 mM added ADP or ATP, but higher concentrations (5 mM) were not inhibitory (see Table 34).

Addition of  $Mg^{2+}$  (2.5 mM) in a series of experiments decreased the accumulation of PEP from malate in the presence of adenine nucleotides by 50 - 80%; the  $O_2$  uptake was increased, as well as the removal of malate and the formation of citrate and  $\alpha$ -oxo-glutarate. The ATP/ADP ratio was about 1, and significant quantities of AMP accumulated.  $Mg^{2+}$  addition had little effect in the absence of added adenine nucleotides. The oxaloacetate concentration was increased 3 - 4 times by the addition of ATP or ADP, independent of the presence of  $Mg^{2+}$ .

Some of the effects described above are illustrated in the experiment recorded in Table 33. The data suggest that contaminant PK was activated by the presence of added  $Mg^{2+}$  and by ADP, added as such or formed from ATP. The activity of PK in the particles was sufficient to



**Table 33.** Effects of added ATP, ADP and  $Mg^{2+}$  on the formation of PEP from malate in pigeon-liver mitochondria.

Mitochondria were prepared as described in METHODS, and were incubated under air at  $30^{\circ}$  for 30 min. The medium contained 15 mM Pi, pH 7.4, and additions as indicated. Tissue dry wt. was 22 mg./cup.

| No.                                      | 1     | 2     | 3     | 4     | 5     | 6     |
|------------------------------------------|-------|-------|-------|-------|-------|-------|
| Additions (mM):                          |       |       |       |       |       |       |
| Malate                                   | 15    | 15    | 15    | 15    | 15    | 15    |
| ATP                                      |       | 1.25  |       |       | 1.25  |       |
| ADP                                      |       |       | 1.25  |       |       | 1.25  |
| $Mg^{2+}$                                |       |       |       | 2.5   | 2.5   | 2.5   |
| Metabolic changes<br>( $\mu$ Moles/cup): |       |       |       |       |       |       |
| $O_2$                                    | -8.4  | -13.0 | -10.4 | -8.6  | -22.0 | -18.1 |
| Malate                                   | -20.6 | -25.2 | -22.6 | -21.0 | -30.1 | -29.4 |
| Compounds found<br>( $\mu$ Moles/cup):   |       |       |       |       |       |       |
| PEP + PGA                                | 11.6  | 17.7  | 14.8  | 11.8  | 9.2   | 8.2   |
| Oxaloacetate                             | 0.13  | 0.54  | 0.39  | 0.09  | 0.44  | 0.47  |
| $\alpha$ -Oxo-glutarate                  | 1.12  | 1.38  | 1.68  | 0.99  | 3.53  | 3.50  |
| Citrate                                  | 1.36  | 1.51  | 1.59  | 1.19  | 3.69  | 4.04  |
| ATP                                      | 0.15  | 4.27  | 3.78  | 0.06  | 1.99  | 1.53  |
| ADP                                      | 0.12  | 0.77  | 0.65  | 0.10  | 1.51  | 1.67  |
| AMP                                      | 0.01  | 0.04  | 0.03  | 0.01  | 0.67  | 0.64  |



account for the difference observed (Section D). However, the failure of the mitochondria to maintain ATP in the presence of  $Mg^{2+}$ , ADP and malate (in agreement with Bartley, 1955) cannot be explained on this basis, since no net loss of  $\Delta P$  occurs in the sequence of reactions: oxaloacetate  $\rightarrow$  PEP  $\rightarrow$  pyruvate. Freshly-prepared liver mitochondria are thought to contain little  $Mg^{2+}$ -activated ATPase (Kielley & Kielley, 1953) but the simplest explanation of the data is that the fall in ATP was due to the combined action of  $Mg^{2+}$ -activated adenylate kinase and ATPase, both outside the inner mitochondrial membrane (Klingenberg & Pfaff, 1966). The experiments do not indicate whether the fall in "phosphorylation potential" decreased the rate of synthesis of PEP.

The results with oxaloacetate as substrate were very similar. In both cases the  $O_2$  uptake was low, presumably because of the absence of effective precursors of acetyl CoA for citrate formation. Since efforts to use fatty acids for this purpose failed (see below), pyruvate was added with malate to saturate the tricarboxylic acid cycle. The results of an experiment, broadly corresponding to that described in Table 33, but involving the addition of both pyruvate and malate or oxaloacetate, are recorded in Table 34. This shows the marked effect of  $Mg^{2+}$  in reducing both the ATP/ADP ratio, especially at high ADP concentrations, and the accumulation of PEP. Pyruvate addition thus did not ameliorate the failure of malate and oxaloacetate to maintain ATP in the presence of  $Mg^{2+}$  ions. The data show that more citrate and  $\alpha$ -oxo-glutarate was formed in the presence of a depressed "phosphorylation potential" or ATP level; the



**Table 34.** Effects of added ADP concentration on PEP formation from malate (or oxaloacetate) + pyruvate in the presence and absence of  $Mg^{2+}$ .

Mitochondria were prepared and incubated at  $30^{\circ}$  for 25 min. under air, as described in METHODS. The medium contained 15 mM Pi, pH 7.4, and 4 mM  $HCO_3^-$ . Tissue dry wt. was 29 mg./cup.

| No.                                    | 1     | 2     | 3     | 4     | 5    | 6    | 7    | 8    | 9     | 10   | 11   | 12   |
|----------------------------------------|-------|-------|-------|-------|------|------|------|------|-------|------|------|------|
| Additions (mM):                        |       |       |       |       |      |      |      |      |       |      |      |      |
| ADP                                    | 1     | 1     | 2.5   | 5     |      | 1    | 2.5  | 5    |       | 2.5  |      | 2.5  |
| Malate                                 | 10    | 10    | 10    | 10    | 10   | 10   | 10   | 10   |       |      |      |      |
| Oxaloacetate                           |       |       |       |       |      |      |      |      | 10    | 10   | 10   | 10   |
| Pyruvate                               | 10    | 10    | 10    | 10    | 10   | 10   | 10   | 10   | 10    | 10   | 10   | 10   |
| $Mg^{2+}$                              |       |       |       |       | 2.5  | 2.5  | 2.5  | 2.5  |       |      | 2.5  | 2.5  |
| Compounds found<br>( $\mu$ Moles/cup): |       |       |       |       |      |      |      |      |       |      |      |      |
| PEP + PGA                              | 9.95  | 27.3  | 25.2  | 23.8  | 10.1 | 12.7 | 6.34 | 5.10 | 18.2  | 26.6 | 16.6 | 2.94 |
| Oxaloacetate                           | 0.03  | 0.07  | 0.20  | 0.35  | 0.06 | 0.29 | 0.34 | 0.30 |       |      |      |      |
| Citrate                                | 2.36  | 2.40  | 2.17  | 4.48  | 2.10 | 5.90 | 5.66 | 5.61 | 1.65  | 3.40 | 6.00 | 9.72 |
| $\alpha$ -Oxo-glutarate                | 1.70  | 3.20  | 4.92  | 11.7  | 1.72 | 12.0 | 11.5 | 10.4 | 3.90  | 3.30 | 3.96 | 7.45 |
| ATP                                    | 0.08  | 3.76  | 9.08  | 18.0  | 0.05 | 2.57 | 4.34 | 5.00 | 0.06  | 8.65 | 0.04 | 1.11 |
| ADP                                    | 0.04  | 0.25  | 0.76  | 0.68  | 0.08 | 1.33 | 3.20 | 5.55 | 0.04  | 0.68 | 0.06 | 2.78 |
| AMP                                    | <0.01 | <0.01 | <0.01 | <0.01 | 0.02 | 0.08 | 0.74 | 6.00 | <0.01 | 0.17 | 0.02 | 4.22 |

Metabolic changes  
( $\mu$ Moles/cup):

|          |       |       |       |       |       |       |       |       |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|
| Malate   | -12.0 | -20.4 | -23.4 | -25.2 | -12.4 | -25.3 | -25.0 | -20.0 |
| Pyruvate | -9.72 | -14.9 | -19.3 | -31.8 | -9.5  | -31.6 | -29.7 | -29.6 |



exception (cup 4) may have been due to the occurrence of high ADP concentrations during part of the incubation, before the 20  $\mu$ Moles of ADP could be phosphorylated. These findings support the postulate of Hathaway & Atkinson (1965) and Shepherd & Garland (1966) that high ATP/ADP ratios may inhibit the activity of citrate synthase, since the oxaloacetate concentrations were in the same range in both series.

An experiment designed to demonstrate whether the non-accumulation of PEP in the presence of  $Mg^{2+}$  was due entirely to PK activity or also to inhibition of synthesis via PEPCK, is recorded in Table 35. Low concentrations of  $Ca^{2+}$  ions (0.5 mM) simulated the effects of  $Mg^{2+}$  with respect to the stimulation of  $O_2$  uptake, increased removal of added substrates, inhibition of PEP accumulation and interference with the maintenance of ATP. Although this concentration of  $Ca^{2+}$  does not inhibit PK to a significant degree (Kachmar & Boyer, 1953), the absence of  $Mg^{2+}$  is markedly inhibitory. PK was assayed in the particle suspension in the absence of added  $Mg^{2+}$  and no activity was found. Under these conditions it may be concluded that the fall in "phosphorylation potential" was responsible for the inhibition of PEP formation, and for the increased synthesis of citrate and  $\alpha$ -oxo-glutarate. In the presence of  $Mg^{2+}$  both mechanisms operate simultaneously. Higher concentrations of  $Ca^{2+}$  grossly inhibited all parameters measured.

At  $4 \times 10^{-5}M$ , DNP inhibited PEP formation from malate and oxaloacetate in the absence of  $Mg^{2+}$  (Table 36, cups 1 - 4). This was accompanied by a fall in ATP and an increase in the formation of citrate.



**Table 35.** Effects of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions on the formation of PEP from pyruvate + Malate in pigeon-liver mitochondria.

Conditions were exactly as described for Table 15. Tissue dry wt. was 24.6 mg./cup.

| No.                                             | 1     | 2     | 3     | 4     | 5     | 6     |
|-------------------------------------------------|-------|-------|-------|-------|-------|-------|
| Additions (mM):                                 |       |       |       |       |       |       |
| Pyruvate                                        | 10    | 10    | 10    | 10    | 10    | 10    |
| Malate                                          | 10    | 10    | 10    | 10    | 10    | 10    |
| ADP                                             |       | 2     |       | 2     |       | 2     |
| $\text{Mg}^{2+}$                                |       |       | 2.5   | 2.5   |       |       |
| $\text{Ca}^{2+}$                                |       |       |       |       | 0.5   | 0.5   |
| Compounds found<br>( $\mu\text{Moles/cup}$ ):   |       |       |       |       |       |       |
| PEP + PGA                                       | 11.8  | 24.7  | 9.1   | 7.4   | 10.4  | 11.4  |
| Oxaloacetate                                    | 0.04  | 0.18  | 0.12  | 0.31  | 0.10  | 0.24  |
| Citrate                                         | 1.28  | 1.08  | 1.42  | 8.00  | 1.21  | 5.82  |
| $\alpha$ -Oxo-glutarate                         | 1.33  | 1.24  | 1.12  | 8.59  | 1.00  | 4.39  |
| ATP                                             | 0.04  | 7.06  | 0.08  | 5.22  | 0.06  | 4.95  |
| ADP                                             | 0.08  | 0.74  | 0.07  | 1.84  | 0.06  | 1.82  |
| AMP                                             | 0.01  | 0.03  | 0.03  | 0.48  | 0.01  | 0.58  |
| Metabolic changes<br>( $\mu\text{Moles/cup}$ ): |       |       |       |       |       |       |
| Pyruvate                                        | -4.2  | -11.5 | -5.7  | -35.4 | -4.6  | -20.1 |
| Malate                                          | -16.1 | -22.9 | -16.0 | -21.0 | -14.8 | -19.5 |
| $\text{O}_2$                                    | -6.1  | -9.65 | -5.95 | -28.0 | -6.3  | -20.0 |



**Table 36.** Effects of DNP and Oligomycin on PEP formation from malate and oxaloacetate in pigeon-liver mitochondria.

Mitochondria were prepared and incubated for 30 min. exactly as described for Table 34. Tissue dry wt. was 18 mg./cup.

| No.                                                   | 1     | 2     | 3    | 4     | 5     | 6    |
|-------------------------------------------------------|-------|-------|------|-------|-------|------|
| <b>Additions (mM):</b>                                |       |       |      |       |       |      |
| Malate                                                | 15    | 15    |      |       | 15    |      |
| Oxaloacetate                                          |       |       | 15   | 15    |       | 15   |
| DNP                                                   |       | 0.04  |      | 0.04  |       |      |
| Oligomycin ( $\mu$ g.)                                |       |       |      |       | 5     | 5    |
| ADP                                                   | 1.25  | 1.25  | 1.25 | 1.25  | 1.25  | 1.25 |
| <b>Compounds formed (<math>\mu</math>Moles/cup):</b>  |       |       |      |       |       |      |
| PEP + PGA                                             | 14.8  | 4.51  | 19.0 | 3.72  | 1.85  | 1.90 |
| Oxaloacetate                                          | 0.60  | 0.37  |      |       | 0.39  |      |
| Citrate                                               | 1.12  | 3.36  | 2.00 | 4.16  | 0.92  | 1.40 |
| $\alpha$ -Oxo-glutarate                               | 1.68  | 4.32  | 1.15 | 5.25  | 1.12  | 0.96 |
| ATP                                                   | 3.78  | 1.40  | 3.97 | 1.25  | 0.04  | 0.08 |
| ADP                                                   | 0.65  | 2.65  | 0.54 | 3.04  | 0.54  | 0.74 |
| AMP                                                   | 0.03  | 0.38  | 0.06 | 0.50  | 3.36  | 3.20 |
| <b>Metabolic changes (<math>\mu</math>Moles/cup):</b> |       |       |      |       |       |      |
| Malate                                                | -24.6 | -28.7 | +5.1 | +3.9  | -13.9 | +5.4 |
| O <sub>2</sub>                                        | -9.4  | -16.5 | -5.7 | -10.8 | -4.6  | -3.1 |



Oligomycin (0.3  $\mu\text{g.}/\text{mg.}$  mitochondrial dry wt.) was also very inhibitory (cups 5 - 6) but in this case the  $\text{O}_2$  uptake and substrate removal were diminished. The same effects were obtained at oligomycin concentrations as low as 0.1  $\mu\text{g.}/\text{mg.}$

From these experiments it was concluded that PEP synthesis in intact mitochondria is sensitive to regulation by the "phosphorylation potential" of the mitochondrial adenine nucleotides, such that high ATP/ADP ratios favour PEP formation while at the same time inhibiting citrate formation and enhancing the activity of pyruvate carboxylase (see below). It was decided to examine more closely the functional localization of PEPCK in relation to its energy supply.

Atractyloside is an inhibitor of mitochondrial reactions involving added or "unbound" adenine nucleotides, and its action may be that of an inhibitor, competitive with adenine nucleotides, of a specific "translocase" catalysing the exchange of "bound" and exogenous adenine nucleotides (see DISCUSSION). This adenine nucleotide-specific enzyme may constitute the site of reaction between the extra- and intra-mitochondrial pools of nucleotides in the intact cell, since guanosine nucleotides are not able to exchange in this manner in intact mitochondria (Klingenberg & Pfaff, 1966). Experiments were carried out with atractyloside (100  $\mu\text{M}$ , in the presence of 15 - 17 mg. dry wt. of particles) to define the location of PEPCK and NuDiKi in relation to this "translocase" (Table 37). Oxaloacetate was used as substrate in order to saturate PEPCK with this <sup>absolute</sup> substrate (no diffusion barriers to oxaloacetate appeared to exist).



**Table 37.** Effects of atractyloside on PEP synthesis from oxaloacetate in pigeon-liver mitochondria.

Mitochondria were prepared and incubated for 10 min. under air, at 20.5°, as described in METHODS. The medium contained 15 mM Pi, pH 7.4, and 10 mM oxaloacetate. The concentrations of additions were as follows: ATP, 1.5 mM; GTP, 0.4 mM; KCN, 5 mM; Oligomycin, 10 µg./cup; atractyloside, 100 µM. Tissue dry wt. was 17 mg./cup.

| No. | Addition                         | PEP + PGA formed<br>(µMoles/cup) |
|-----|----------------------------------|----------------------------------|
| 1.  | none                             | 2.52                             |
| 2.  | ATP                              | 3.35                             |
| 3.  | Atractyloside                    | 2.42                             |
| 4.  | ATP + atractyloside              | 2.47                             |
| 5.  | KCN                              | 0.11                             |
| 6.  | KCN + ATP                        | 0.92                             |
| 7.  | KCN + ATP + atractyloside        | 0.12                             |
| 8.  | Oligomycin                       | 0.50                             |
| 9.  | Oligomycin + ATP                 | 1.38                             |
| 10. | Oligomycin + ATP + atractyloside | 0.52                             |
| 11. | GTP                              | 2.36                             |
| 12. | GTP + atractyloside              | 2.41                             |



The stimulation of PEP formation by added ATP was eliminated by atractyloside (cups 2 and 4), which did not affect the ATP<sup>in</sup>-dependent phosphorylation (cups 1 and 3). PEP synthesis was totally prevented when 4 mM KCN was added to suppress endogenous ATP synthesis; atractyloside again eliminated the PEP formation that occurred in the presence of KCN + ATP (cups 5, 6 and 7). The same result was obtained when oligomycin was added, both to prevent endogenous ATP synthesis and to inhibit the hydrolysis of added ATP (cups 8, 9 and 10). Added GTP or ITP did not affect the rate of PEP formation, nor was there an effect of atractyloside (cups 11 and 12). PEPCK and NuDiKi both appear to be located within the atractyloside-sensitive barrier; this is confirmed by the fact that no added metal ions were required for PEP synthesis in situ, whereas the solubilized PEPCK has an absolute requirement for  $Mn^{2+}$  or  $Mg^{2+}$ . Endogenous "bound" metals are presumably involved (Klingenberg & Pfaff, 1966).

The inhibition of PEP synthesis from malate by oligomycin (Table 36) shows that high-energy intermediates of oxidative phosphorylation cannot be used to energize the formation of PEP. From this, and from the effects of DNP and of added adenine nucleotides in the absence of  $Mg^{2+}$ , it may be concluded that the immediate energy donor for PEP synthesis from malate and oxaloacetate was ATP, derived either from oxidative phosphorylation of endogenous or added ADP, or from added ATP itself. The rate of substrate-level phosphorylation during the standard incubations, when the great bulk of substrate was converted into PEP and  $\alpha$ -oxo-glutarate formation was very limited, could not have been sufficient to supply the



GTP required. This suggests that the results of Bartley (1955) with regard to the kinetics of incorporation of  $^{32}\text{Pi}$  into ATP and PEP during PEP formation from malate may have been influenced by compartmentation of adenine nucleotide pools, although this would not explain why the  $^{32}\text{Pi}$  incorporation pattern was different during PEP formation from  $\alpha$ -oxo-glutarate (Bartley, 1954). It is possible that the difference in the true net rates of synthesis from the two substrates may account for the discrepancy; another possibility is that hitherto undiscovered subtleties may exist in the nucleotide specificity of certain reactions of oxidative phosphorylation (see Low, et al., 1963).

Oxamate (6 mM) did not inhibit PEP formation from malate or oxaloacetate, in agreement with Seubert & Huth (1965). L-tryptophan also had no effect (see Foster et al., 1966).

Since PEP synthesis from malate and oxaloacetate could thus occur, using GTP supplied via the action of NuDiKi, the question arose whether this was the obligatory pathway of phosphoryl transfer. This is the obverse of the view held by Azzone & Ernster (1961) and Nordlie & Lardy (1963,a) relating to possible compartmentation of energy transfer between succinic thiokinase and PEPCK in rat and guinea-pig liver mitochondria, respectively. PEP synthesis from  $\alpha$ -oxo-glutarate was accordingly examined, in the presence and absence of oxaloacetate or malate.

##### 5. $\alpha$ -Oxo-glutarate as precursor of PEP in pigeon-liver mitochondria.

The oxidation of  $\alpha$ -oxo-glutarate (5 mM) was relatively slow (compared with that of pyruvate) in a phosphate medium containing 1.25 mM



ADP and 0.5 mM or no  $Mg^{2+}$  ions. It was doubled by the addition of  $5 - 10 \times 10^{-5} M$  DNP (Table 38), with a fall in ATP and an increase in the concentrations of both malate and oxaloacetate. PEP formation was decreased.  $\sim P$  synthesized during the stimulated oxidation of  $\alpha$ -oxo-glutarate in the presence of DNP was thus available to the DNP-activated ATPase via the NuDiKi reaction. When ADP was omitted, as in the experiments of Stanbury & Mudge (1954), the rates were lower, but a biphasic pattern became discernible. This difference may be explained in terms of competition between added ADP and oxaloacetate for GTP. When malate was incubated with  $\alpha$ -oxo-glutarate and ADP, in the presence of increasing concentrations of DNP, such that  $\alpha$ -oxo-glutarate oxidation was increased, there was only a slight stimulation of PEP formation over that in the presence of malate alone (Table 39).

These results suggest that no functional relationship between the substrate-level phosphorylation and PEPCK exists in pigeon-liver mitochondria, and that the nucleotides are able freely to interact in the mitochondrial matrix. According to Klingenberg (1964), the oxidative phosphorylation system in intact mitochondria is in a state of dynamic equilibrium, such that the true measure of the "energy status", and the determinant of the respiratory rate, is not the absolute concentration of ATP but the "phosphorylation potential" or  $[ATP] / [ADP] \times [P_i]$ . This may well explain the effect of  $P_i$  on the formation of PEP from malate and pyruvate, since increasing the  $P_i$  concentration would tend to be associated with increasing ATP/ADP ratios. Reference to Table 30 shows that this was in fact the



**Table 38.** Effects of DNP on the formation of PEP from  $\alpha$ -oxo-glutarate in pigeon-liver mitochondria.

Mitochondria were prepared and incubated for 30 min. at 30° under air, as in METHODS. The medium contained 15 mM Pi, pH 7.4, 0.625 mM  $Mg^{2+}$  and 10 mM  $\alpha$ -oxo-glutarate. Tissue dry wt. was 20 mg./cup.

| No.                                      | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
|------------------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Additions (mM):                          |       |       |       |       |       |       |       |       |
| ADP                                      | 1.25  | 1.25  | 1.25  | 1.25  |       |       |       |       |
| DNP                                      |       | 0.02  | 0.05  | 0.10  |       | 0.02  | 0.05  | 0.10  |
| Compounds found<br>( $\mu$ Moles/cup):   |       |       |       |       |       |       |       |       |
| PEP + PGA                                | 3.39  | 4.37  | 1.93  | 0.88  | 2.25  | 2.64  | 1.02  | 1.59  |
| Oxaloacetate                             | 0.06  | 0.14  | 0.24  | 0.20  |       |       |       |       |
| Malate                                   | 0.23  | 0.47  | 2.42  | 1.40  | 0.06  | 0.16  | 1.43  | 1.67  |
| ATP                                      | 4.09  | 4.03  | 1.33  | 0.10  |       |       |       |       |
| ADP                                      | 0.85  | 0.95  | 2.82  | 1.10  |       |       |       |       |
| Metabolic changes<br>( $\mu$ Moles/cup): |       |       |       |       |       |       |       |       |
| $\alpha$ -Oxo-glutarate                  | -5.02 | -6.42 | -10.9 | -10.9 | -7.34 | -9.91 | -4.94 | -6.12 |
| O <sub>2</sub>                           | -7.05 | -9.4  | -15.8 | -16.1 |       |       |       |       |



Table 39. Effect of  $\alpha$ -oxo-glutarate and DNP addition on the formation of PEP from malate in pigeon-liver mitochondria.

Mitochondria were prepared and incubated at 30° for 30 min. as in METHODS. The gas phase was O<sub>2</sub>. The medium contained 15 mM Pi, pH 7.4, 0.625 mM Mg<sup>2+</sup>, 4 mM HCO<sub>3</sub><sup>-</sup>, and 1 mM ATP. The dry wt. was 31 mg/cup.

| No.                                      | 1     | 2     | 3                | 4     | 5     | 6     | 7     | 8     | 9     |
|------------------------------------------|-------|-------|------------------|-------|-------|-------|-------|-------|-------|
| Additions (mM):                          |       |       |                  |       |       |       |       |       |       |
| Malate                                   | 10    | 10    | 10               |       |       |       | 10    | 10    | 10    |
| $\alpha$ -Oxo-glutarate                  |       |       |                  | 5     | 5     | 5     | 5     | 5     | 5     |
| DNP                                      |       | 0.05  | 0.1              |       | 0.05  | 0.1   |       | 0.05  | 0.1   |
| Metabolic changes<br>( $\mu$ Moles/cup): |       |       |                  |       |       |       |       |       |       |
| PEP + PGA                                | +23.1 | +6.71 | +3.32            | +8.00 | +2.25 | +1.7  | +27.2 | +8.04 | +4.77 |
| O <sub>2</sub>                           | -17.4 | -32.6 | <del>-30.4</del> | -19.7 | -34.7 | -29.1 | -21.6 | -28.2 | -34.9 |



case, although the causal relationship is not obligatory.

It is difficult to reconcile this view of the integrated control of PEP synthesis by nucleotides, with the results of Nordlie & Lardy (1963,a) where DNP or a glucose-hexokinase trap (both agents likely to depress the ATP/ADP ratio) increased the formation of PEP from  $\alpha$ -oxo-glutarate in guinea-pig liver mitochondria in the absence of added adenine nucleotides. This implies either that compartmentation of nucleotides occurred or that there may have been disequilibrium in the NuDiKi system under these conditions. The latter could have been due to inhibition of NuDiKi by ADP (see Section E) but this would require that addition of adenine nucleotides should exaggerate the effect. This was not so (Table 38). Unfortunately, these authors did not supply details of the substrate balance in their experiments, which would have permitted calculation of what proportion of GTP molecules was used for PEP formation. As previously mentioned, such calculations fail to take into account the turnover of PEP in the presence of added  $Mg^{2+}$  ions. It is thus possible that species differences account for these discrepancies. M.B. Wilson (personal communication) has observed a 5 - 7-fold increase in PEP synthesis from citrate or  $\alpha$ -oxo-glutarate in guinea-pig liver mitochondria when uncoupling concentrations of palmitate (0.75 mM) were added.

#### 6. PEP synthesis from pyruvate in pigeon-liver mitochondria.

In the presence of 20 mM Pi, 4 mM  $HCO_3^-$  and 0.5 mM ATP, and in the absence of  $Mg^{2+}$ , PEP was formed from pyruvate at rates about half of those attained in the presence of malate (Table 40). About 50% of pyruvate



**Table 40. Effects of ATP, ADP,  $Mg^{2+}$  and DNP on the formation of PEP from pyruvate in pigeon-liver mitochondria.**

Mitochondria were prepared and incubated for 30 min. at  $30^{\circ}$  under  $O_2$ , as described in METHODS. The medium contained 20 mM Pi, pH 7.4, 4 mM  $HCO_3^-$  and 10 mM pyruvate. Tissue dry wt. was 28 mg./cup.

| No.                                      | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    |
|------------------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Additions (mM):                          |       |       |       |       |       |       |       |       |       |       |       |       |
| ATP                                      |       |       | 0.5   |       |       | 0.5   |       |       | 0.5   | 0.5   |       |       |
| ADP                                      |       |       |       | 2.5   |       |       | 2.5   |       |       |       | 2.5   | 2.5   |
| DNP                                      |       |       |       |       | 0.05  |       |       | 0.05  | 0.05  | 0.05  | 0.05  | 0.05  |
| $Mg^{2+}$                                |       | 2.5   |       |       |       | 2.5   | 2.5   | 2.5   |       | 2.5   |       | 2.5   |
| Compounds found<br>( $\mu$ Moles/cup):   |       |       |       |       |       |       |       |       |       |       |       |       |
| PEP + PGA                                | 5.46  | 4.73  | 12.6  | 12.2  | 0.39  | 4.00  | 1.74  | 0.01  | 0.22  | 0.02  | 0.04  | 0.01  |
| Oxaloacetate                             | 0.10  | 0.10  | 0.19  | 0.14  | 0.01  | 0.17  | 0.11  | 0.01  | 0.09  | 0.09  | 0.09  | 0.07  |
| Malate                                   | 0.64  | 0.62  | 1.91  | 3.90  | 0.18  | 3.73  | 1.89  | 0.10  | 0.47  | 0.20  | 0.17  | 0.11  |
| $\alpha$ -Oxo-glutarate                  | 1.94  | 1.83  | 2.90  | 4.20  | 1.08  | 3.52  | 2.70  | 0.55  | 1.49  | 1.00  | 0.70  | 0.43  |
| Citrate                                  | 1.24  | 1.30  | 2.24  | 3.14  | 1.12  | 2.54  | 2.24  | 0.76  | 0.99  | 0.85  | 0.65  | 0.52  |
| ATP                                      |       |       | 1.70  | 6.82  |       | 1.40  | 2.77  |       | 0.47  | 0.21  | 0.54  | 0.08  |
| ADP                                      |       |       | 0.65  | 1.38  |       | 0.95  | 2.62  |       | 1.12  | 0.78  | 2.37  | 0.90  |
| AMP                                      |       |       | 0.03  | 0.03  |       | 0.05  | 0.79  |       | 0.40  | 0.84  | 2.85  | 3.80  |
| Metabolic changes<br>( $\mu$ Moles/cup): |       |       |       |       |       |       |       |       |       |       |       |       |
| Pyruvate                                 | -12.7 | -13.3 | -26.5 | -34.4 | -20.1 | -29.6 | -29.0 | -19.5 | -20.4 | -20.6 | -22.1 | -21.9 |
| $O_2$                                    | -5.05 | -5.55 | -14.1 | -15.1 | -14.1 | -21.5 | -22.8 | -13.0 | -20.8 | -19.9 | -18.9 | -15.7 |



removed was converted into PEP. The rate was halved in the absence of added ATP, as was the  $O_2$  uptake, the malate and oxaloacetate concentration and the rate of substrate removal. Addition of 2.5 mM  $Mg^{2+}$  markedly inhibited PEP formation, especially at higher ADP concentrations. The level of oxaloacetate was not changed. There was a significant fall in the ATP/ADP ratio. The effects were similar to those of Table 33, where malate was the substrate.  $5 \times 10^{-5}$  M DNP (in the absence of  $Mg^{2+}$ ) increased the  $O_2$  uptake and the rate of pyruvate removal, but completely prevented the accumulation of PEP whether adenine nucleotides were added or not. The effect of DNP differed from that of  $Mg^{2+}$  in that the formation of malate was inhibited - this was correlated with much lower ATP concentrations.

When pyruvate was incubated with increasing concentrations of DNP, the synthesis of PEP was more readily affected than that of malate (Fig. 15). The data indicate that PEPCK is more sensitive than pyruvate carboxylase to a fall in the "phosphorylation potential". According to Keech & Utter (1963), an ADP/ATP ratio of 10 inhibits pure pyruvate carboxylase by only 46%. Despite the evidence of control by ADP advanced by Berry (1965) for rat liver mitochondria, the twin role of pyruvate carboxylase in both the anabolic and catabolic metabolism of pyruvate may demand that it be less sensitive to shut-down in times of energy deficiency than PEPCK, a purely anabolic enzyme.

Addition of DNP together with pyruvate in the presence and absence of  $\alpha$ -oxo-glutarate gave the same result as that shown for malate in



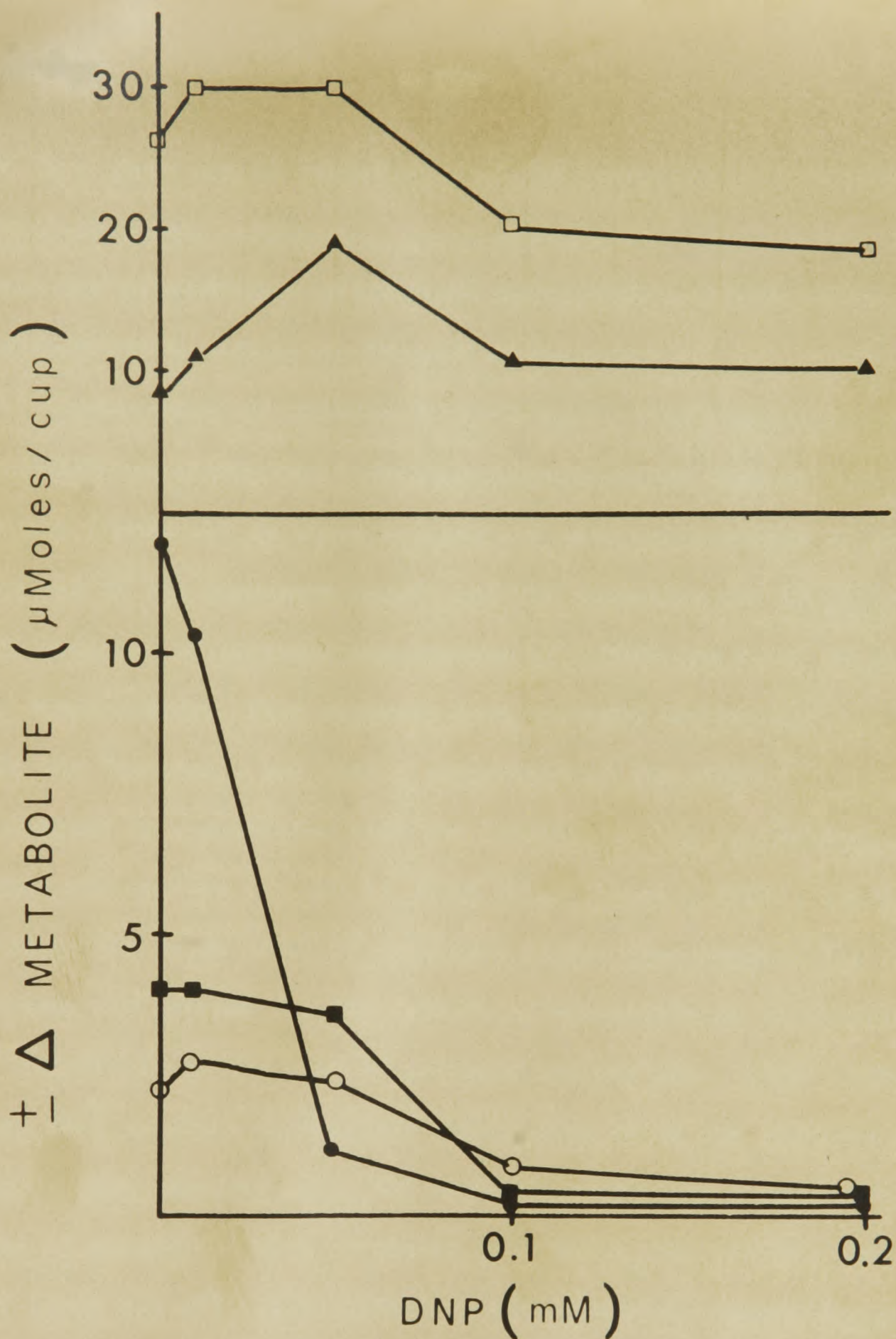


Fig 15. Effect of DNP concentration on metabolism of pyruvate in pigeon-liver mitochondria.

Mitochondria were prepared and incubated under O<sub>2</sub> for 30 min. at 30°, as described in METHODS. The medium contained 15 mM Pi, pH 7.4, 4 mM HCO<sub>3</sub>, 1.25 mM ADP and 10 mM pyruvate. Tissue dry wt. was 24 mg./cup. □—□ Pyruvate removal; ▲—▲ O<sub>2</sub> uptake (both in μMoles/cup); ●—● PEP + PGA, ■—■ ATP, ○—○ Malate (all in μMoles/cup).



Table 39 - there was no stimulation of PEP formation at increased rates of  $\alpha$ -oxo-glutarate oxidation. In agreement with Soubert & Huth (1965), oxamate was an inhibitor of pyruvate carboxylation - both malate and PEP formation were decreased by 75% at 6 mM oxamate.

The results of experiments designed to test whether pyruvate carboxylase was within the "attractyloside-sensitive barrier" were not as clear-cut (Table 41) as those carried out with oxaloacetate as substrate. 51 and 64% of the increased malate and PEP formation due to ATP addition in the presence of pyruvate was removed by 0.0625 and 0.125 mM attractyloside, respectively. This suggests that pyruvate carboxylase was indeed inside the "barrier", but that a proportion of the added ATP was being translocated in competition with the inhibitor. The apparent  $K_m$  of pyruvate carboxylase for ATP is about 30  $\mu$ M (Keech & Utter, 1963), while that of NuDiKi is an order of magnitude higher (see Section E). This difference may account for the failure of attractyloside to inhibit ATP entry completely in the case of pyruvate carboxylase. (This argument assumes that pyruvate carboxylase rather than PEPCK was limiting for PEP synthesis from pyruvate under the standard conditions - see below). The effects of oligomycin were in accord with these conclusions. Added GDP or GTP (both 0.4 mM) had no effect on mitochondrial metabolism, but were hydrolysed slowly during the incubation, such that only half of the original form was left after 30 min.



**Table 41.** Effects of atractyloside, ATP, GTP, GDP and Oligomycin on PEP synthesis from pyruvate in pigeon-liver mitochondria.

Mitochondria were prepared and incubated at 20° for 20 min. under air, as described in METHODS. The medium contained 15 mM Pi, pH 7.4, 4 mM HCO<sub>3</sub> and 10 mM pyruvate. Tissue dry wt. was 21 mg./cup. The concentrations of added reagents were: ATP, 1.4 mM; GTP, 0.4 mM; GDP, 0.4 mM; oligomycin, 10 µg./cup; atractyloside, (as indicated).

| No. | Additions                                    | PEP formed<br>(µMoles/cup) |
|-----|----------------------------------------------|----------------------------|
| 1.  | None                                         | 2.73                       |
| 2.  | ATP                                          | 5.26                       |
| 3.  | Atractyloside (62.5 µM)                      | 2.59                       |
| 4.  | ATP + atractyloside (62.5 µM)                | 3.97                       |
| 5.  | ATP + atractyloside (125 µM)                 | 3.64                       |
| 6.  | Oligomycin                                   | 0.11                       |
| 7.  | ATP + oligomycin                             | 0.95                       |
| 8.  | ATP + oligomycin + atractyloside<br>(125 µM) | 0.28                       |
| 9.  | GTP                                          | 2.59                       |
| 10. | GDP                                          | 2.60                       |



7. The role of the oxaloacetate concentration in the regulation of PEP synthesis.

It was not possible to study the distribution of oxaloacetate during the incubations because of the low concentration present. Assuming that it was evenly distributed between medium and mitochondria, the ratios of malate/oxaloacetate varied between 20 : 1 and 2 : 1 (when these compounds were not added as substrates) e.g. Table 40. The oxaloacetate concentrations were highest when malate was the substrate (Tables 33 and 34), and were lower in the presence of pyruvate (Table 40), pyruvate + malate (Table 34) or  $\alpha$ -oxo-glutarate (Table 38). This correlates with the ability of these compounds to form PEP (Table 32) and suggests that the limiting factor in the rate of PEP synthesis from these substrates was their ability to maintain the oxaloacetate concentration in the face of rapid PEPCK activity. This would support the conclusion (Chap. 1) that pyruvate carboxylase is rate-limiting for PEP (and carbohydrate) synthesis from pyruvate  
^ in pigeon-liver homogenates. The formation of oxaloacetate from  $\alpha$ -oxo-glutarate was enhanced by addition of DNP, but the unfavourable nucleotide ratios prevented the synthesis of PEP (Table 38). This applies also to the effects of adenine nucleotide addition, which resulted in an increase in the oxaloacetate concentration independent of the presence of  $Mg^{2+}$  (Tables 33, 34 and 40), but which was associated with increased PEP synthesis only when the ATP/ADP ratios were high. An explanation for the effects of AMP addition in pigeon-liver homogenates (Chap. 3, Section C) is also afforded. Here there was no inhibition of pyruvate carboxylase,



since malate formation was increased, but PEP synthesis was inhibited because of the high concentration of ADP.

Succinate (10 mM) was added to mitochondria in an experiment designed to test whether, under conditions where a favourable energy status might be expected, the synthesis of PEP would be affected by a fall in the oxaloacetate concentration. This was based on the known effects of succinate oxidation on intra-mitochondrial NADH (Krebs et al., (1961). Succinate addition decreased the oxaloacetate concentration by 50% in both cases, and PEP formation was diminished, although to a lesser extent (Table 42). This confirms the conclusion that the oxaloacetate concentration was a regulatory factor for PEPCK activity in situ, even if no estimate can be made of the effective concentration of the substrate at the site of the enzyme (see DISCUSSION).

#### 8. Fatty acid oxidation in pigeon-liver mitochondria.

Surprisingly, butyrate, octanoate and oleate (5, 0.4 and 0.1 mM respectively) were not oxidised by these mitochondria, despite the following additions, singly or in combination:

- i) 0.5% Bovine serum albumen (Bode & Klingenberg, 1965);
- ii) 1 mM D,L-carnitine (Garland et al., 1965);
- iii) 1 - 2 mM ATP (Kennedy & Lehninger, 1949);
- iv) 0.5 - 2 mM EDTA (van den Bergh, 1966);
- v) 0.5 - 2 mM malate (Kennedy & Lehninger, 1949).

This conclusion was based on the non-appearance of ketone bodies and the



**Table 42.** Effect of succinate addition on PEP synthesis from malate and  $\alpha$ -oxo-glutarate in pigeon-liver mitochondria.

Mitochondria were prepared and incubated at 30° for 30 min. under air, as described in METHODS. The medium contained 15 mM Pi, pH 7.4, 1 mM ADP, 4 mM HCO<sub>3</sub> and 0.625 mM Mg<sup>2+</sup>. Tissue dry wt. was 19 mg./cup.

| No.                                    | 1    | 2    | 3    | 4    | 5    |
|----------------------------------------|------|------|------|------|------|
| Additions (mM):                        |      |      |      |      |      |
| Malate                                 | 10   | 10   |      |      |      |
| $\alpha$ -Oxo-glutarate                |      |      | 10   | 10   |      |
| Succinate                              |      | 10   |      | 10   | 10   |
| Compounds found<br>( $\mu$ Moles/cup): |      |      |      |      |      |
| PEP + PGA                              | 14.4 | 10.4 | 6.22 | 4.55 | 8.91 |
| Oxaloacetate                           | 0.18 | 0.08 | 0.22 | 0.11 | 0.09 |
| Malate                                 | 21.3 | 42.4 | 1.37 | 25.5 | 24.6 |



failure of the fatty acids to stimulate the  $O_2$  uptake. Reduction of the  $P_i$  concentration (tested in case activation of fatty acids in pigeon-liver mitochondria proceeded exclusively over the  $P_i$ -sensitive GTP-linked enzyme of Rossi & Gibson (1964)) was without effect. Butyrate was, however, oxidised in pigeon-liver homogenates and slices (unpublished work), and it was concluded that the activating enzymes were all in the cytoplasm or that they were sensitive to the procedures employed. The subject was not further investigated.



### DISCUSSION

It may be concluded from these studies that the mitochondria, which contain PEPCK, are the site of PEP formation in pigeon liver. They also contain pyruvate carboxylase, the enzyme converting pyruvate (and all its precursors) into oxaloacetate. Two enzyme systems forming GTP, NuDiKi and the  $\alpha$ -oxo-glutarate oxidase system, are also present. The complement of these enzymes is constant during starvation and refeeding.

The control of the net rate of PEP synthesis depends on the regulation both of the reactions forming this metabolite, and of those removing it in the cytoplasm. The regulatory mechanisms appear to be as follows:

i) The conversion of oxaloacetate into PEP is controlled in part by the "phosphorylation potential" of the mitochondrial adenine nucleotides. It is most likely that this effect is exerted indirectly via the equilibrium reaction catalysed by NuDiKi, through an effect on the ratio GTP/GDP. Scholz & Bucher (1966) have shown that the overall tissue GTP/GDP ratio in perfused rat liver follows the ATP/ADP ratio very closely during an aerobic to anaerobic transition. The nucleotides react freely with each other in the matrix space of the mitochondria and there appears to be no special relationship of PEPCK to the substrate-level phosphorylation. The GTP/GDP ratio probably influences the activity of PEPCK by variations in the degree of substrate saturation (GTP) and competitive inhibition (GDP) (see Section C).



A second possibility is that NuDiKi may be inhibited by ADP when the concentration of the latter rises relative to that of the other substrates, due to the formation of an "abortive complex" (Section E). Because of the high activity of the enzyme in pigeon-liver mitochondria, and because of the fact that a similar effect of the "phosphorylation potential" of the adenine nucleotides was found when  $\alpha$ -oxo-glutarate was the precursor in the presence of DNP (inhibition of NuDiKi under these circumstances would favour PEP formation by conserving GTP), this explanation is not favoured.

A third possibility is that energy is required for the maintenance of functional mitochondrial compartments, such that the movements of oxaloacetate are controlled (see Tager, 1966). This concept has arisen because of the need for energy (in the form of ATP or high energy intermediates of oxidative phosphorylation) for the transfer of reducing equivalents from malate to  $\alpha$ -oxo-glutarate +  $\text{NH}_3$  in rat-liver mitochondria (Tager, 1963). This effect is exerted on malate oxidation rather than on transhydrogenation of NADH to NADPH. The relief of oxaloacetate-inhibited succinate oxidation by ATP under conditions when the measured overall oxaloacetate concentration does not change (Schollmeyer & Klingenberg, 1961), was also considered to involve an ATP-linked translocation of oxaloacetate from the site of succinate dehydrogenase. Thus it is possible that energy is also required to "translocate" oxaloacetate to the site of PEPCK, and to increase the rate of malate oxidation, since oxaloacetate is a potent inhibitor of mitochondrial malate dehydrogenase (Siegel &



Englard, 1960). However, explanation of the results obtained in the present work does not require an assumption of oxaloacetate compartmentation in the mitochondria.

The oxaloacetate concentration appeared to be directly related to the rate of PEP synthesis, excepting when the ATP/ADP ratio was unfavourable. It is evident from the effects of succinate addition (Table 42) and from the effects of high concentrations of DNP in the presence of  $\alpha$ -oxo-glutarate (Table 38) that the intra-mitochondrial NADH concentration affects the steady-state oxaloacetate concentration. Another factor may be the activity of citrate synthase, although this is not supported by the data of Table 35, where the oxaloacetate concentration was not decreased when "de-inhibition" of citrate synthase had occurred.

Seubert & Huth (1965) have proposed that variations in cytoplasmic oxaloacetate concentrations could regulate the activity of PEPCK in rat liver; in a subsequent paper (Henning *et al.*, 1966) this concept was withdrawn in favour of emphasis on the amount of PEPCK in the cytoplasm, which permits "accelerated gluconeogenesis even at lowered oxaloacetate levels" such as occur in diabetic rat liver (Wieland & Löffler, 1963). The role of nucleotides in the control of PEP synthesis in rat liver has not yet been investigated.

ii) The conversion of pyruvate into oxaloacetate, catalysed by pyruvate carboxylase, is also controlled, but to a less refined degree, by the "phosphorylation potential" of the adenine nucleotides, due to inhibition of the enzyme when the ADP concentration rises. It is likely



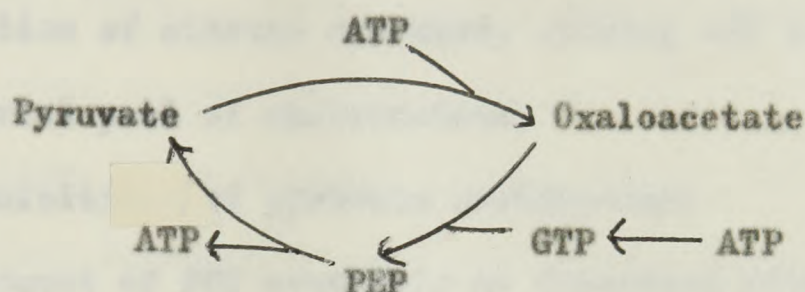
that variations in the mitochondrial levels of acetyl CoA, CoA and NADH may also regulate the activity of pyruvate carboxylase, both directly and by effects on pyruvate dehydrogenase (D.G. P.B. Nicholls & Garland, in the press), although no evidence for this has been obtained in the studies reported here.

According to Shepherd & Garland (1966), the oxidation of fatty acids in rat-liver mitochondria is accompanied by inhibition of citrate synthase thought to be due to an increase in the ATP/ADP ratio. The locus of the inhibition is the "allosteric" effect of ATP on this enzyme (Hathaway & Atkinson, 1965). The concentration of acetyl CoA is increased during fatty acid oxidation (Tubbs & Garland, 1964; Garland et al., 1965). This combination of factors would favour pyruvate carboxylation at the expense of pyruvate oxidation; it would also inhibit the formation of citrate (which removes oxaloacetate in competition with PEPCK) and would promote PEP synthesis by the effects previously outlined. Although such a theory involves extrapolation from one species to another, it does furnish a reasonable explanation for the association of increased gluconeogenesis and ketogenesis which is seen in such states as diabetes and starvation (see also Chap. 3 - DISCUSSION). In the perfused rat liver, Exton & Park (1966) have shown that  $^{14}\text{C}$ -lactate is converted quantitatively to glucose in the presence of a high rate of respiration; this might imply that pyruvate oxidation is completely suppressed in the mitochondria under these conditions, although the data can also be interpreted to support the extra-mitochondrial operation of both pyruvate carboxylase



and PEPCK in rat liver (Henning et al., 1966).

iii) The activity of PK is an important determinant of the net rate of PEP synthesis in isolated mitochondria and presumably also in the intact cell. Since cyclical activity of the pathway:



constitutes an energy-wasting "futile cycle" of the type described in Chap. 3, mechanisms are required by which the activity of PK can be controlled in the circumstances where rapid gluconeogenesis from oxaloacetate is occurring. In pigeon liver this is not achieved by variations in the overall activity of PK (e.g. Krebs & Eggleston, 1965 - although even in this case (rat liver) the total activity of PK always exceeds that of PEPCK). Other mechanisms were looked for (Section D) but no satisfactory proposal can yet be made. The inhibitory effect of ATP and the requirement for ADP would be in line with the general thesis that a high ATP/ADP ratio favours gluconeogenesis in a co-ordinate way. The distribution of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions may have a bearing on the control of PK activity; movements of  $\text{Ca}^{2+}$ , for instance, could have reciprocal effects on intra-mitochondrial pyruvate carboxylase (Utter & Keech, 1963) and extra-mitochondrial PK. Siebert et al., (1965) have analysed in detail some of the factors controlling PK activity in intact kidney tissue.

It would thus appear that the ATP/ADP ratio, both in the mitochond-



rial and cytoplasmic compartments, is a very important determinant of the rate of carbohydrate synthesis, although part of its effect is due to AMP, related to the other adenosine polyphosphates by the action of adenylate kinase. The control by ATP expresses itself in a number of ways:

- a) Inhibition of citrate synthase, closing off a major "leak" from the mitochondrial pool of oxaloacetate;
- b) "De-inhibition" of pyruvate carboxylase;
- c) Enhancement of PEP synthesis as described above;
- d) Inhibition of PK, preventing a "leak" at the level of PEP;
- e) Enhancement of the net conversion of FDP into fructose 6-P; (see Chap. 3);
- f) Promotion of glycogen synthesis by an effect on the UTP/UDP ratio (see Chap. 1).

All these effects may be regarded as the manifestations at the enzymatic level of the energy requirement of anabolism, and are certainly examples of "primitive" or inherent control mechanisms of liver cells, although they may have much wider significance for the physiological control of glycolysis and gluconeogenesis in the liver. They bear a remarkable similarity to the properties of reconstituted enzyme systems reported by Mendicino & Utter (1962).

The major difficulty in this view of hepatic carbohydrate metabolism is the occurrence of a "switch-over" of principal storage product from glycogen to triglyceride, which occurs at a certain stage during carbohydrate feeding. This implies that the ATP/ADP ratio must



be depressed in order to permit reversal of many of the reactions previously favoured by the high energy status and the low AMP concentrations. No such differences have yet been observed in studies of the adenine nucleotide content of rat liver under various conditions (Soling *et al.*, 1966; Underwood, 1965). In fact, rapid gluconeogenesis from lactate in the perfused liver is accompanied by a fall in the steady-state ATP/ADP ratio (Hems, Ross, Berry & Krebs, in the press), while Hohorst *et al.*, (1964) found that the ATP/ADP ratio was much depressed in alloxan-diabetic rat livers. Under these conditions, it might be argued that mechanisms other than those listed above are responsible for the increased gluconeogenesis observed, such that the supply of ATP might be limiting for gluconeogenesis. However, imposing an additional stress on the liver (urea synthesis) does not diminish the rate of carbohydrate synthesis from lactate (Hems, Ross, Berry & Krebs, in the press).

#### The compartmentation of adenine nucleotides in the cell.

At this point it may be profitable to discuss current concepts on the intracellular compartmentation of nucleotides. It has been known for some time that washed isolated mitochondria contain "bound" adenine and other nucleotides. Recently Heldt & Klingenberg (1965) have demonstrated the existence of a large variety of uridine, guanosine and cytidine nucleotides by sensitive chromatographic and ratio-isotope techniques. The content of adenine nucleotides is far greater than all the others combined (except for the  $\text{NAD}^+$ -NADH and  $\text{NADP}^+$ -NADPH series) and the concentration was estimated to be in the region of 6 mM (Klingenberg & Pfaff,



1966). The same workers have shown that 40 - 70% of the mitochondrial water (which they assumed to represent the "cristae space") is readily permeable to many low-molecular substances including all nucleotides. This penetration is proportional to the external concentration, is nearly instantaneous and readily reversible by washing, and is not inhibited by atractyloside. (This was confirmed by O'Brien & Brierley, 1965). At the same time, a slower "exchange" of internal and external ATP and ADP takes place (involving the whole molecules) with kinetic and physical characteristics of an enzymic reaction. This is specifically inhibited by atractyloside. The exchange appears to work on the "swing-door" principle in that no accumulation of adenine nucleotides occurs (but see Carafoli et al., 1965). The reaction is specific to adenine nucleotides, and guanyline nucleotides do not exchange (see Section F).

Heldt et al., (1964,a) have shown that endogenous ADP is the primary acceptor for  $^{32}\text{Pi}$  in oxidative phosphorylation, while Heldt et al., (1964,b) demonstrated that endogenous GDP was the primary acceptor of  $^{32}\text{Pi}$  in substrate-level phosphorylation in rat liver mitochondria. This suggests that the "translocation" is an essential intermediate step in the utilization of external ATP or ADP for any mitochondrial reaction. Klingenberg & Pfaff (1966) showed that at low temperatures the "translocase" step was rate-limiting for oxidative phosphorylation of external ADP. Should this be the case in intact mitochondria, it is feasible that the intra-mitochondrial ATP/ADP ratio could be significantly different from that of the cytoplasm. Frunder et al., (1961) obtained presumptive



evidence that such a difference did exist in fresh liver homogenates. From the known tissue "concentrations" of adenine nucleotides (3-4 mM - Hohorst et al., 1959) it may be inferred that the mitochondria (i.e. the cristae and matrix spaces combined) will not contain significantly more adenine nucleotides than the rest of the cell.

[Note: It must be emphasized that the above concepts derive largely from the work of one laboratory, and have not yet been confirmed in toto by other workers. Bruni (1966) believes that atractyloside inhibits oxidative phosphorylation of ADP by competing with ATP or ADP for a binding site in the mitochondrial multi-enzyme system which serves for various mitochondrial reactions dependent on added adenine nucleotides, including oxidative phosphorylation. Kemp & Slater (1964) and Chappell & Crofts (1965), on the other hand, have supported most of the proposals of Klingenberg and his co-workers.]

Another factor that may be important is the effect of the  $Mg^{2+}$  concentration in a particular compartment. Some of the enzymes shown to be sensitive to ATP, e.g. PFK (Passonneau & Lowry, 1964) and fumarase (Cohen & Penner, 1965) are less susceptible to the ATP-Mg complex. The free ATP concentration could be significantly different in cellular compartments.

It is feasible that a large part of the ATP measured in whole tissues by freeze-clamping and perchloric acid extraction (a standard technique) may be inactive in the sense that it is not available to enzymic reactions (see Ling, 1962 for a description of ATP as the



"cardinal adsorbant" of the cell). The analytical figures based on the amounts of metabolites extracted from a tissue and referred to its fresh weight, provide an average value of the compounds bound at many sites, as well as the true concentrations in various spatially-separate compartments. Thus Scholz & Bucher (1966) have shown that the CTP/CDP ratio changes from 30 to 3 in a few seconds following anoxia in the perfused rat liver, while the ATP/ADP ratio only falls from 5 to 2.

These considerations indicate that the mechanisms of regulation of metabolic processes revealed by studies with disrupted systems may have great importance in intact cells in vivo, despite the contradictory results obtained with the technique of metabolite assay in frozen-arrested tissues. Final verification will require intensive studies of the intracellular physiology of enzymes, e.g. their exact distribution, the occurrence and effects of reversible binding to particles and membranes, conformational changes and inter-conversions of separate forms, and the effective concentrations of substrates and modifiers in well-defined compartments of the cell.



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