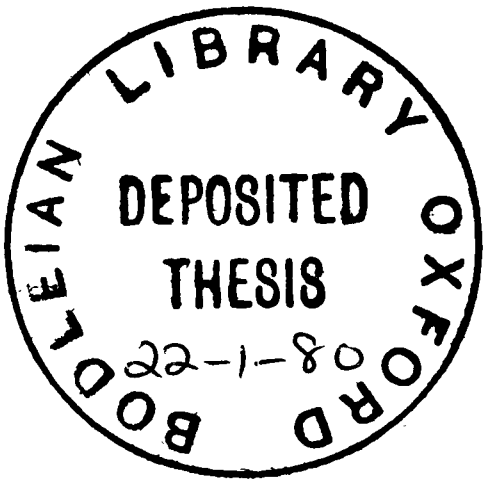
# COMPARATIVE STUDIES ON THE CITRIC ACID CYCLE IN MUSCLE

JENNIFER M. PAUL

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## ABSTRACT

Muscles throughout the animal kingdom vary widely in physiological function and metabolic characteristics. The citric acid cycle (or TCA cycle) plays a very important role in energy production in muscle. However there exists no simple means of estimating the quantitative significance of the TCA cycle in different muscles from animals across the animal kingdom.

The measurement of maximum catalytic activities of some enzymes may provide a reasonably rapid method for the indication of the maximum flux through metabolic pathways. However, only some'key' enzymes can play this role of 'rate-indicator'. These enzymes must catalyse reactions which are far displaced from equilibrium *in vivo*, and which are externally regulated. The theory behind this experimental approach to the regulation of metabolism is explained in Chapter One. The maximum activities *in vitro* of these 'rate-indicator' enzymes must also be tested against measured maximum rates of operation of the metabolic pathway in which they occur.

Using the above criteria, the work in this thesis demonstrates that 2-oxoglutarate dehydrogenase can be used as an indicator of maximum flux through the TCA cycle in muscles from vertebrates and invetebrates, and that pyruvate dehydrogenase can be similarly used for pyruvate oxidation in vertebrate muscles (Chapter Four). The investigations into the optimal conditions for extraction and assay of these enzymes are discussed in Chapter Three.

A comparative survey of the maximum catalytic activities *in vitro* of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase in muscles throughout the animal kingdom was carried out. Knowledge of the quantitative nature of the TCA cycle enabled the physiological function and metabolic characteristics of these muscles to be discussed in detail (Chapters Four and Five).

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

The abbreviations of enzymes used in the text are shown in the list of enzymes shown on p. v.

| | |
|---|---|
| acetyl CoA | acetyl coenzyme A |
| ADP | adenosine 5'-diphosphate |
| $Al_2O_3$ | alumina (aluminium oxide) |
| AMP | adenosine 5'-monophosphate |
| AR | analytical reagent |
| ATP | adenosine 5'-triphosphate |
| ATPase | adenosine 5'-triphosphatase |
| A-V | arteriovenous (difference) |
| $^oC$ | degrees Celsius |
| $Ca^{2+}$ | calcium ion |
| $CaCl_2$ | calcium chloride |
| cal | calorie |
| cAMP | adenosine 3':5'-phosphate |
| CoA | coenzyme A (reduced) |
| $CO_2$ | carbon dioxide |
| CP | creatine phosphate |
| DEAE | diethylaminoethyl cellulose |
| $\Delta G$ | Gibbs free energy change |
| $\Delta G^o$ | Gibbs standard free energy change |
| $\Delta G^{o'}$ | Gibbs standard free energy change at given pH |
| DHAP | dihydroxyacetone phosphate |
| DTNB | 5,5'-dithiobis-(2-nitro benzoic acid) |
| EDTA | diaminoethanetetra-acetic acid |
| EGTA | ethylene glycol bis-($\beta$-aminoethyl ether)-N,N'-tetraacetic acid |
| F6P | fructose-6-phosphate |
| FbP | fructose-bis-phosphate |
| G6P | glucose-6-phosphate |
| GAP | glyceraldehyde-3-phosphate |
| $H^+$ | hydrogen ion (proton) |
| HHQ | 2-heptyl-4-hydroxy-quinoline-N-oxide |
| $J_{max}$ | maximum flux through a reaction or pathway <u>in vivo</u> |
| K | degrees Kelvin |
| $K^+$ | potassium ion |
| $K_{eq}$ | equilibrium constant |

| | |
|---|---|
| $K_i$ | inhibitor constant |
| KCl | potassium chloride |
| KCN | potassium cyanide |
| $KHCO_3$ | potassium bicarbonate |
| l | litre |
| $Mg^{2+}$ | magnesium ion |
| $MgCl_2$ | magnesium chloride |
| $MgSO_4$ | magnesium sulphate |
| $Mn^{2+}$ | manganese ion |
| MSH | 2-mercaptoethanol |
| $NAD^+$ | nicotinamide-adenine dinucleotide, oxidised |
| NADH | nicotinamide-adenine dinucleotide, reduced |
| $NADP^+$ | nicotinamide-adenine dinucleotide phosphate, oxidised |
| NADPH | nicotinamide-adenine dinucleotide phosphate, reduced |
| $NH_4$ | ammonium ion |
| $O_2$ | oxygen |
| OAA | oxaloacetate |
| pAABS | p(p-amino azo)benzene sulphonic acid |
| PEP | phosphoenolpyruvate |
| 2PGA | 2-phosphoglycerate |
| 3PGA | 3-phosphoglycerate |
| $P_i$ | inorganic phosphate |
| R | universal gas constant (1.987 $cal.mol^{-1}.K^{-1}$) |
| R.Q. | respiratory quotient, i.e. $CO_2$ produced/$O_2$ consumed |
| TCA cycle | tricarboxylic acid cycle |
| TPP | thiamine pyrophosphate |
| TRA | triethanolamine-hydrochloride |
| TRIS | 2-amino-2-hydroxymethylpropane-1,3-diol |
| $V_{max}$ | maximum catalytic activity <u>in vitro</u> |
| $V_f$ | rate of forward reaction <u>in vivo</u> |
| $V_r$ | rate of reverse reaction <u>in vivo</u> |

## ENZYMES

| ENZYME (ABBREVIATION USED IN TEXT) | E.C. NO. | SYSTEMATIC NAME |
|---|---|---|
| aconitase | 4.2.1.3 | citrate (isocitrate) hydro-lyase |
| aldolase (aldol.) | 4.1.2.13 | D-Fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase |
| arylamine acetyltransferase (AAAT) | 2.3.1.5 | Acetyl-CoA:arylamine N-acetyl transferase |
| ATP-citrate lyase | 4.1.3.8 | ATP:citrate oxaloacetate lyas |
| carnitine palmitoyltransferase (CPT) | 2.3.1.21 | Palmitoyl-CoA:L-carnitine O-palmitoyltransferase |
| citrate synthase (CS) | 4.1.3.7 | citrate oxaloacetate lyase |
| creatine phosphokinase (CPK) | 2.7.3.2 | ATP:creatine N-phospho-transferase |
| cytochrome oxidase | 1.9.3.1 | Ferrocytochrome c:oxygen oxidoreductase |
| diglyceride lipase (DGL) | 3.1.1.34 | Triacylglycero-protein acylhydrolase |
| enolase (enol.) | 4.2.1.11 | 2-Phospho-D-glycerate hydrolase |
| glucose-6-phosphate dehydrogenase (G6PDH) | 1.1.1.49 | D-Glucose-6-phosphate: $NADP^+$ 1-oxidoreductase |
| glucosephosphate isomerase (GPI) | 5.3.1.9 | D-glucose-6-phosphate ketol-isomerase |
| glutamate-oxaloacetate transaminase | 2.6.1.1 | L-aspartate:2-oxoglutarate aminotransferase |
| glutamate-pyruvate transaminase | 2.6.1.2 | L-alanine:2-oxoglutarate aminotransferase |
| glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | 1.2.1.12 | D-Glyceraldehyde-3-phosphate :$NAD^+$ oxidoreductase (phosphorylating) |
| glycerol-3-phosphate dehydrogenase (G3PDH) | 1.1.1.8 | sn-Glycerol-3-phosphate: $NAD^+$ 2-oxidoreductase |
| hexokinase | 2.7.1.1 | ATP:D-hexose 6-phosphotrans-ferase |
| isocitrate dehydrogenase, $NAD^+$-linked (NAD-ICDH) | 1.1.1.41 | threo-D$_s$-Isocitrate:$NAD^+$ oxidoreductase (decarboxyla-ting) |

| ENZYME (ABBREVIATION USED IN TEXT) | E.C. NO. | SYSTEMATIC NAME |
|---|---|---|
| Isocitrate dehydrogenase, $NADP^+$-linked (NADP-ICDH) | 1.1.1.42 | threo-$D_s$-Isocitrate:$NADP^+$ oxidoreductase (decarboxylating) |
| lactate dehydrogenase (LDH) | 1.1.1.27 | L-Lactate:$NAD^+$ oxidoreductase |
| malate dehydrogenase (MDH) | 1.1.1.37 | L-Malate:$NAD^+$ oxidoreductase |
| oxoglutarate dehydrogenase (OGDH) | 1.2.4.2 | 2-Oxoglutarate: lipoamide oxidoreductase (decarboxylating and acceptor-succinylating) |
| phosphoenolpyruvate carboxykinase (PEPCK) | 4.1.1.49 | ATP: oxaloacetate carboxylyase (transphosphorylating) |
| phosphofructokinase (PFK) | 2.7.1.11 | ATP: D-fructose-6-phosphate 1-phosphotransferase |
| phosphoglycerate kinase (PGK) | 2.7.2.3 | ATP: 3-phospho-D-glycerate 1-phosphotransferase |
| phosphoglycerate mutase (PGM) | 5.4.2.1 | D-Phosphoglycerate 2,3-phosphomutase |
| phosphorylase | 2.4.1.1 | 1,4-$\alpha$-D-Glucan:orthophosphate $\alpha$-D-glucosyltransferase |
| proline dehydrogenase | 1.5.1.2 | L-proline: $NAD(P)^+$ 5-oxidoreductase |
| pyruvate carboxylase | 6.4.1.1 | Pyruvate:carbon-dioxide ligase (ADP forming) |
| pyruvate dehydrogenase (PDH) | 1.2.4.1 | Pyruvate: lipoamide oxidoreductase (decarboxylating and acceptor-acetylating) |
| pyruvate dehydrogenase kinase (PDH kinase) | 2.7.1.99 | ATP: [pyruvate dehydrogenase (lipoamide)] phosphokinase |
| pyruvate dehydrogenase phosphatase ($PDH_b$-phosphatase) | 3.1.3.43 | [Pyruvate dehydrogenase (lipoamide)] -phosphate phosphohydrolase |
| pyruvate kinase (PK) | 2.7.1.40 | ATP: pyruvate 2-0-phosphotransferase |
| succinate dehydrogenase (SDH) | 1.3.99.1 | Succinate: (acceptor) oxidoreductase |
| succinyl CoA synthetase | 6.2.1.4 | Succinate:CoA ligase (GDP-forming) |
| triglyceride lipase (TGL) | 3.1.1.3 | Triacylglycerol acylhydrolas |
| triosephosphate isomerase (TIM) | 5.3.1.1 | D-Glyceraldehyde-3-phosphate ketol-isomerase |

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# CHAPTER ONE

## GENERAL INTRODUCTION

### SECTION 1: THE TRICARBOXYLIC ACID CYCLE

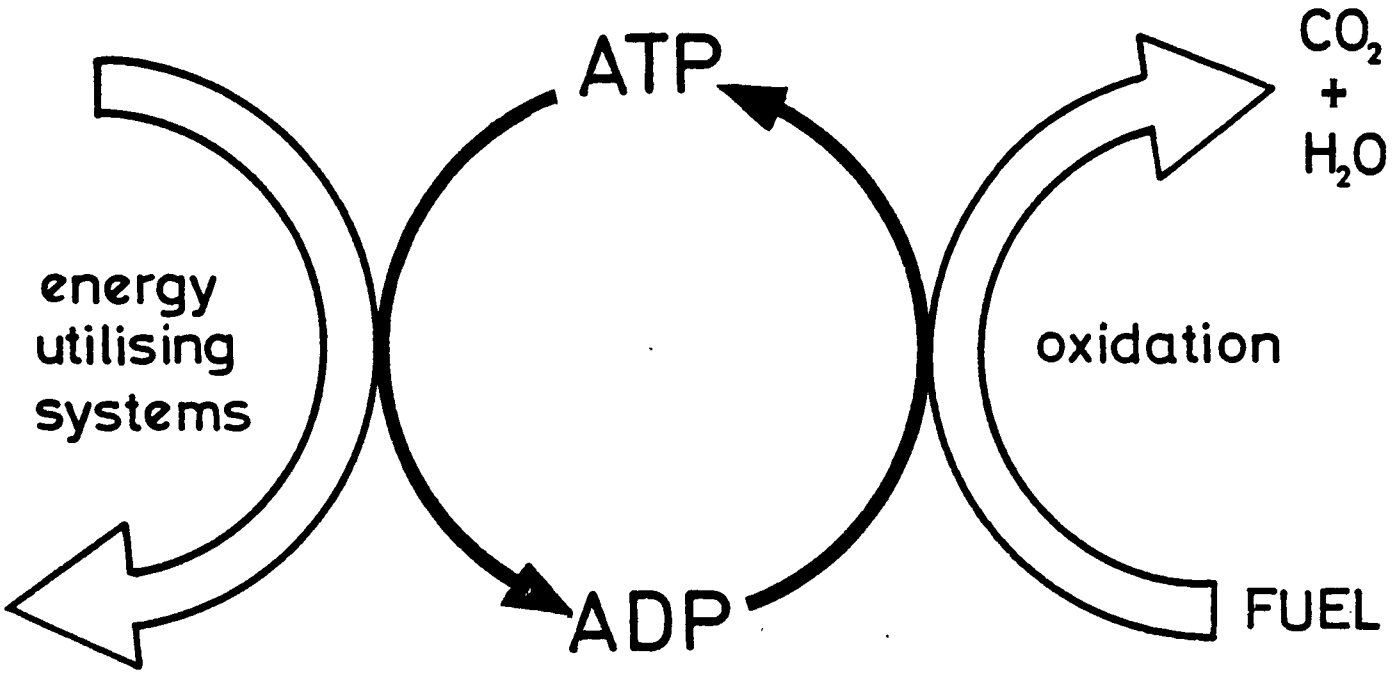
#### 1) INTRODUCTION

Energy transfer in the living cell usually involves the use of adenosine triphosphate (ATP) as an intermediate carrier of energy. Some of the energy produced in the oxidative reactions of catabolism is conserved in the formation of ATP. The hydrolysis of the terminal phosphate bond of ATP is coupled to the energy requiring processes of the cell, such as those involving electrical, chemical or mechanical work (see Fig. 1).

The work done by, for example, a muscle cell varies considerably with the type of muscle. Thus the power output of insect flight muscles is very large (Sacktor, 1970; Weis-Fogh, 1961), whereas the smooth muscle of invertebrates is very small. These requirements for energy must necessarily be satisfied by a supply of ATP, though this is not stored in the cell; its concentration in a large range of muscles is 5-7 mM (Beis & Newsholme, 1975). For muscular contraction to continue for more than a few seconds, the level of ATP in the muscle must continually be restored, as the amount of ATP in muscle is very small relative to the rates of ATP hydrolysis measured during contraction (10-2000 $\mu$mol.min$^{-1}$g$^{-1}$ weight of fresh muscle).

In fact, the level of ATP in the muscle cell does not change appreciably during contraction, even though the rate of utilisation of

Figure 1



ATP may increase by many fold (e.g. at least by a hundred-fold in insect flight muscle, Sacktor & Hurlbut, 1966). The reason for this constant ATP level in contracting muscle in not known. It has been suggested that as ATP is involved in many reactions of the cell, a decrease in its concentration could decrease the activity of many cellular processes that require ATP. However this is unsatisfactory, as for many enzymes the intracellular ATP concentration is considerably greater than the $K_m$ for ATP, so small changes in the concentration of ATP would not affect these processes.

A possible explanation may be that a constant ATP/ADP concentration ratio (and not the absolute value for ATP levels) is required for the maximum kinetic efficiency of ATP utilising pathways (Newsholme, 1976a). Many enzymes whose substrate is ATP are competitively inhibited by ADP and vice versa and this makes them sensitive to changes in the ATP/ADP concentration ratio of the cell. For an overall ATP generating process such as glycolysis, a maximum rate of operation is required during periods of high demand for ATP, e.g. when ATP is being rapidly hydrolysed by muscle. However, some enzymes involved in that process are inhibited by a low ATP/ADP concentration ratio (e.g. hexokinase), and this would reduce the rate of the process. Others would be inhibited by a high ATP/ADP concentration ratio (e.g. phosphoglycerate kinase), so that in order to maintain a high flux through glycolysis, a relatively constant ATP/ADP concentration ratio must be maintained. In order to maintain a high degree of constancy, ATP utilisation is linked to ATP formation by regulatory mechanisms that are very sensitive and that transfer information very rapidly.

Another mechanism for the maintenance of constant levels of ATP and ADP is the phosphagen/phosphagen kinase system. In anaerobic muscles, the phosphagen provides a store of 'energy rich' phosphate which can regenerate ATP from ADP during muscle contraction. The role of the phosphagen/phos-

phagen kinase system in aerobic muscle is not so clear. However, recent evidence suggests that it might play a role in decreasing transient changes in ATP and ADP when the rate of ATP turnover is modified (Newsholme et al., 1978). In heart muscle, the localisation of creatine kinase tends to support this theory. Creatine kinase in heart muscle is found in the mitochondrial intermembrane space and closely associated with the myofibrils, i.e. near the adenine nucleotide translocator and at the site of myofibrillar ATPase. Large increases in the concentration of ATP would inhibit the translocator (by competing with ADP for entry into the mitochondria, Beis & Newsholme, 1976), and large increases in the concentration of ADP would inhibit the myofibrillar ATPase (Maruyama & Pringle, 1967), and thus the creatine kinase is located at two sites where the maintenance of stable concentrations of ATP and ADP is of major importance.

The production of ATP in the cell occurs through the oxidation of metabolic fuels such as carbohydrate and fat. The biochemical details of the main metabolic pathways responsible for the degradation of fuels are given in most biochemistry texts. Some of the more important pathways are outlined in Figs. 2 and 3. Carbohydrate is the major energy source which produces ATP anaerobically, and this is accomplished via the glycolytic pathway. Some adaptations to anaerobiosis in marine invertebrates involve the utilisation of the amino acids aspartate and glutamate with the formation of succinate as an end product (Hochachka et al., 1973).

The amino acid proline is used in aerobic metabolism as a fuel for flight in some insects (Bursell, 1963, 1965, 1966, 1967. For review, see Sacktor, 1970).

For complete oxidation of carbohydrate, the glycolytically produced pyruvate must be converted to acetyl CoA and oxidised via the tricarboxylic acid (TCA) cycle. The pathway for the complete oxidation of fatty acids and ketone bodies is also via acetyl CoA and the TCA cycle. The regulation

Figure 2 THE TRICARBOXYLIC ACID CYCLE AND ELECTRON TRANSPORT CHAIN

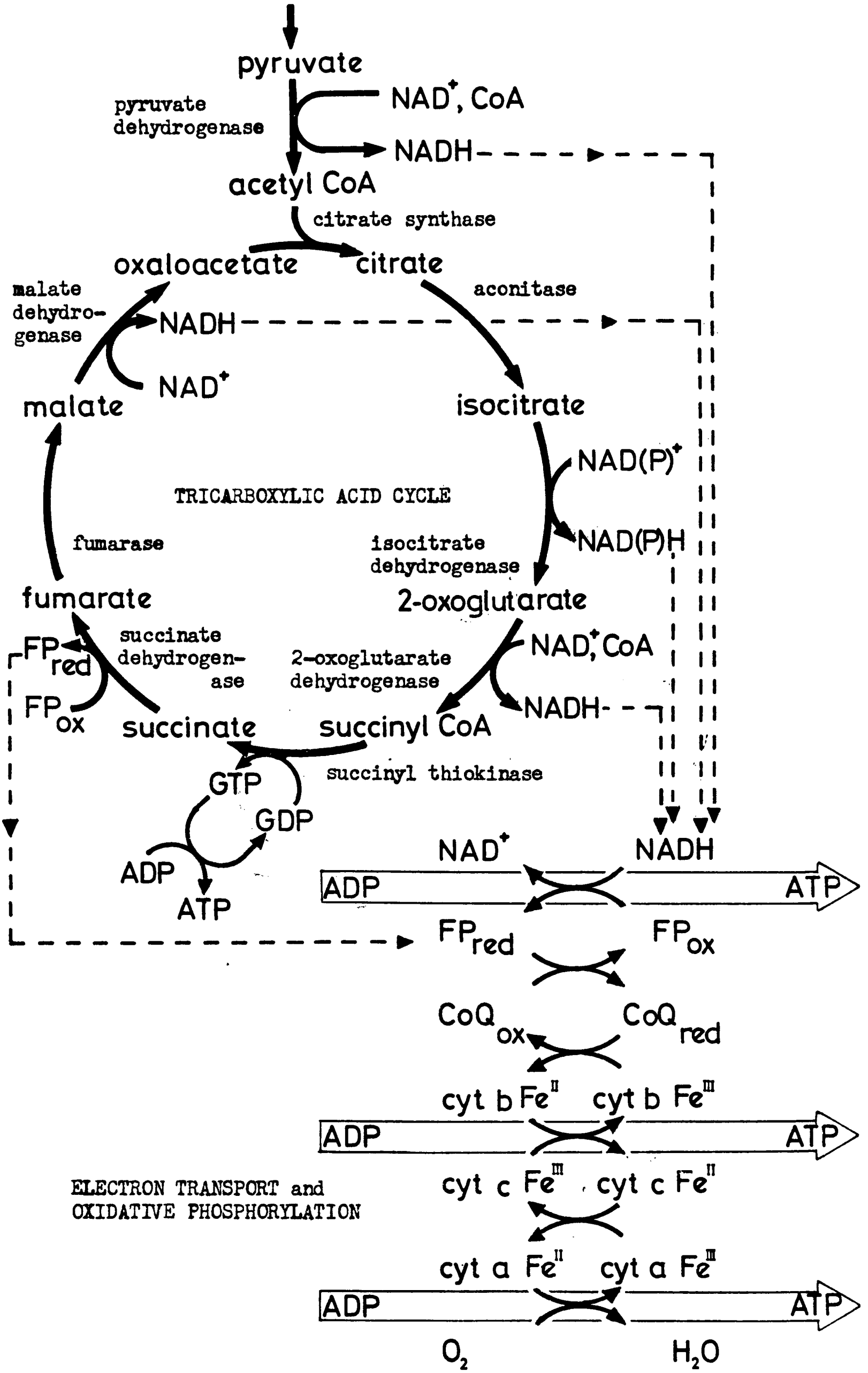
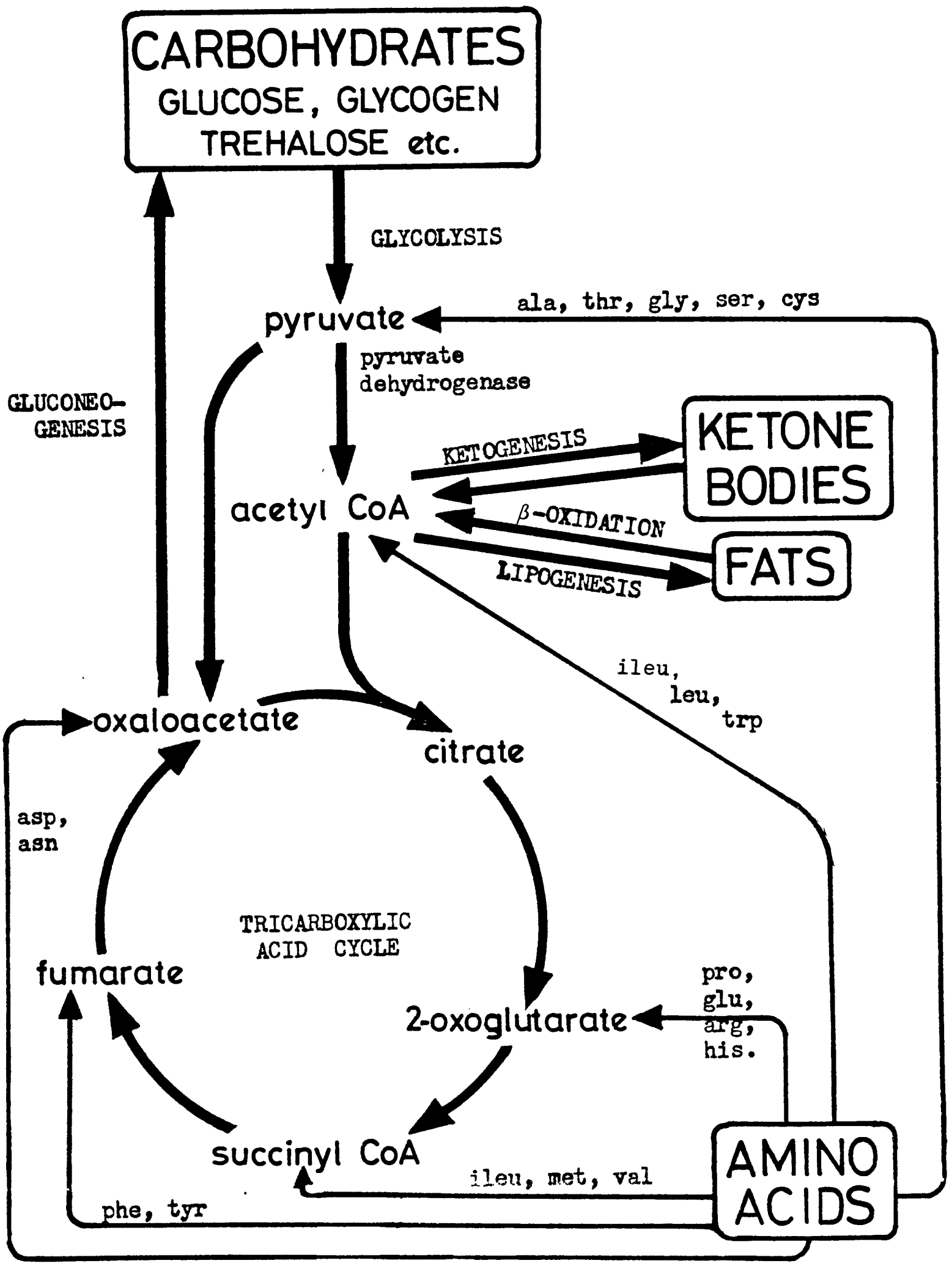


Figure 3 METABOLIC INTERRELATIONSHIPS BETWEEN FUELS



of these pathways of fuel degradation to satisfy the ATP demands of the cell is carried out in various ways. For example, a small decrease in the concentration of ATP leads to an increase in the rate of glycolysis via the relief of inhibition of one of the "key" enzymes of the pathway, phosphofructokinase, via changes in the concentrations of AMP and $NH_4^+$ and possibly other regulators. The theory of feedback regulation of "key" enzymes of a metabolic pathway by specific metabolic regulators is produced by an approach to the study of metabolic control described by Newsholme & Gevers (1967), Newsholme (1970) and Newsholme & Start (1973).

The final common pathway for the oxidation of various fuels is the TCA cycle (also called the Citric Acid cycle and the Krebs cycle). This cycle may be said to lie at the centre of intermediary metabolism; it serves both catabolic and anabolic functions. Many of the intermediates and some of the enzymes of the cycle are distributed both in the cytoplasm and in the mitochondrion (Greville, 1969), but the full operation of the cycle takes place in the mitochondrion.

The reactions utilising the TCA cycle can be considered under four general headings.

(a) The energy producing role of the TCA cycle.

(b) The biosynthetic role of the TCA cycle.

(c) The involvement of the cycle in amino acid oxidation.

(d) Other reactions involving the TCA cycle.

## 2) FUNCTIONS OF THE TCA CYCLE

### (a) The energy producing role of the TCA cycle

The major energy producing role of the TCA cycle is the oxidation of acetate, "activated" as acetyl CoA, to carbon dioxide and water. The reactions of the cycle together with the electron transport chain are shown

in Fig. 2. The hydrogen atoms from the TCA cycle, carried as reduced co-enzymes, are fed into the electron transport chain and oxidised to water; the energy obtained from this oxidation is conserved in the production of ATP. The acetyl CoA which enters the cycle may be supplied as the break-down product of various fuels. Fig. 3 shows how carbohydrates, fatty acids and ketone bodies enter the TCA cycle as acetyl CoA (see Section Two on fuel utilisation).

(b) The biosynthetic role of the TCA cycle

Various intermediates of the TCA cycle can be removed to serve as biosynthetic precursors or to take part in other reactions.

1) Oxaloacetate may be removed by the enzyme phosphoenolpyruvate carboxykinase, which in some muscles catalyses the conversion of oxaloacetate to phosphoenolpyruvate. This reaction also occurs in the gluconeogenic tissues (liver and kidney) and in adipose tissue.

2) Acetyl CoA is the substrate for ketone body formation and cholesterol biosynthesis in the liver, and for fat biosynthesis in adipose tissue.

3) Succinyl CoA is the substrate for porphyrin biosynthesis in the liver.

4) Citrate may be converted to acetyl CoA by ATP-citrate lyase. The distribution of this enzyme is not well known, but it occurs mainly in adipose tissue and liver as part of a system for the transfer of acetyl units across the mitochondrial membrane (see Chapter 2).

5) 2-oxoglutarate and oxaloacetate may serve as precursors for amino acids in taking part in the following transamination reactions:

2-oxoglutarate + alanine $\rightleftharpoons$ glutamate + pyruvate

oxaloacetate + alanine $\rightleftharpoons$ aspartate + pyruvate

These reactions occur in most tissues.

6) Malate may be removed by NADP-linked malic enzyme to form pyruvate, in the gluconeogenic tissues and adipose tissue.

malate + $NADP^+$ $\longrightarrow$ pyruvate + NADPH + $CO_2$ + $H^+$

In order to maintain the steady state levels, residues cannot be removed from the TCA cycle without replacing them. Various reactions exist which can be involved in replacing TCA cycle intermediates; these are termed anaplerotic reactions (see (d) below).

(c) <u>The involvement of the TCA cycle in amino acid oxidation</u>

Amino acids may be oxidised to yield energy after they have been deaminated. The role of the TCA cycle in the oxidation of amino acids varies according to which amino acid is involved. Amino acid oxidation may involve only partial oxidation or full oxidation to carbon dioxide and water. If the amino acid gives rise to acetyl CoA or pyruvate (see Table I), it may be fully oxidised to carbon dioxide and $H^+$ via the TCA cycle. However, many amino acids give rise to other TCA cycle intermediates (e.g. glutamate, histidine, arginine and proline to 2-oxoglutarate. See Table 1). Contrary to the assumption of many textbooks, the carbon skeleton of these intermediates cannot be oxidised by the TCA cycle, which oxidises only the carbon skeleton of the acetate moiety of acetyl CoA. The fate of amino acids giving rise to TCA cycle intermediates in muscle is discussed by Goldstein & Newsholme (1976).

It is known that six amino acids can be oxidised by muscle to carbon dioxide and water: leucine, isoleucine, valine, aspartate, glutamate and alanine (Miller, 1961; Odessey & Goldberg, 1972). Alanine and leucine give

TABLE I

PRODUCTS OF BREAKDOWN OF AMINO ACIDS

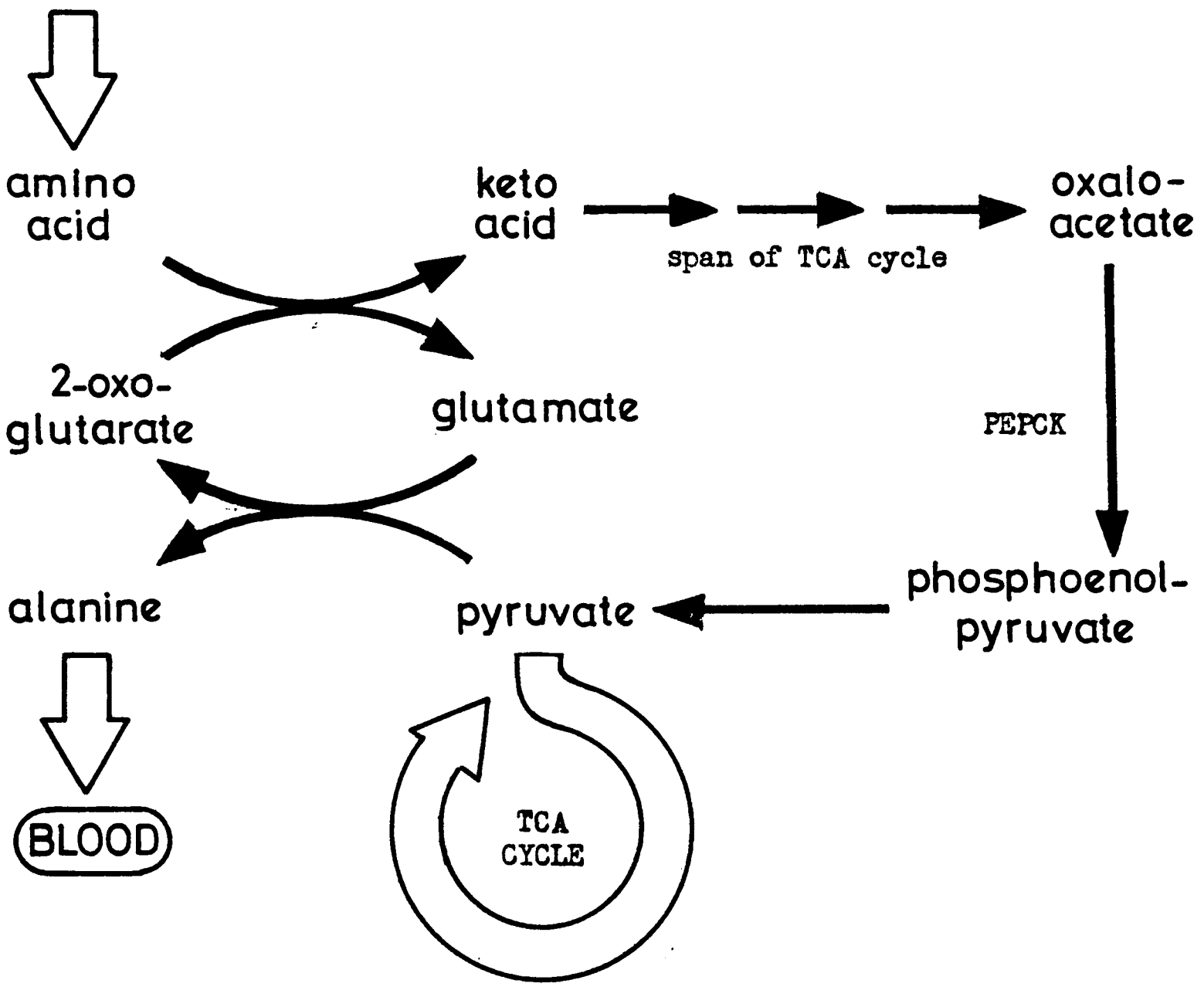
| Amino acids giving rise to pyruvate or acetyl CoA | | Amino acids giving rise to other TCA cycle intermediates | |
|---|---|---|---|
| Acid | Product | Acid | Product |
| alanine | pyruvate | asparagine | oxaloacetate |
| cysteine | " | aspartate | |
| glycine | " | phenylalanine | fumarate |
| serine | " | tyrosine | " |
| threonine | " | isoleucine | succinyl CoA |
| hydroxyproline | " | methionine | " |
| isoleucine | acetyl CoA | valine | " |
| leucine | acetyl CoA | glutamine | 2-oxoglutarate |
| tryptophan | " | glutamate | |
| phenylalanine | acetoacetyl CoA | histidine | " |
| tyrosine | " | arginine | " |
| leucine | " | proline | " |
| lysine | " | | |
| tryptophan | " | | |

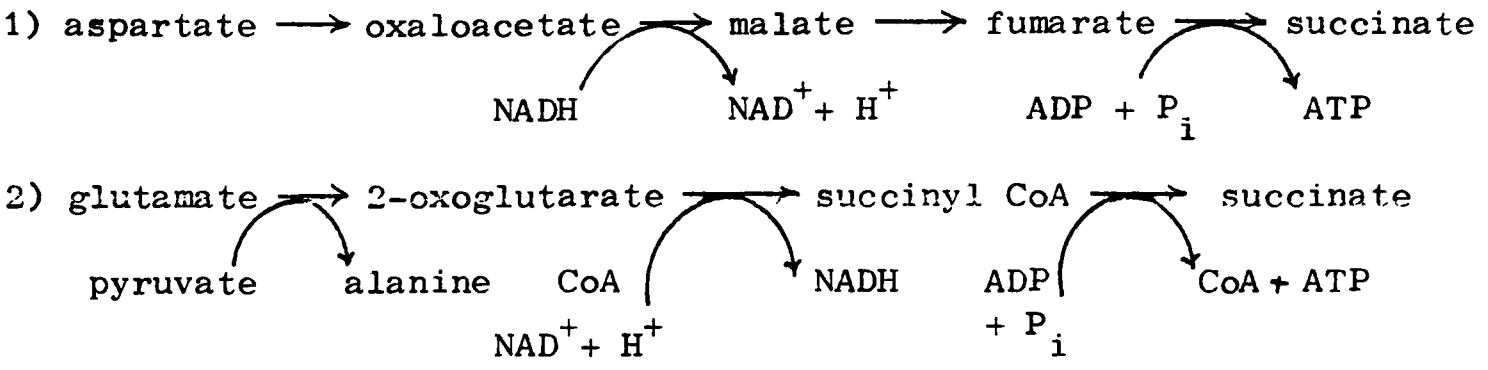
rise to pyruvate and acetyl CoA, respectively and therefore it can be easily seen how they are completely oxidised by the TCA cycle. Aspartate, glutamate, valine and a portion of the isoleucine residues enter the cycle at some point in the span from 2-oxoglutarate to oxaloacetate - the 2-oxoglutarate-oxaloacetate span. As the carbon of these cycle intermediates cannot be oxidised directly in the cycle, the resultant oxaloacetate must therefore be removed from the cycle. It has been proposed that phosphoenol pyruvate carboxykinase could fulfill this function (Goldstein & Newsholme, 1976; Snell & Duff, 1977; Newsholme & Williams, 1978). The pyruvate produced via the pyruvate kinase reaction may then either be oxidised via acetyl CoA and the TCA cycle, or transaminated to alanine and released from the muscle (see Fig. 4 and Goldstein & Newsholme, 1976). This theory is supported by the fact that alanine (together with glutamine) is released by muscle, for example when branched chain amino acids are being oxidised by incubated diaphragms (Odessey et al., 1974) and when branched chain and other amino acids are oxidised in the perfused hindlimb of the rat (Ruderman & Berger, 1974).

The involvement of the TCA cycle with the catabolism of amino acids is therefore two-fold. Some amino acids may contribute to the energy producing role of the cycle, e.g. acetyl CoA $\longrightarrow$ $CO_2 + H^+ + CoA$. Others may utilise only the 2-oxoglutarate-oxaloacetate span, and may be converted to a gluconeogenic precursor, (e.g. alanine). In this case, the TCA cycle can be considered as serving a biosynthetic function.

Some amino acids have been adopted for use as a fuel, i.e. to supply energy for muscular activity. Aspartate and glutamate have been mentioned before as fuels for anaerobic metabolism in some marine invertebrates. The pathways postulated for the utilisation of these amino acids are as follows (Hochachka et al., 1973).

Figure 4 PATHWAY PROPOSED FOR THE OXIDATION OF SOME AMINO ACIDS IN MUSCLE



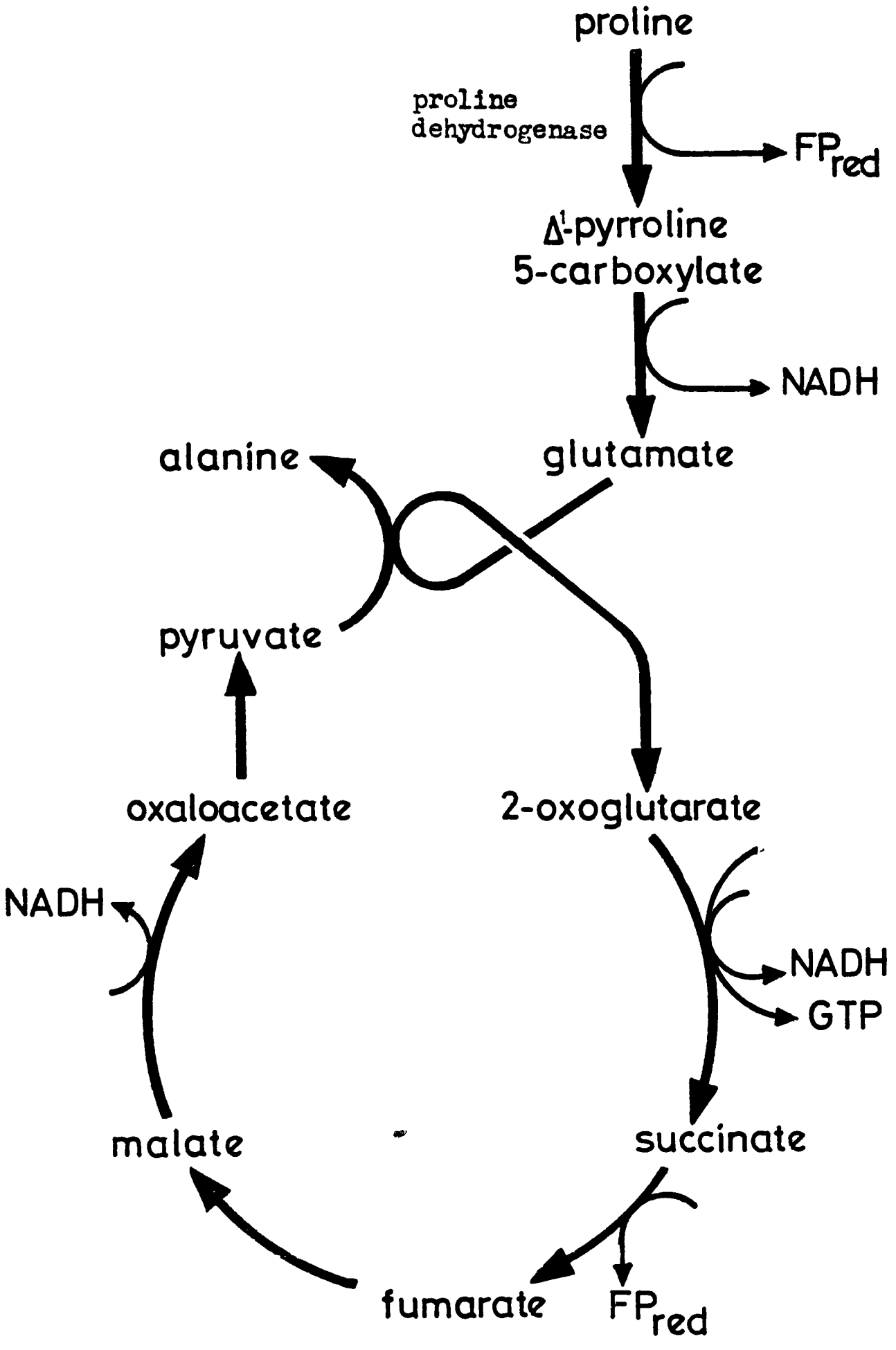



This pathway utilises the span of the TCA cycle from 2-oxoglutarate to succinate in the forward direction.

Most of the aspartate carbon (70%) is eliminated as succinate, and 17% accumulates as alanine.

In insects, various free amino acids are found in high concentration in the haemolymph. One particular amino acid that seems to play an important role is proline. Originally, the ability to oxidise proline in many insects was seen as a mechanism to increase the level of TCA cycle intermediates (Sacktor & Childress, 1967). It was suggested that proline is converted into glutamate which can enter the TCA cycle as 2-oxoglutarate. However, in the tsetse fly, Bursell (1963) has shown that proline may serve as a fuel during the initial period of flight. Subsequent investigations revealed that proline is converted into alanine via the pathway outlined in Fig. 5. The maximal acitvities of the first enzyme of this pathway, proline dehydrogenase, have been measured (Crabtree & Newsholme, 1970). Estimates of the rate of utilisation of proline from oxygen uptake data suggest that the maximum activity of proline dehydrogenase may provide an indication of flux through this pathway (Crabtree & Newsholme, 1975, p. 450). In those flight muscles in which the activity of proline dehydrogenase is high (e.g. tsetse fly and cockchafer) this pathway may be quantitatively important in energy production.

Figure 5 PATHWAY OF PROLINE UTILISATION IN TSETSE FLY FLIGHT MUSCLE



(d) Other reactions involving the TCA cycle

As mentioned before, intermediates cannot be removed from the TCA cycle without being replenished, if the steady state levels of these intermediates are to be maintained. For example, depletion of intermediates could lead to a decrease in the concentration of oxaloacetate which may effect the rate of operation of the TCA cycle (see properties of citrate synthase on pp.58,69). Various reactions have been proposed as serving anaplerotic functions, but the main reactions supplying oxaloacetate in animal tissues are pyruvate carboxylase and various transamination reactions. Pyruvate carboxylase catalyses the reaction shown below:

$$\text{pyruvate} + \text{ATP} + CO_2 \longrightarrow \text{oxaloacetate} + \text{ADP} + P_i$$

High activities of this enzyme are found in mitochondria of tissues capable of gluconeogenesis (liver and kidneys) and also in adipose tissue, but low activities are present in other tissues (e.g. brain and muscle excluding insect flight muscle, which has appreciable activities). In the gluconeogenic tissues, pyruvate carboxylase is acting mainly as a gluconeogenic enzyme, providing oxaloacetate for conversion to phosphoenolpyruvate.

Oxaloacetate, together with 2-oxoglutarate, is also involved in the transaminase reactions, and they may be replenished as TCA cycle intermediates in this way.

$$\text{glutamate} + \text{pyruvate} \longrightarrow \text{2-oxoglutarate} + \text{alanine}$$

$$\text{aspartate} + \text{pyruvate} \longrightarrow \text{oxaloacetate} + \text{alanine}$$

These reactions may also be involved in the malate-aspartate shuttle, a means of transporting reducing equivalents across the mitochondrial membrane in adipose tissue. This involves the enzyme glutamate - aspartate transaminase which catalyses the following reaction.

$$\text{oxaloacetate} + \text{glutamate} \longrightarrow \text{aspartate} + \text{2-oxoglutarate}$$

Thus in many tissues, the interpretation of the roles of these intermediates in the TCA cycle may be complicated by the occurrence of several different pathways.

It must be stressed that, in most tissues (i.e. excluding beetle and tsetse fly flight muscle) the major energy producing role of the TCA cycle probably involves only the oxidation of the acetate moiety of acetyl CoA. Reactions that supply or deplete intermediates of the cycle do not involve complete oxidation of the carbon skeleton of the intermediate involved. However, in some tissues (e.g. in liver and kidney) these reactions may occur to an extent which complicates the interpretation of the function of the TCA cycle; the ATP yield from the utilisation of the 2-oxoglutarate-oxaloacetate span of the cycle raises a problem about the concerted control of both spans of the cycle. Even in muscle, the oxidation of amino acids and the occurrence of the transamination reactions suggest that the 2-oxoglutarate-oxaloacetate span of the cycle is used to some extent and this may be responsible for a significant amount of ATP generation.

## 3) THE IMPORTANCE OF THE TCA CYCLE IN MUSCLE

It can be seen from the above discussion that the TCA cycle serves both a catabolic and an anabolic role. However, in muscle there is negligible flux through the gluconeogenic pathway and the role of the TCA cycle is primarily that of energy production. Some muscles can of course utilise carbohydrate anaerobically by converting glucose or glycogen to lactate.

However, greater efficiency is obtained by utilising the TCA cycle and molecular oxygen, i.e. more ATP is produced per substrate molecule Table II shows the ATP yields from various fuels. It can be seen that complete oxidation to $CO_2$ and $H_2O$ yields higher amounts of ATP. In some

TABLE II

ATP Yields from the Breakdown of

Various Metabolic Fuels

| Fuel | Conditions | Moles ATP produced/ Moles substrate utilised |
|---|---|---|
| glucose | complete oxidation to $CO_2 + H_2O$ | 38 |
| glucose | lactate production | 2 |
| glycogen | complete oxidation to $CO_2 + H_2O$ | 39 |
| glycogen | lactate production | 3 |
| glycogen | succinate production | 4 |
| palmitate | complete oxidation to $CO_2 + H_2O$ | 129 |
| acetoacetate | complete oxidation to $CO_2 + H_2O$ | 24 |
| proline | oxidation to alanine | 14 |

specialised muscle, such as insect flight muscle, the importance of the oxidative supply of energy and hence the TCA cycle is emphasised by the large increase in oxygen consumption on initiation of activity. Some blowflies consume oxygen at a rate of about 1000 $\mu$l.min$^{-1}$g$^{-1}$ of fresh weight of muscle on initiation of flight, thus elevating their basal rates approximately 100 fold (Davis & Fraenkel,1940). There are also many examples of vertebrate muscles in which the TCA cycle is important; perhaps the most extensively studied vertebrate muscles are pigeon pectoral muscle and rat heart.

In order to investigate the importance of the TCA cycle in muscle tissue, it is first necessary to discuss the utilisation of fuels by muscle of different types in order to establish which pathways are quantitatively important for ATP production.

## SECTION 2: COMPARATIVE STUDIES ON FUEL UTILISATION IN MUSCLE

### 1) INTRODUCTION

The preceding section introduced the possible ways in which the breakdown of fuels involved the ultimate use of the TCA cycle, It was pointed out that in working muscle, the TCA cycle was operating primarily to provide energy from the oxidation of fuels. In order to examine the relationships between fuel utilisation and energy production in muscle, quantitative information concerning the rates of fuel utilisation in different muscles is essential.

Exogenous fuel sources available to muscle are carbohydrates, fatty acids and amino acids. These are transported in the blood. Endogenous fuel stores are also available to muscle consisting of triglycerides,

glycogen and the amino acid constituents of muscle proteins.

## 2) EXPERIMENTAL INVESTIGATION OF FUEL UTILISATION

It is known that various fuels are available to muscle from various animals. In many cases, it is not known which of these fuels are utilised for the formation of energy during muscular contraction, or the maximum capacity of the various energy producing pathways. There are several methods that can be used to indicate the type of fuel utilised but most evidence is of an indirect nature.

Two major problems in studying fuel utilisation in muscle are as follows:

1) Muscles may use different fuels when at rest and during contraction and also at different times during contraction,

2) It is difficult to find a suitable preparation of working muscle.

While *in vivo* or whole animal studies are designed to investigate fuel utilisation in working muscle, the biochemical details of the utilisation may have to be elucidated with techniques *in vitro*.

A critical and extensive review of the methods used in investigating fuel utilisation is given by Crabtree & Newsholme (1975). A brief review of the techniques is given here.

### (i) In vivo techniques

a) Determination of respiratory quotient. The respiratory quotient is calculated after the uptake of oxygen and the release of carbon dioxide by the whole animal have been measured. The quotient (R.Q.) is given by the ratio, $CO_2$ released/ $O_2$ taken up. The R.Q. is unity if carbohydrate is the source of fuel and 0.7 if lipids are oxidised. The R.Q. for protein oxidation depends upon the amino acid involved, but is intermediate between

0.7 and 1.0. The major problem of this method is that, since the R.Q. of the whole animal is measured, the contribution of the metabolism of muscle to the R.Q. is sometimes difficult to determine.

In the case of insect flight muscle, however, the 50 to 100 fold increase in both oxygen uptake and carbon dioxide production on initiation of flight allows the reasonable assumption that the flight muscles are primarily responsible for the increase in metabolism. Thus the method has provided much useful information on fuel utilisation in insect flight muscle (see Sacktor, 1970).

It may be possible to modify the method for a particular muscle by measuring the arteriovenous (A-V) differences of carbon dioxide and oxygen across the tissue. However, serious drawbacks of the methods are as follows:

1) It does not differentiate between different classes of carbohydrate (e.g. glycogen, glucose) or fat (e.g. triglycerides, fatty acids).

2) It does not supply quantitative information about the rate of utilisation of the fuels.

b) Arteriovenous differences in the whole animal. A second approach is the measurement of A-V differences of fuels across a muscle or groups of muscle. This method has been used extensively in man (Drummond, 1967; Ekelund, 1969), but the technical difficulties of catheterisation make it an unsuitable method for many animals. It also suffers from the disadvantage that it is rarely possible for it to be used for measuring uptake or output across a specific muscle; many muscles are usually involved. These difficulties mean that the method is not suitable for a comparative survey of all muscle types across the animal kingdom.

(ii) In vitro techniques

a) Perfusion and incubation experiments. Measurement of the

utilisation of a fuel can be performed more accurately by using isolated preparations of organs or tissues and either perfusing or incubating them *in vitro*. Information can be gained about the rates of utilisation of exogenous fuels (added to the perfusate or incubation medium) or that of endogenous fuels in the tissue. The following muscle preparations have been perfused: rat heart (Evans *et al.*, 1963; Cavert, 1956; Opie, 1962) rat hindlimb (Ruderman *et al.*, 1971; Rennie & Holloszy, 1977) and the insect half thorax (Candy, 1970). However, the latter two preparations suffer a severe disadvantage - the amount of work performed by the muscle under *in vitro* conditions is far different from that performed *in vivo* (Rennie & Holloszy, 1977). The oxygen uptake of the working preparation of Ruderman was 14.3 $\mu$mol $O_2$. $min^{-1}$.$15g^{-1}$ muscle but that of Rennie & Holloszy was 31.5 $\mu$mol $O_2$.$min^{-1}15g^{-1}$.

In addition, muscle fibres of different types (e.g. red and white) will be present and it may not be possible to distinguish on the basis of such measurements the fuel utilised by the different types of fibre.

Incubation of excised muscle tissue (e.g. rat diaphragm, rat soleus) has been carried out with a few preparations which are easily dissected without extensive damage to muscle fibres, and which are thin enough to allow diffusion of oxygen and fuels. However, only a few muscles are amenable to these methods because of the difficulties encountered in obtaining physiologically viable intact muscle preparations which can be supplied satisfactorily with $O_2$ and fuel. Thus the methods are not suitable for more widespread application in a comparative survey of muscle types across the animal kingdom.

b) Tissue slices, homogenates and isolated cells. Tissue slices have often been used for the study of the metabolism of tissues

other than muscle (e.g. kidney, brain); the slice preparation is thin enough for adequate diffusion of gases and metabolites to take place (Krebs, 1963, 1964). Liver slices have also been used, but have been found unsatisfactory in studies on gluconeogenesis; both enzymes and co-factors leak out of the slices (Krebs, 1970). Slices of muscle tissue have not been used, as the damage to cells would be extensive, and the preparation would suffer the same disadvantage as liver slices.

Homogenates of various tissues have been used for metabolic studies, but measured rates of fuels are always lower than those provided by perfusion techniques and thus quantitative data are not available. Isolated cells such as hepatocytes (Berry & Friend, 1969) and isolated, beating heart cells (Seraydarian et al., 1970) could be used for metabolic studies; indeed, hepatocytes are already proving useful in this field. However, the heart cells have not yet been widely used for studies on fuel utilisation. It remains to be seen whether heart cells can carry out as much mechanical work as the intact perfused heart.

c) Measurement of enzyme activities. Measurement of activities in vitro of enzymes specific to different metabolic pathways of fuel utilisation has been carried out to gain information about the operation of such pathways in various tissues. These measurements can be performed quickly and relatively easily and are therefore suitable for a comparative investigation.

This approach has been used by some workers to compare the importance of pathways of fuel utilisation in different tissues (e.g. Beenakkers, 1963, 1969; Pette, 1966; Bass et al., 1969). However, the activities of most of the enzymes selected in these investigations cannot be used to provide quantitative information about the rates of operation of these pathways. The enzymes chosen catalysed reactions which are readily reversible in vivo

(i.e. near-equilibrium reactions) and quantitative information can be obtained only from the activities of enzymes which catalyse reactions which are far removed from equilibrium *in vivo* (i.e. non-equilibrium reactions) and even then such activities may not always be useful (see p. 58).

This enzymatic approach has been used in this thesis and a fuller discussion of the theory underlying the importance of enzymes catalysing non-equilibrium reactions and their identification will be given in Section 3. A brief account of the method is given here.

After the identification of "non-equilibrium" enzymes which might give an indication of the rate of operation of fuel-utilising pathways, these enzymes are then assayed under optimal conditions of, for example, pH, co-factor and substrate concentrations, and in the presence of activators required for maximal activity. The use of sensitive recording spectrophotometers enables low activities to be detected so that measurements can be done on small amounts of tissue (10-50 mg). This means that a wide variety of different muscle types from different animals may be investigated.

## 3) A SURVEY OF DIFFERENT MUSCLE TYPES

Before discussing the different fuel requirements of muscle, a brief description of different types of muscle will be given.

### (i) Classification of muscle types

Muscle tissue is characterised by its ability to perform mechanical work and the fundamental molecular mechanism seems to be the same in all muscles. However, functionally and structurally, muscles are very diverse. All muscles are composed of fibres which may be single elongated cells or syncytia. The classification of these muscle fibres or muscles can be carried out on the basis of several criteria. A brief description of four ways of classifying muscles will be given.

a) Striations. Muscles can be classified according to the presence or absence of cross striations along the length of the fibre. This reflects the degree of organisation of the contractile apparatus. Thus, in vertebrates the cardiac and skeletal muscles are striated, and the unstriated or smooth muscle is found in the blood vessels and viscera. In other phyla, striated muscles are usually found in situations where fast action is required, e.g. insect flight muscles. However, the correlation between the striated nature of the muscle and the time of contraction is not precise as some smooth muscles may have a fast contraction time, e.g. the muscles controlling the nictitating membrane of the eye of the cat. Speed of contraction may however be correlated with myofibrillar ATPase activities (see tissue typing (c) ).

b) Visible colour. Muscle fibres vary in colour from red to white, but it is possible to classify the extremes as red and white fibres. Most vertebrate muscles are a mixture of red and white fibres, though muscles can be identified in which one type of fibre predominates, e.g. in the rat, the soleus muscle is mainly red, the gastrocnemius is mainly white. The colour reflects the concentration of the myoglobin in the fibre. (In most species, the role of the myoglobin is primarily to facilitate the diffusion of oxygen through the muscle cell to the mitochondria. However, in diving mammals such as whales, myoglobin is present at higher concentrations and serves as a reservoir for oxygen.) "Redness" of muscle has been correlated with many other characteristics (e.g. see Table III). This, and other early work, gave rise to the view that red muscles were associated with prolonged activity and slow speeds of contraction whereas white muscles were associated with short-lived and vigorous activity (see Beatty and Bocek, 1970; Needham 1971 for review).

However, as we shall be dealing with other vertebrate and invertebrate muscle types, this type of classification has only a limited value.

c) Tissue typing by cytochemical means. This method of classifying muscle fibre type depends on the intensity of a stain used cytochemically to localise glycolytic or oxidative enzymes, or myoglobin. In 1962, Engel suggested the use of a stain for myofibrillar ATPase after alkaline preincubation (Engel, 1962). This method separates muscle fibres into two fairly well defined groups. He proposed the names type I and II fibres for those fibres staining light and dark, respectively. These corresponded to the red (type I) and the white (type II) fibres referred to above. By adding a stain for a mitochondrial enzyme, a sub-division of the type II fibres is sometimes possible: type IIA with high activities of, for example, succinate dehydrogenase, and type IIB with low activities of succinate dehydrogenase. However, this subdivision of the type II fibres is sometimes made on different criteria in different animals, and does not represent a clear-cut boundary. Table III summarises some of the more frequently used criteria for classifying muscle fibres, with their nomenclature.

However, this type of classification, in common with the previous two types, suffers from overlap of types as there is a continuous spectrum of these properties across the types of muscle.

Furthermore, the above three ways of classifying muscles have been based on criteria from vertebrate systems. When these classifications are applied to other muscle types (e.g. insect flight muscle) confusion is caused by the different specialisations of these muscles, and therefore these classifications are not appropriate to a comparative survey of muscle types from many different phyla.

TABLE III

A SUMMARY OF SOME OF MORE FREQUENTLY USED CLASSIFICATIONS OF MUSCLE FIBRE

This table is taken from Saltin et al. (1977) where the full references of authors using these classifications can be found.

| BASE FOR CLASSIFICATION | NOMENCLATURE | | |
|---|---|---|---|
| 1) Myoglobin or visible colour | Red | Intermediate | White |
| 2) Mitochondrial enzymes | High oxidative (HO) | Oxidative (O) | Low oxidative (LO) |
| 3) Glycolytic enzymes | Low glycolytic (LG) | Glycolytic (G) | High glycolytic (HG) |
| 4) Lipids | High content (C) | Intermediate content (B) | Low content (A) |
| 5) Myofibrillar ATPase | Slow twitch (ST) (type I) | Fast twitch (FTa)(type IIa) | Fast twitch (FTb)(type IIb) |
| 6) Combination of 2), 3) & 5) | Slow, oxidative | Fast, oxidative, glycolytic | Fast, glycolytic |

d) Physiological function. It is possible to group muscles under the three following groups according to their physiological contraction properties.

a) continuously contracting muscles (vertebrate heart, diaphragm, and postural muscles; red muscle of trout)

b) muscles undergoing sustained contraction from time to time (other vertebrate red skeletal muscles, insect flight muscles).

c) muscles undergoing intermittent and short term contractions (e.g. cat gastrocnemius, locust leg exterior muscles)

This classification will be used to survey muscle types as it may be applied throughout the animal kingdom.

ii) Specialisation of muscle types according to their physiological function

a) Continuously contracting muscles (e.g. heart, postural muscles, diaphragm). The energy demand in these muscles is relatively steady with only small changes in flux through the energy producing pathways. As a result there is a continual demand for fuel which is met via a well developed blood supply. This ensures that the tissue is well oxygenated which allows energy demands to be met by the more efficient oxidative metabolism. (See Table I).

In vertebrates, these muscles may be striated (e.g. skeletal muscle) or smooth (e.g. blood vessels) and they are usually red. The degree of energy demand varies, however, with the power output of the muscle and the rates of fuel utilisation vary accordingly. Thus the oxygen uptake and rate of fuel utilisation would be expected to be greater in heart than in diaphragm.

A sudden increase in energy demand on the heart may not be met

immediately by an increase in blood-flow due to the time lag required for vasodilation, and the energy demand could exceed the necessary oxygen and fuel supply. Thus, in heart, a sudden increase in work load results in a transient breakdown of endogenous glycogen and increase in lactate output (Daw et al., 1967; Evans, 1934). Normally, however, the heart is well supplied with oxygen, i.e. it has a very good blood supply and an indication of the efficiency of the blood supply is the low myoglobin content.

The heart can usually oxidise not only exogenous glucose, fatty acids and ketone bodies but also exogenous lactate produced by other anaerobic tissues and carried in the blood, and, if necessary, it can oxidise endogenous triglyceride as fuel for its activity (for reviews, see Opie, 1968; Randle et al., 1966).

In the fed state in vivo, the heart utilises both glucose and fatty acids and ketone bodies. However, in the isolated perfused heart, severe inhibition of glucose utilisation is caused by the simultaneous perfusion of fatty acids at levels in the perfusate equivalent to those present in the blood during starvation. This preferential use of fatty acids is also supported by experiments on man in vivo, where A-V differences across the heart shown significant linear relationships between myocardial extraction of carbohydrate and plasma free fatty acid levels (Lassers et al., 1971).

b) Muscles undergoing sustained activity from time to time. In these muscles, which have a sustained high energy demand interspersed with periods of rest, the fuels utilised could well vary between resting and active conditions. However, at the onset of activity, e.g. on the initiation of flight in birds, anaerobic breakdown of glycogen to lactate may be important. During the sustained period of activity however, a steady state will be reached where the oxygen and fuel supply provided by the blood will be

sufficient to support oxidative metabolism, and satisfy the energy demands of the muscle at work. In some muscles where the power output is very high, the energy demand will be correspondingly great. Examples of this sort of muscle are the pectoral muscle of birds and insect flight muscle.

Pigeon pectoral muscle, which contains mainly type I fibres, is deep red in colour (high myoglobin content) is highly vascularised and has a high mitochondrial content. (Ashurst, 1969). George and Jyoti in 1955 studied the histology of pigeon pectoral muscle and showed that fat globules were visible. Indeed, fat was present to 4.5% net weight of the muscles and in experiments where the pigeon was electrically stimulated to fly, the muscle showed a depletion of lipid. They concluded that fat was a major fuel for flight. (George & Jyoti, 1955).

Insect flight muscle is also capable of sustained high-energy demand activity. It contains neither myoglobin nor haemoglobin, as the oxygen is supplied directly to the muscles by an extensive tracheolar system. Weis-Fogh (1967a) has calculated that diffusion (which is several thousand fold greater in the gas than the liquid phase) coupled with abdominal and thoracic pumping, ensures that oxygen is not rate-limiting in vivo. This is remarkable considering the enormous increase in oxygen uptake on initiation of flight. Fuel is supplied to insect flight muscle via the haemolymph. The circulation of this body fluid is not as efficient as in the vertebrate closed blood system, but a high concentration of fuel in the haemolymph ensures adequate diffusion into the flight muscle.

Early studies which measured respiratory quotients of insects during flight showed that some orders of insects, such as Diptera and Hymenoptera, had an R.Q. of unity (indicating carbohydrate as fuel) while others, such as Orthoptera and Lepidoptera, had R.Q. s approaching 0.7 indicating that fat is the preferred fuel (for review see Sacktor, 1965). However, locusts,

roaches, aphids and some Lepidoptera may use both lipid and carbohydrate as fuels for flight, and the use of amino acids as fuels in some insects has already been mentioned. In the desert locust, Shistocerca gregaria, carbohydrate is used in the initial stages of flight while lipid is the major fuel for prolonged flight (Weis-Fogh, 1952). In addition, the work of Van Handel and Nayar (1972) indicates that Lepidopterans may use carbohydrate as well as fat as energy sources for flight. Finally, measurement of the activities of hexokinase and phosphofructokinase in the flight muscles of several moths show activities similar to those of the flight muscles of Dipterans and Hymenopterans. These activities suggest that the flight muscles of these Lepidopterans can utilise carbohydrates (as glucose and/or trehalose) to support flight (see Crabtree & Newsholme 1975, also for review).

In mammalian skeletal muscle of this type, both carbohydrate and fat are used as fuels. The inhibition of carbohydrate utilisation by the availability of fatty acids in the heart was described in the previous subsection. A similar effect has been demonstrated in skeletal muscle (e.g. isolated diaphragm and soleus muscles and perfused hindlimb of the rat) (Newsholme & Randle, 1964; Randle et al., 1964; Cuendet et al., 1975; Rennie and Holloszy, 1976, 1977).

Thus both fat and carbohydrate may be used as a fuel by muscles in this category. The factors controlling the interrelationship of fat and carbohydrate utilisation are complex. A brief mention is given below of fuel utilisation under conditions of starvation and sustained exercise, but a fuller account of this and other effects on the regulation of the utilisation of glucose is given in Newsholme (1976b, 1977).

c) Muscles undergoing intermittent and short term contraction. The energy demand in these muscles is immediate and sudden, and is usually met

by the anaerobic degradation of endogenous glycogen to lactate and by the breakdown of creatine or arginine phosphate. As this process is relatively inefficient in terms of ATP yield, the glycogenolytic capacity of the muscle is high to compensate for this inefficiency (Crabtree & Newsholme, 1972a).

Vertebrate white skeletal muscles, capable of bursts of vigorous activity, come into this category, and consist mainly of white or type IIB fibres (see Table III). Thus, the muscle fibres have high glycogenolytic capacity and low oxidative capacity. Insect skeletal muscles other than flight muscles are included in this group of muscles undergoing intermittent and short term contraction. These muscles may use trehalose (see Fig. 3) in addition to glycogen. As mentioned before, carbohydrate is the major fuel which can be utilised anaerobically for energy production in muscle.

The pectoral muscle of the domestic fowl is an example of white vertebrate muscle which can usefully be compared to pigeon pectoral muscle described in the previous section. In the domestic fowl, the pectoral muscle is only used for escape flight (as in the game birds) and not for sustained flight. The fibres are mainly white; myoglobin content is as low as 0.006% as opposed to 0.25% net weight in pigeon pectoral muscle; there is a poor blood supply and endogenous glycogen is used as a fuel for energy (Laurie, 1956; George & Berger, 1966).

To provide a muscle with an efficient supply of well oxygenated blood exerts a load on the heart and lungs of an animal. The lack of such specialisation in white muscle represents an economic saving in the animal as a whole, as mechanical activity is only required relatively infrequently (e.g. during escape flight) for short periods of time. Fatigue occurs in these birds after a very short burst of activity and this is consistent with their reliance on anaerobic metabolism since the endogenous glycogen store is rapidly utilised.

## 4) FUEL UTILISATION AND THE PHYSIOLOGICAL STATE OF THE ANIMAL

It has been shown in the above discussion that, depending on the availability of the different fuels used, the supply of oxygen and the type of activity carried out by the muscle, different muscles may depend on one or more types of fuel for the production of ATP. Thus sustained activity requires aerobic metabolism of fuels and bursts of activity can be supported by anaerobic glycolysis from carbohydrates. Fuel utilisation in the whole animal is also affected by the type of food, starvation and exercise.

Fuels are supplied in the diet and in most cases, this is ingested as carbohydrate and absorbed as glucose. (However, carnivores probably ingest a higher proportion of protein which is absorbed as amino acids.) In ruminants, all ingested carbohydrate is converted to volatile fatty acids by the action of bacteria in the rumen. These fuels may be used immediately for energy production or, as feeding is usually intermittent, they can be stored in the form of fat (in adipose tissue) or glycogen (in the liver and in muscle).

Fat is an important storage fuel in terrestrial animals; it is the most economic form of stored energy. This is partly because 1g of lipid (dry weight) yields twice as much chemical energy than does 1g (dry weight) of carbohydrate. In addition, while lipid may be stored "dry" due to its hydrophobic nature, carbohydrate must be stored with a large amount of associated water. These two factors contribute to the fact that fat is almost 8-fold more efficient than carbohydrate as a biological energy store (Weis-Fogh, 1967 b). This makes it an important fuel during sustained exercise in animals, e.g. in the constant search for food and especially in migration. It may be an important fuel, therefore, in insects such as the locust and some moths, some birds and fish such as the salmon. Migratory birds synthesise enormous quantities of fat which is then consumed during flight (Odum, 1965).

Fat is also stored for use in hibernation and may be mobilised in other situations when the animal is not feeding, i.e. in starvation. In man, during starvation or prolonged exercise, the R.Q. approaches 0.7 indicating that fat is the major fuel of the body. During rest and especially after a carbohydrate meal, the R.Q. approaches 1.0 indicating that carbohydrate is the major fuel of the body (Newsholme, 1976b).

During starvation, the energy requirements of some tissues (e.g. muscle, liver and kidney cortex) can be met from the oxidation of fatty acids. Some tissues, however, require glucose; e.g. brain and other nervous tissue, at least in the early part of starvation, erythrocytes, white cells, testis, retina of eye and kidney medulla. Hence during starvation carbohydrate must be conserved for use by these tissues. Consequently, physiological mechanisms exist which ensure that, under conditions of starvation (or sustained exercise which can be considered as a form of starvation), lipid oxidation supplements and/or replaces carbohydrate oxidation by muscle (see 3a and 3b above).

In man, the amount of glucose required by the brain is about 120g per day and the requirement of the obligatory anaerobic tissues is about 40g per day (Cahill & Owen, 1968). Glycogen stores are restricted (about 0.2% total reserve of fat in the average human subject i.e. 70 g) and it will be mobilised between meals or in the early stages of starvation in the resting subject (e.g. during the overnight fast). Further starvation will require de novo synthesis of glucose from endogenous precursors in the liver (in early stages) and kidney ( during prolonged starvation) at least in man. The main precursors for gluconeogenesis are lactate, glycerol and the amino acids; these are derived from anaerobic glycolysis in blood cells, lipolysis in adipose tissue and protein degradation in skeletal muscle, respectively.

In the early stages of starvation in man, gluconeogenesis provides approximately 160 g of glucose per day for the various tissues. During further starvation, the glucose requirement by the brain is gradually reduced, due to a gradual increase in the rate of oxidation of ketone bodies, so that the rate of gluconeogenesis is gradually reduced. The rate of glucose production from amino acids during prolonged starvation of obese subjects decreases from about 90g per day initially to about 15 g per day after five to six weeks starvation, whereas glucose production from lactate and glycerol is unchanged (Owen et al., 1969).

Most of the amino acids (60%) released by muscle during starvation consist of alanine and glutamine despite the fact that these amino acids constitute less than 10% of the residues in muscle protein. This release of alanine and glutamine also accompanies the oxidation of amino acids in preparations of muscle tissue incubated in vitro (see Section 1, 2c, above), and the amount released by muscle increases during starvation (Felig & Wahren, 1974). In the scheme proposed in Section 1, 2c), the pyruvate produced from the partial oxidation of amino acids may either enter the TCA cycle via the production of acetyl CoA by pyruvate dehydrogenase or be transaminated to alanine and released from the muscle. During starvation, pyruvate dehydrogenase is inhibited (see Garland et al., 1964; Wieland et al., 1971) and so the fate of the pyruvate is limited to transamination to alanine.

As mentioned before, sustained exercise may be considered as a form of starvation and this is because similar mechanisms may be involved in the control of fuel utilisation. Upon the onset of exercise, muscle glycogen is the major fuel consumed. As exercise continues steadily, there is a progressive decline in the use of glycogen and both glucose uptake and fatty acid oxidation by the muscles increase. The contributions of glucose, fatty acids and endogenous fuels in increasing lengths of exercise are

TABLE IV

PERCENTAGE CONTRIBUTION OF FUELS TO THE OXYGEN UPTAKE OF THE LEG MUSCLES OF EXERCISING MAN

Taken from Felig & Wahren (1975)

| TIME min. | GLUCOSE % | FATTY ACIDS % | ENDOGENOUS FUELS % |
|---|---|---|---|
| 10-40 | 30 | 40 | 30 |
| 90 | 41 | 37 | 22 |
| 180 | 36 | 50 | 14 |
| 240 | 30 | 62 | 8 |

demonstrated in Table IV.

Thus there is an increasing dependence on fatty acids, a continual requirement for glucose and a decreasing dependence on endogenous fuels. The glucose is provided by the liver from glycogen breakdown and from gluconeogenesis. In the resting state, 25% of the glucose produced by the liver comes from the gluconeogenic precursors (alanine, lactate, glycerol and amino acids) and 75% is from glycogenolysis. As total glucose production increases during prolonged exercise, the relative contribution of gluconeogenesis rises to 45% which corresponds to a three-fold rise in the absolute rate of gluconeogenesis (Ahlborg et al., 1974). Ahlborg in 1974 measured the gluconeogenic substrates across the splanchnic vascular bed during exercise and his data show that during prolonged exercise lactate contributes 44% of the glucose production by liver, glycerol contributes 32% and the amino acids contribute 18%.

## 5) SUMMARY

It can be seen that the three main types of muscle, classified according to their physiological function and having different metabolic requirements, will show different rates of fuel utilisation in different metabolic pathways. In order to understand the operation of these muscles, it is important to have quantitative data on the rates of operation of these pathways. For instance, in some highly specialised muscles, such as insect flight muscle, which is highly adapted to high rates of aerobic respiration, problems arise in the understanding of the control of these pathways. Indeed, in comparative surveys of muscles throughout the animal kingdom, other such specialisations may be discovered, and knowledge of the rates of operation of metabolic pathways may throw new light on mechanisms of control. However, as has been shown, most methods of investigation of

fuel utilisation in muscle require much time and effort in the setting up of suitable preparations, and these methods do not lend themselves well to use in comparative surveys. Thus we have comparatively little information on the rates of fuel utilisation in many vertebrate and invertebrate muscles. The exceptions to this are the perfused working rat heart and in skeletal muscle in man, on whom many arteriovenous difference studies have been carried out.

Maximum enzyme activity measurements have been introduced as a relatively simple experimental method for investigating comparative surveys of rates of operation of metabolic pathways. However, it is necessary to elaborate on the theoretical basis of this experimental approach, before it can be seen which enzymes should be investigated.

## SECTION 3: THE THEORY UNDERLYING THE ENZYMATIC APPROACH TO FUEL UTILISATION

### 1. INTRODUCTION

On p.16, it was stated that quantitative information about the maximum rates of utilisation of fuels could be gained from the *in vitro* maximum activities of some enzymes catalysing non-equilibrium reactions *in vivo*. As this enzymatic approach is used in this thesis, the theory underlying the approach will be discussed in more detail.

In near-equilibrium reactions, the rates of both the forward and backward reactions are much greater than the net flux through the pathway and the enzymes are not saturated with substrate. In the assay of such enzymes *in vitro*, the enzymes are saturated with substrate and the measurement of the rate of the reaction in performed in one direction only. Consequently, the maximum catalytic activity *in vitro* will be much higher than the maximum rate of the pathway. In non-equilibrium reactions, the rate of

the reverse reaction is negligible (compared to that of the forward rate) so that the rate of catalysis in the forward direction will be equal to the rate of flux through the pathway. Furthermore, in some non-equilibrium reactions (flux generating steps, see later discussion) the enzyme is saturated with substrate or nearly saturated, so that the maximum activity measured *in vitro* may be similar to the maximum rate of the pathway *in vivo*, and can be used as an indicator of flux through that pathway.

## 2) TYPES OF REACTIONS IN METABOLIC PATHWAYS

In order to understand the control of the flux through metabolic pathways, it is important to define the types of reaction involved. In the living cell, most metabolic pathways are normally maintained in a steady state. This steady state is characterised by a constant flux through the pathway, constant concentrations of the intermediates of the pathway and a constant ability to do work. The control of metabolic pathways concerns the maintenance of the properties of the system when it changes from one steady state to another.

The enzyme catalysed reactions which comprise a metabolic pathway are such that they maintain the above properties. It is established that these reactions consist of two types: near-equilibrium reactions and non-equilibrium reactions. It can be seen that if all the reactions of a pathway were at equilibrium, the intermediates of the pathway would be maintained at a constant level but there would be no net flux nor ability to do work. Similarly, while there is a constant flux through the system, all the reactions of the pathway will be removed from equilibrium. However there is a functional difference in that some reactions are very close to equilibrium and some are very far removed from equilibrium. The latter play an important role in providing directionality for the flux through the pathway.

A specialised type of non-equilibrium reaction of a metabolic pathway is the flux generating step (see Newsholme & Crabtree, 1973). This can be defined as having the following properties:

1) It is the first non-equilibrium reaction of a pathway in which the enzyme is saturated with the pathway substrate. (The pathway substrate is that substrate which represents the flow of residues through the pathway.) This reaction is therefore pathway substrate-independent.
2) It generates flux to which the other reactions must respond.
3) It must be regulated by external factors (e.g. allosteric effectors, co-substrates).

Thus, flux generating steps can be seen to maintain the properties of the steady state of metabolic pathways. For example, if the first step were not saturated with the pathway substrate, the flux would decrease whenever the substrate concentration decreased and the steady state would not be maintained. From these properties it is also necessary to redefine a metabolic pathway as a sequence which is initiated by one flux generating step and terminates at a reaction preceding another flux generating step or at a reaction where the end product is dissipated from the system.

The other reactions of a metabolic pathway (i.e. near-equilibrium reactions and non-equilibrium reactions) also play important roles in the control of metabolic pathways. Their properties may be outlined as follows:

a) Non-equilibrium reactions.

1) A non-equilibrium reaction provides unidirectionality in a pathway.
2) It transmits the flux generated by the flux generating step by responding to changes in the pathway substrate (i.e. it is a substrate-dependent reaction).

3) It can be regulated by external factors (e.g. co-factors, allosteric regulators).

b) Near-equilibrium reactions

1) A near-equilibrium reaction transmits flux generated by the flux generating step (i.e. it is a pathway substrate-dependent reaction).

2) It can also be regulated by external factors (e.g. co-substrates, co-factors, products, co-products) but it is unlikely that the flux will be regulated by allosteric effectors.

3) Flux through the reaction is very sensitive to changes in the concentrations of regulators (co-substrates and the pathway substrate).

Thus, while both near-equilibrium and non-equilibrium reactions other than flux generating steps are pathway substrate-dependent, flux generating steps are pathway substrate-independent.

While all these reactions are regulated, the non-equilibrium and the near-equilibrium reactions play different roles in the metabolic pathway. Regulation of non-equilibrium reactions is more likely to change the flux through the pathway, and therefore these reactions may play a role in the control of the pathway. The regulation of near-equilibrium reactions may serve other functions, e.g. in reducing large fluctuations in the metabolite levels *in vivo*. The flux through a near-equilibrium reaction is very sensitive to changes in the concentration of its substrates and products compared with reactions that are non-equilibrium. Under some conditions, near-equilibrium reactions can accommodate large changes in flux with only small changes in the concentration of the pathway substrate. This is discussed more fully in Crabtree & Newsholme (1978) and Crabtree (1976b).

In general, simple reversible binding mechanisms can give at best only a 1:1 relationship between effector concentration and the rate of a reaction. This ratio can be improved to a certain extent by a degree of

co-operativity in the enzyme (i.e. a change from hyperbolic to sigmoidal response of activity to effector concentration.) Higher sensitivity to effector concentration can be achieved by substrate cycling or by an interconversion cycle (Newsholme, 1978; Newsholme & Crabtree, 1973, 1976).

These theoretical principles form the basis of an experimental approach to metabolic regulation, which is explained in Newsholme & Start (1973) and Crabtree & Newsholme (1975), and which includes the enzymatic approach used in this thesis. A brief description of the experimental approach will be given before its application to pyruvate oxidation and the TCA cycle is muscle is discussed.

## 3) AN EXPERIMENTAL APPROACH TO THE REGULATION OF METABOLIC PATHWAYS

One approach to the regulation of metabolic pathways, based on the principles outlined above, consists of the following procedure:

(i) Identification of the equilibrium nature of the reactions (i.e. whether near equilibrium or non-equilibrium).

(ii) Identification of the flux generating steps.

(iii) Identification of those reactions which are regulated by factors other than their pathway substrates, i.e. subject to external regulation.

(iv) The properties of the enzymes catalysing flux generating steps and externally regulated reactions are investigated in detail *in vitro*.

(v) On the basis of these properties a theory of metabolic control is formulated.

This approach depends on knowledge of the details of the biochemistry and physiology of the pathway. One serious drawback to many studies of metabolic regulation is that the properties of the enzymes are investigated

*in vitro* without identifying the equilibrium nature of the reactions catalysed by those enzymes in the metabolic pathway. One of the dangers in this approach is that, while evidence for inhibition or activation of an enzyme may be easy to obtain *in vitro*, a theory of control based on this evidence would be misleading, as the inhibition (or activation) may not be significant *in vivo*. This sort of error arises because such an approach fails to predict the influence of changes in the activity of the enzyme on the flux through the pathway.

(i) Identification of the equilibrium nature of the reactions.

There are three main ways of identifying the equilibrium nature of a reaction:

(a) Comparison of mass action ratio with the equilibrium constant

(b) Measurement of the forward and backward rates of the reaction *in vivo*.

(c) Measurement of the maximum catalytic activity of the enzymes *in vitro* and comparison with each other or with maximum flux.

These methods are based on different theoretical considerations of a reaction. Method (a) derives from a thermodynamic approach, methods (b) and (c) derive from a kinetic approach.

(a) Thermodynamic approach: Comparison of mass action ratio with the equilibrium constant

The ratio of the concentration of products to those of substrates, as measured in a tissue, is known as the mass action ratio and is designated by the symbol, $\Gamma$ (Bücher & Rüssman, 1964). This is related to the free energy change of a reaction ($\Delta G$) in the following way:

$$\Delta G = -RT \ln \frac{K}{\Gamma}$$

where R is the universal gas constant, T is the absolute temperature of the reaction and K is the equilibrium constant of the reaction (see Appendix 1 for the derivation of this relationship). The ratio of $K/\Gamma$ has been used

as a measure of displacement from equilibrium (Bücher & Rüssman, 1964; Rolleston & Newsholme, 1967). This is because when $K/\Gamma$ is unity, $\Delta G$ is zero, so that the reaction is at equilibrium. When the value of $K/\Gamma$ is greater than unity, $\Delta G$ is negative, so that the reaction is displaced from equilibrium. Thus it has been customary to use the ratio $K/\Gamma$ as an indication of the displacement from equilibrium. However, it is perhaps more logical, on thermodynamic grounds, to calculate the value of $\Delta G$ and use this to indicate the equilibrium nature. If $\Delta G$ is numerically small, the reaction is near-equilibrium, and if $\Delta G$ is numerically large, the reaction is non-equilibrium. Newsholme & Crabtree in 1976 suggest that a $K/\Gamma$ ratio of 5 is a reasonable empirical division between near-equilibrium and non-equilibrium reactions. This value gives rise to a free energy change of -991 cal. $mol^{-1}$ (at $37^{o}C$) or -953 $cal.mol^{-1}$ (at $25^{o}C$). Thus, a reaction whose $\Delta G$ is numerically smaller than approximately 1 $kcal.mol^{-1}$ can be considered near-equilibrium, and a reaction whose $\Delta G$ is numerically greater than approximately 1 $kcal.mol^{-1}$ can be considered non-equilibrium. Thus, from a knowledge of the values of the equilibrium constant of a reaction and the mass action ratio, the values of $\Delta G$ can be calculated, which will give an indication of the equilibrium nature of that reaction.

(b) Kinetic approach: Measurement of forward and backward rates of a reaction in vivo

If the rates of the forward and the reverse direction of a reaction can be measured in vivo, (i.e. the actual rates rather than the rate constants) the ratio of the rates can provide a measure of the displacement of the reaction from equilibrium. It can be shown that, if $V_f$ is the rate in the forward direction, and $V_r$ is the rate in the reverse direction, then

$$\frac{V_f}{V_r} = K/\Gamma \quad \text{(see appendix II)}$$

$K/\Gamma$ has already been shown to be measure of the displacement from equilibrium (see above).

The forward and reverse rates of a reaction can be measured *in vivo* by using isotopic methods. However, there are very few studies using this approach. Randle and co-workers have done this for the isocitrate dehydrogenase reaction in perfused rat heart. (Randle *et al.*, 1970). The flux through the TCA cycle and hence the flux through this reaction (J) was measured using $C^{14}$-acetate, and $V_r$ was measured by following the incorporation of $C^{14}$-bicarbonate into isocitrate. The rate of the forward reaction, $V_f$, was calculated from the relationship $J = V_f - V_r$ and it was shown that $V_r$ was 46% of $V_f$, thus indicating a reaction close to equilibrium. ($K/\Gamma$ approximately 2 and $\Delta G$ approximately -0.4 $kcal.mol^{-1}$). This method has not been widely used to investigate the equilibrium nature of reactions.

(c) *Measurement of the maximum catalytic activity of the enzyme in vitro*

A non-equilibrium reaction in a metabolic pathway arises because the enzyme which catalyses this reaction is not sufficiently active compared with the other reactions of the pathway to bring the substrates and products to equilibrium. This provides a means of identifying the equilibrium nature of the reactions in a pathway. If the maximum catalytic activities of all the enzymes of a pathway are measured *in vitro*, the enzymes can be divided into two classes: low activity and high activity enzymes. The enzymes with low activity may be considered to catalyse non-equilibrium reactions in the cell.

However, a clearer picture may be seen if it is possible to measure the maximum flux through the pathway. A comparison of the maximum catalytic activities ($V_{max}$) with the maximum flux through the pathway can give an indication of the equilibrium nature of the reaction (Crabtree & Newsholme,

1975). Thus, if $V_{max}$ is approximately equal to the maximum flux, the reaction is considered to be far displaced from equilibrium, and if $V_{max}$ is much greater than the maximum flux, the reaction is considered to be near equilibrium. However, the latter need not necessarily be true if the enzyme is inhibited to a great extent *in vivo*. Ideally data on maximal enzyme activities should be supported by mass action ratio data in order to relate to the situation *in vivo* as closely as possible.

Other problems arise from extrapolation from the studies *in vitro* to the situation in the cell. Firstly, it is important to assay the enzymes under optimal conditions to measure the $V_{max}$, and for this, some preliminary information on the properties of each enzyme is essential before this method is attempted. Although it is desirable that the enzyme is extracted in a crude homogenate, in which the properties of the enzyme relevant to metabolic control are likely to be preserved, it may be necessary to purify the enzyme to some extent to assay its activity satisfactorily. Secondly, an enzyme may catalyse an equilibrium reaction under one set of conditions but under other conditions it may be inhibited sufficiently to catalyse a non-equilibrium reaction. If it is possible to obtain mass action ratio data for each set of conditions, this complementary information may help elucidate such a situation.

The use of maximum enzyme activities has been used extensively to study the control of many metabolic pathways. A good example of the application of this approach is the study of control of glycolysis (see Tables V and VI and Newsholme & Start, 1973).

The maximum activities of the glycolytic enzymes from a variety of tissues (measured *in vitro* under optimum conditions) are presented in Table VII with estimated values of glycolytic flux. (The tables V and VI are taken from Newsholme & Start (1973) and table VII is taken from Crabtree & Newsholme, 1972a).

TABLE V

Maximal activities of glycolytic enzymes in different tissues.

The enzyme activities are expressed as $\mu mol.min^{-1}.g^{-1}$ (fresh weight).

This table is taken from Newsholme & Start (1973).

| Reactions catalysed by: | Enzyme Activities | | | |
|---|---|---|---|---|
| | Brain | Heart | Skeletal Muscle | Erythrocytes |
| Hexokinase | 17 | 7 | 1.5 | 0.3 |
| Phosphoglucoisomerase | 80 | 65 | 176 | 5.6 |
| Phosphofructokinase | 24 | 14 | 56 | 1.8 |
| Aldolase | 15 | 24 | 78 | 0.7 |
| Triosephosphate isomerase | 415 | 580 | 2,650 | 97 |
| Glyceraldehyde-3-phosphate dehydrogenase | 105 | 135 | 440 | 17.1 |
| Phosphoglycerate kinase | 610 | 74 | 169 | 25.6 |
| Phosphoglycerate mutase | 122 | 27 | 100 | 8.6 |
| Enolase | 47 | 15 | 158 | 1.6 |
| Pyruvate kinase | 164 | 145 | 387 | 4.6 |
| Lactate dehydrogenase | 100 | 386 | 366 | 20.4 |

TABLE VI

A Comparison of Apparent Equilibrium Constants with Mass-action ratios for the reactions of Glycolysis

This table is taken from Newsholme & Start (1973)

| Reactions catalysed by: | Apparent equilibrium constant (K') | Mass-action ratios ( $\Gamma$) | | | | Units of K' and $\Gamma$ * |
|---|---|---|---|---|---|---|
| | | Brain | Heart | Erythrocytes | Ascites tumour | |
| Hexokinase | $3.9\text{-}5.5 \times 10^{3}$ | 0.04 | 0.08 | $7.6 \times 10^{-4}$ | $2.6 \times 10^{-2}$ | |
| Phosphoglucoisomerase | 0.36-0.47 | 0.22 | 0.24 | 0.41 | 0.23 | |
| Phosphofructokinase | $0.9\text{-}1.2 \times 10^{3}$ | 0.13 | 0.03 | $4.4 \times 10^{-2}$ | 0.63 | |
| Aldolase | $6.8\text{-}13.0 \times 10^{-5}$ | $2.4 \times 10^{-6}$ | $9 \times 10^{-6}$ | $1.4 \times 10^{-5}$ | $2.9 \times 10^{-5}$ | M |
| Triosephosphate isomerase | $3.6\text{-}4.5 \times 10^{-2}$ | - | 0.24 | 0.35 | 1.5 | |
| Glyceraldehyde-3-phosphate dehydrogenase plus phosphoglycerate kinase | $0.2\text{-}1.5 \times 10^{3}$ | 53 | 9 | 124 | 17.8 | $M^{-1}$ |
| Phosphoglycerate mutase | 0.1-0.2 | 0.1 | 0.12 | 0.15 | 0.43 | |
| Enolase | 2.8-4.6 | 3.6 | 1.4 | 1.7 | 1.6 | |
| Pyruvate kinase | $2\text{-}20 \times 10^{3}$ | 5.4 | 40 | 51 | 2.8 | |

*Unless otherwise stated, K' and $\Gamma$ are dimensionless.

TABLE VII

Rate of Carbohydrate Utilisation by Intact Muscles

This table is taken from Crabtree & Newsholme (1972a). The full legend and the sources of the data may be found in the original table.

| Animal | Method of Estimation | Rate of carbohydrate utilisation necessary to account for the measured metabolic rate (as $\mu$mol of $C_6$ unit.$min^{-1}$.$g^{-1}$ of fresh muscle at 25°C) | Enzyme activity ($\mu$mol.$min^{-1}$.$g^{-1}$ of fresh muscle at 25°C) | |
|---|---|---|---|---|
| | | | Hexokinase | Phosphorylase |
| Locust (*Schistocerca gregaria*) | Oxygen uptake of flying insect | 14.0 | 11.5 | 7.5 |
| Cockroach (*Periplaneta americana*) | Oxygen uptake of flying insect | 15.0 | 18.0 | 30.0 |
| Honey-bee (*Apis mellifera*) | Oxygen uptake of flying insect | 32.0 | 29.0 | 4.0 |
| Blowfly (*Lucilia sericata*) | Oxygen uptake of flying | 59.0 | 35.0 | 55.0 |
| Trout (*Salmo gairdneri*) | Oxygen uptake during continuous swimming | 1.2 | 2.6 | 14.0 |
| Frog (*Rana temporaria*) | Isolated sartorius muscle incubated anaerobically: rate of lactate production | 13.0 | 1.3 | 29.0 |
| Black duck (*Anas rubripes*) | Oxygen uptake of flying bird | 1.5 | 3.8 | 50.0 |
| Rat heart | Perfused, *in vitro*, working against 10 cm $H_2O$ ventricular pressure: glucose uptake measured | 1.2 | 6.1 | 12.0 |
| Mouse skeletal muscle | Electrically stimulated *in situ*: lactate production measured | 30.0 | 3.9 | 34 |

If the relative activities of glycolytic enzymes are considered, it appears that the enzymes hexokinase, phosphofructokinase, aldolase and in most cases enolase have low activities in comparison with the remainder of the glycolytic enzymes. Thus, from the criterion of maximum enzyme activity, these enzymes should be classified as catalysing non-equilibrium reactions. However, from the data on mass action ratios of these enzymes (Table VI) only hexokinase, phosphofructokinase and pyruvate kinase can be definitely classed as non-equilibrium enzymes (suggesting that the assay of the activities of enolase and aldolase in vitro does not give a good indication of their maximum activities).

The flux through glycolysis can be estimated from rates of carbohydrate utilisation calculated from oxygen uptake, production of lactate or utilisation of glycogen. When this value of the flux is compared with the maximum activities of hexokinase and phosphorylase (see Table VII) it can be seen that there is reasonable agreement between the rates. Hexokinase activity is similar to the flux in cases when glucose may be oxidised and phosphorylase activity is similar to the flux in cases when glycogen may be utilised. Thus the hexokinase and phosphorylase reactions are non-equilibrium reactions limiting the rate of glycolysis from glucose and glycogen respectively.

It follows from this approach that if the comparison of activities with the flux can be used to demonstrate the non-equilibrium nature of a reaction, then the maximum activity of the enzyme (measured in vitro) catalysing that reaction can be used as an indicator of the flux through that pathway. This is therefore a very important tool for quantitative evaluation of maximum flux through fuel utilising pathways.

(ii) Identification of flux generating steps

Flux generating steps are non-equilibrium reactions, and therefore the identification of the equilibrium nature of the reaction must be carried out before identifying flux generating steps. Flux generating steps may be identified in two ways. Firstly, the $K_m$ of the enzyme for the pathway substrate may be measured *in vitro* and compared with the substrate concentration *in vivo*. If the concentration of the pathway substrate *in vivo* is much higher than the $K_m$ (perhaps between 2-5 times higher) it may be assumed that the enzyme catalysing the reaction is saturated with substrate. This is one of the properties of flux generating steps (see above).

Secondly, the concentration of the pathway substrate may be changed in the preparation or animal under investigation and the flux through the reaction monitored. If there is no change in flux, then again it may be assumed that the enzyme catalysing the reaction is saturated with substrate.

(iii) Identification of those reactions which are externally regulated

If a reaction is regulated by factors other than its pathway substrate it is said to be subject to secondary or external regulation. As can be seen from the definitions on pp.29-30, all types of reactions may be subject to this type of regulation, though only non-equilibrium reactions would be expected to be regulated by allosteric effectors. A metabolic pathway may therefore contain several externally regulated reactions, or it may contain only one - the first reaction, the flux generating step. (In which case, the other reactions would follow a change in flux by responding to a change in the level of pathway substrate). It is therefore important to identify how many of these externally regulated reactions occur in a metabolic pathway, before proposing a theory of control.

This may be done by altering the flux through a pathway *in vivo* and following the change in concentration of the pathway substrate of the reaction to be investigated. If the pathway substrate concentration changes in the opposite direction to the change of flux (i.e. it increases when the flux through the pathway decreases and vice versa) then some modulator apart from the pathway substrate must be altering the flux through the pathway.

(iv) *Studies of the properties of the enzymes catalysing externally regulated reactions*

The purpose of such study is to identify the effector (or effectors) which will modify the activity *in vitro* and which are likely to control the enzyme *in vivo*. For near-equilibrium reactions, these effectors may be the co-substrates, the products or the co-products, or all of these. But for non-equilibrium reactions, the effector may be a co-substrate or an allosteric effector, and the latter may prove difficult to identify. Often this is a process of trial and error, but the nature of the effector may be predicted from an understanding of the role of the metabolic pathway.

Although purification of the enzyme may be necessary for accurate assays for kinetic analysis, this may remove or conceal important control properties. It is important to carry out initial studies with crude extracts of the enzyme, using a suitable medium in which the maximum catalytic activity of the enzyme is retained.

(v) *Formulation of a theory of metabolic control and the evidence supporting it*

Having identified the enzymes which catalyse flux generating steps and those which are subject to secondary regulation and having investigated their properties, one can next postulate a theory of control. This must

accommodate itself to most of the accepted physiological facts relating to that particular pathway, and predict experimentally, testable consequences. The theory has to be based on the properties of enzymes measured *in vitro* and the extrapolation from this to the situation *in vivo* may sometimes present problems. One of these problems is compartmentation. It is not always possible to predict the conditions likely to prevail in the microenvironment of the appropriate sites on the enzymes. Also, the enzymes are frequently present in the cell at concentrations much higher than those used in current experimental assays. For example, it was found that at low concentrations of citrate synthase, (concentrations normally used in the assay), ATP inhibited the enzyme (Smith & Williamson, 1971). However, studies performed on yeast cells made permeable with toluene allowed the properties of this enzyme to be studied *in situ*, and no such inhibition could be shown (Weitzmann & Hewson, 1973). Thus, properties of enzymes discovered *in vitro* may not always be applicable *in vivo*.

## 4) CONCLUSION

In this section, the theoretical basis of the enzymatic approach to the investigation of rates of fuel utilisation has been explained. This enzymatic approach, the measurement of maximum catalytic activities (or $V_{max}$) of enzymes *in vitro*, is part of an experimental approach to metabolic control originally introduced by Newsholme & Start (1973). The application of this method to glycolysis has been outlined, but the approach has also been used to obtain information concerning the flux through the following pathways in muscle: lipolysis and fatty acid oxidation (Crabtree & Newsholme, 1972b), proline oxidation (Crabtree & Newsholme, 1970) and the TCA cycle (Alp *et al.*, 1976; Rowan & Newsholme, 1979).

Although much work has been done on the control of pyruvate oxidation and the TCA cycle (for example, the work of groups associated with Williamson, J.R., Randle, P.J. and Hansford, R.G.) this has not involved a rigorous application of the enzymatic approach explained in this section. The importance of these two pathways, and the problems inherent in their control (especially in such specialised muscle as insect flight muscle) has already been stressed in Section One.

In the next section, the extent to which the experimental approach to metabolic regulation has been applied to pyruvate oxidation and the TCA cycle will be reviewed.

## SECTION FOUR: ENZYMES OF PYRUVATE OXIDATION AND THE TCA CYCLE IN MUSCLE

### 1) INTRODUCTION

This section will be concerned with the application of the experimental approach to metabolic regulation to the processes of pyruvate oxidation and the TCA cycle. As described earlier, the requirement for energy during contraction in muscle raises problems in the understanding of the control of the major energy producing pathways, i.e. pyruvate oxidation and the TCA cycle.

The importance of the TCA cycle in muscle has already been stressed (pp. 10-11). Pyruvate oxidation is the process linking glycolysis with the TCA cycle, and therefore constitutes the route by which carbohydrate, a major fuel for muscle contraction, enters the TCA cycle. This process consists of two reactions: pyruvate transport into the mitochondrion and the conversion of pyruvate to acetyl CoA, catalysed by the enzyme pyruvate dehydrogenase (PDH). During contraction, the flux through the PDH reaction may be greatly increased, especially if carbohydrate is the sole fuel for aerobic metabolism. It is therefore very important to look at the equilibrium nature, and hence the control, of this reaction.

It is also important to note that an assessment of the theory of control of pyruvate oxidation and the TCA cycle is necessary to understand properties of the enzymes, which may vindicate their use *in vitro* as indicators of flux.

### 2) PYRUVATE OXIDATION

#### (i) The equilibrium nature of the PDH reaction

Data on the equilibrium nature of the PDH are scarce. Information about the equilibrium nature of a reaction can be gained by comparison of the mass action ratio with the $K_{eq}$, by measurement of the forward and reverse rates of a reaction *in vivo* or by comparison of the maximum activity *in vitro*

TABLE VIII

COMPARISON OF MASS ACTION RATIO DATA OF THE PYRUVATE DEHYDROGENASE REACTION WITH THE EQUILIBRIUM CONSTANT

The equilibrium constant for the reaction

$$\text{pyruvate}^- + \text{CoA} + \text{NAD}^+ \longrightarrow \text{acetyl CoA} + CO_2 + \text{NADH}$$

is calculated from the $\Delta G^{o'}$ of - 9400 $cal.mol^{-1}$ (see Appendix V and Krebs & Kornberg, 1957). The mass action ratio data were calculated from Rowan (1975) for insect flight muscle and Siess et al.(1977) for rat liver mitochondria separated from isolated hepatocytes by the digitonin method. The concentration of $CO_2$ is taken to be $1.12 \times 10^{-3}$M (Williamson et al., 1969). For insect flight muscle, all the metabolites are assumed to be in the mitochondria (boundary condition A, see p. 45). Mass action ratios calculated under boundary condition B are shown in brackets. The range of values of the mass action ratios for rat liver is due to the different estimates of the mitochondrial free concentration ratio of $NAD^+/NADH$ given by Siess et al. (1977).

| Tissue | Conditions | Mass action ratio | $K_{eq}$ | $\Delta G$ at 25°C $kcal.mol^{-1}$ |
|---|---|---|---|---|
| Rat liver | mitochondria separated from hepatocytes | $4.0$ to $0.27 \times 10^{-7}$ | $8.4 \times 10^{6}$ | -19.3 to -19.8 |
| Locust flight muscle | at rest | $4.3 \times 10^{-1}$ (1.2) | $8.4 \times 10^{6}$ | - 9.9 (-9.3) |
| | 3 mins. flight | $3.4 \times 10^{-2}$ ($9.6 \times 10^{-2}$) | | -11.4 (-10.8) |

with the maximum flux *in vivo* (see pp. 33-36 ).

a) Mass action ratio versus $K_{eq}$: Mass action ratio data for PDH are difficult to interpret due to the problem of compartmentation. That is, although tissue levels of the participants of the reaction can be measured, the distribution between mitochondria is not known (e.g. for $CO_2$, pyruvate, acetyl CoA and $H^+$). However, a technique has been developed for the measurement of cytoplasmic and mitochondrial levels of metabolites in isolated rat liver cells (see Zuurendonk & Tager, 1974). Mitochondria are separated from isolated hepatocytes by treatment of the cells with digitonin, followed by rapid centrifugation. Evidence was provided that the extraction technique caused minimum change in the concentrations of the metabolites investigated from those *in vivo*. This is the best method so far developed for the measurement of intramitochondrial concentrations of metabolites under physiological conditions. (This technique will be referred to as the digitonin method). Presumably, the technique may eventually be applied to muscle, although the technical difficulties are greater.

It is possible to calculate the mass action ratio for the PDH reaction from the *in vivo* concentrations of the substrates and products within rat liver mitochondria separated by the digitonin method. The data are taken from Siess *et al*. (1977), with the assumption that the mitochondrial free concentration ratio of $NAD^+$/NADH can be estimated from the concentrations of the substrates and products of the following reactions: malate dehydrogenase, β-hydroxybutyrate dehydrogenase or glutamate dehydrogenase. This calculated mass action ratio is shown in Table VIII. For comparison, the mass action ratio has also been calculated for the enzyme from locust flight muscle (data from Rowan, 1975; Rowan & Newsholme, 1979). For this calculation, tissue concentrations of metabolites are converted to mitochondrial concentrations using conversion factors and assumptions which are explained in subsection (ii) below, and in Appendix IV.

As the data in Table VIII show, $\Delta G$ is very large. For rat liver, $\Delta G$ is approximately -19 kcal.mol$^{-1}$ and for locust flight muscle, $\Delta G$ is approximately -10 kcal.mol$^{-1}$. It therefore seems very likely that the reaction is far displaced from equilibrium. Interestingly, the mass action ratio for the reaction in locust flight muscle does not differ whether measured metabolites are assumed to be evenly distributed between mitochondria and cytoplasm, or whether they are assumed to be localised totally within the mitochondria. These boundary conditions are discussed below.

b) Measurement of $V_f$ and $V_r$ *in vivo*: This technique has apparently not been applied to the PDH reaction. The overall reaction catalysed by PDH can be separated into three component reactions. (See Denton *et al.*, 1975, for review). The first reaction, the decarboxylation, is considered to be irreversible. This would indicate that the overall reaction catalysed by the complex is also irreversible. However, the individual rates in the forward and reverse directions through the reaction *in vivo* have not been measured.

c) Comparison of the maximum activity *in vitro* with the maximum flux *in vivo*: Data on the maximum flux through PDH in brain, and its maximum activity *in vitro* are presented by Cremer & Teal (1974) and Blass *et al.* (1975). Cremer and Teal estimate the flux through the PDH reaction *in vivo* to be 0.52 to 0.94 $\mu$mol.min$^{-1}$.g$^{-1}$ from isotopic data, and the maximum *in vitro* catalytic activity was 1.0 $\mu$mol.min$^{-1}$.g$^{-1}$. Similarly, Blass estimated 1.2 to 2.3 $\mu$mol.min$^{-1}$.g$^{-1}$ for the flux through the reaction from oxygen uptake data and 2.0 to 3.7 $\mu$mol.min$^{-1}$.g$^{-1}$ for the maximum activity of PDH *in vitro*. This suggests that the reaction is far displaced from equilibrium in brain. The maximum catalytic activity ($V_{max}$) of PDH *in vitro* has been measured in the present study, in various different muscles and these data can therefore be used as further evidence in the assessment of the equilibrium nature of the PDH reaction. (see Chapter Four).

(ii) Is the PDH reaction a flux generating step?

The identification of a flux generating step involves the comparison of the concentration of the pathway substrate with the $K_m$ value of the enzyme for that substrate (see p.38). Although the distribution of pyruvate between mitochondria and cytoplasm is not known, it is possible to calculate the intramitochondrial concentration of pyruvate from measured tissue levels. The concentration can be calculated under two boundary conditions and it can be assumed that the real concentration lies somewhere between the two extremes. The first boundary condition, A, assumed that all the measured pyruvate is in the mitochondria. A conversion factor is required for the matrix volume of mitochondria. The other boundary condition, B, assumed that the pyruvate is evenly distributed between the mitochondria and cytoplasm, i.e. throughout the whole intracellular water. (The assumption is also made that there is no pyruvate in the extracellular water). Boundary condition A gives a higher estimate of the concentration than boundary condition B.

Comparison of calculated intramitochondrial concentrations of pyruvate with the $K_m$ values of PDH for pyruvate is shown in Table IX. It can be seen that considerable variation occurs in the estimates both of the $K_m$ of PDH for pyruvate (17 to 100 $\mu$M) and of calculated intramitochondrial concentrations of pyruvate (47 to 4540 $\mu$M). However, if the $K_m$ value and the concentration of pyruvate are compared in the same tissue PDH approaches saturation with pyruvate, so that the reaction may be a flux generating step. If pyruvate is localised mainly within the mitochondria, then the evidence supporting this conclusion is stronger. Evidence from mitochondria separated from rat hepatocytes by the digitonin method shows that pyruvate is approximately two-fold more concentrated in the mitochondria than in the cytoplasm (Siess *et al.*, 1977). If PDH does catalyse a flux generating step, then the reaction must be regulated by external factors, and it is therefore necessary to investigate the properties of PDH in order to identify such factors.

TABLE IX

PYRUVATE DEHYDROGENASE: COMPARISON OF $K_m$ VALUES FOR PYRUVATE WITH CALCULATED INTRAMITOCHONDRIAL CONCENTRATIONS OF PYRUVATE

The intramitochondrial concentrations have been calculated from tissue concentrations, unless otherwise stated, using the conversion factors in Appendix IV, and assuming boundary condition A. The concentration of pyruvate under boundary condition B is given in brackets.

| SOURCE | CONDITIONS | $K_m$ $\mu$M | CONCENTRATION OF PYRUVATE $\mu$M |
|---|---|---|---|
| Insect flight muscle | (blowfly) | 100[1] | |
| | (locust) at rest | | 1333(500)[2], 480[3] |
| | " during flight | | 2000(700)[2] |
| Pig heart | purified enzyme | 20[4], 37[5], 40[6], 25[7] | |
| | ", in presence of $PP_i$ | 44[7] | |
| Rat heart | perfused, not working | | 470(47)[8] |
| | perfused, working | | 4540(454)[9], 2800(280)[10] |
| Rat liver | | | 1080[11*] |
| Rat kidney | partially purified PDH | 27[12] | |
| Rat brain | partially purified PDH | 17[12] | |

1) Hansford (1974)
2) Sacktor & Wormser-Shavit (1966)
3) Rowan (1975)
4) Scriba & Holzer (1961)
5) Denton et al. (1975)
6) Nawa et al. (1960)
7) Walsh et al. (1976)
8) Randle et al. (1970)
9) Hiltunen & Hassinen (1977)
10) Opie & Owen (1975)
11) Siess et al. (1977)
12) Blass & Lewis (1973)

* This value was obtained from mitochondria separated from isolated hepatocytes, by the digitonin method.

(iii) Properties of PDH

The activity of PDH is regulated by many factors other than the concentration of pathway substrate. (If the reaction is a flux generating step, the activity of PDH cannot, of course, be regulated by the pathway substrate.) The regulation of PDH in mammalian tissue is reviewed in Denton et al. (1975). A brief outline of the properties will be given here.

PDH from many animal tissues, including yeast and many mammals, exists as a multi-enzyme complex, with three enzymes catalysing the reaction sequence shown in Figure 6. Mammalian PDHs are inhibited by acetyl CoA (competitively with CoA), by NADH (competitively with $NAD^+$) and by acetoin (competitively with pyruvate). (Garland & Randle, 1964a; Wieland et al., 1969; Bremer, 1969; Tsai et al., 1973; Cooper et al., 1974). In addition, the PDH complex is regulated by a phosphorylation - dephosphorylation cycle. Linn and co-workers showed that the enzyme exists in two forms: an inactive, phosphorylated form and an active, dephosphorylated form (Linn et al., 1972). The interconversion of these two forms (known as $PDH_a$ and $PDH_b$) is catalysed by two enzymes: a kinase, which is tightly bound to the PDH complex, and a phosphatase, which is less tightly bound. PDH kinase, in the presence of $Mg.ATP^{2-}$, catalyses the phosphorylation and inactivation of the complex, and $PDH_b$-phosphatase, in the presence of $Mg^{2+}$ and $Ca^{2+}$, catalyses the dephosphorylation and reactivation of the complex.

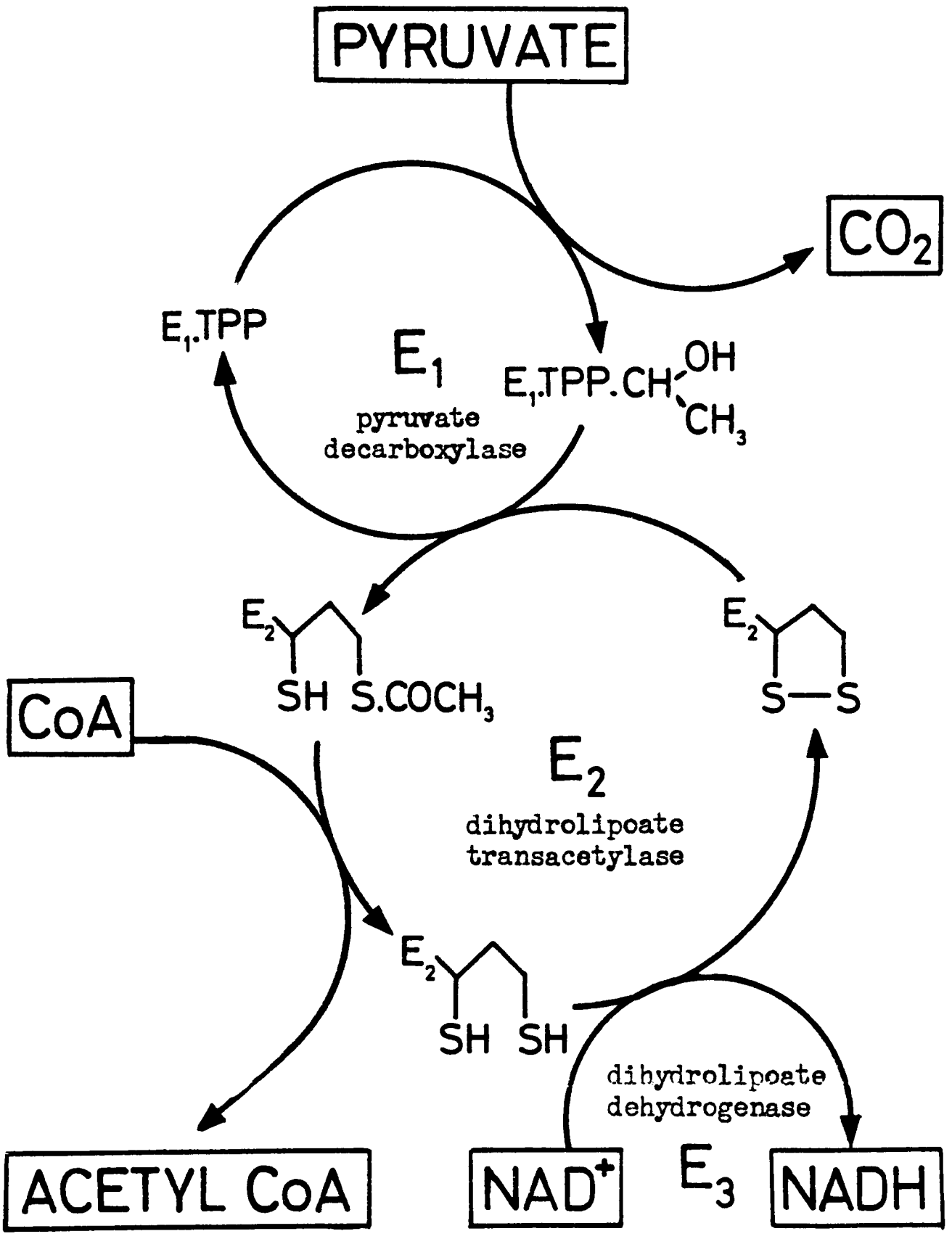
As $Mg.ATP^{2-}$ activates the kinase, and ADP is a competitive inhibitor (especially potent in the presence of $K^+$), it would be expected that the ATP/ADP concentration ratio could control the activity of the kinase, rather than the absolute concentration of either. Indeed, Roche and Reed (1974) working with a purified preparation of PDH kinase, showed that varying the concentrations of ADP and ATP over a three fold range, while

Figure 6 REACTIONS OF THE PYRUVATE DEHYDROGENASE COMPLEX

Overall reaction:

$$\text{pyruvate}^- + \text{CoA} + \text{NAD}^+ \longrightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH}$$

Reaction mechanism:



maintaining a fixed concentration ratio of ATP/ADP, did not affect the steady state activity of the kinase. However, the highest concentration ratio of ATP/ADP which they used was 0.5, while Siess et al.(1977) using the digitonin method showed that the intramitochondrial concentration ratio of ATP/ADP occurring in rat liver is approximately 2.0.

The substrates and effectors of the overall reaction, the phosphatase and the kinase are shown in Tables X and XI. Hansford (1977) presents evidence that both regulation of the kinase and the phosphatase, and end-product inhibition of the overall reaction of the complex may reduce PDH activity at the same time; thus evidence has been obtained in incubated rat heart mitochondria that oxidation of palmitoyl carnitine causes the inhibition of PDH not only by phosphorylation, but also by end-product inhibition.

The interconversion of PDH by the kinase and the phosphatase is known to occur with the enzyme from Neurospora crassa (Wieland et al., 1972 and Harding et al.,1970) and with the enzyme from mammalian tissues (in pig heart, Wieland & Siess, 1970, in pig liver and kidney Linn et al., 1969, and in rat adipose tissue, Jungas, 1971) but not with the enzyme from Escherichia coli (Schwartz & Reed, 1970).

However, the existence of a phosphorylation interconversion cycle in insect flight muscle has not been established. There is a report of interconversion in insect PDH in Candy & Kilby (1975, p. 66) of work by Chiang & Sacktor (1973), but this work does not appear to have been published. There are very few reports on the properties of insect flight muscle PDH, but Hansford (1974) reports inhibition of blowfly flight muscle PDH by NADH, and suggests that ADP may activate PDH. In addition, Hansford postulates inhibition by acetyl CoA by analogy with the mammalian enzyme (Hansford, 1972a).

TABLE X

KINETIC CONSTANTS FOR SUBSTRATES AND EFFECTORS OF THE PIG HEART PDH REACTION

The data are taken from Cooper *et al*. (1974) and Walsh *et al*. (1976)

| SUBSTRATE OR EFFECTOR | $K_m$ $\mu M$ | $K_i$ $\mu M$ |
|---|---|---|
| pyruvate | 36 | - |
| CoA | 2 | - |
| $NAD^+$ | 53 | - |
| thiamine pyrophosphate | 4* | - |
| $Mg^{2+}$ | 5 | - |
| acetyl CoA | - | 15 |
| NADH | - | 50 |
| acetoin | 430 | 71 |
| glyoxylate | 747 | 500 |
| sodium pyrophosphate | - | 225 |

*The $K_m$ depends on the pyruvate concentration. The value shown is the minimum value at saturating pyruvate concentration.

TABLE XI

EFFECTORS OF PIG HEART PDH KINASE AND PDHP PHOSPHATASE REACTIONS

The data is taken from Cooper *et al.* (1974), Cooper *et al.* (1975), Denton *et al.* (1972), Kerbey *et al.*(1976) and Linn *et al.* (1969a, b). Where the data is available, $K_m$ or $K_i$ values are given in parentheses.

| REACTION | SUBSTRATE | ACTIVATOR | INHIBITOR |
|---|---|---|---|
| Kinase | ATP (20 $\mu$M) | acetyl CoA | ADP (28 $\mu$M) (competitive with ATP) |
| | | NADH | pyruvate (635 $\mu$M) (uncompetitive) |
| | | acetoin | CoA |
| | | | $Mg^{2+}$ (1300 $\mu$M) |
| | | | $Ca^{2+}$ (1 $\mu$M) |
| | | | sodium pyrophosphate |
| | | | TPP |
| Phosphatase | $Mg^{2+}$ (1000 $\mu$M) | | |
| | $Ca^{2+}$ (0.7 $\mu$M) | | |

(iv) Evidence for the theory of control of PDH

From the properties above, one can propose a theory of control of the activity of mammalian PDH by the following effectors:

a) Acetyl CoA/CoA concentration ratio

b) ATP/ADP concentration ratio

c) $NAD^+$/NADH concentration ratio

d) $Mg^{2+}$ and $Ca^{2+}$ concentrations.

In order to provide evidence for the theory of control of PDH by the factors given above, the direction of change of the concentrations of these factors during muscle contraction must be investigated. However, this sort of evidence is difficult to acquire, since all the factors which are proposed to control the activity of PDH (i.e. the concentration ratios of acetyl CoA/CoA, $NAD^+$/NADH and ATP/ADP and the concentrations of $Mg^{2+}$ and $Ca^{2+}$) refer to concentrations within the mitochondria. It is not known how the mitochondrial ratios and concentrations of these metabolites change during the contraction of muscle. However, there is information which may throw light on this problem. Most of this information comes from two types of work: mitochondria from various sources have been incubated in different respiratory states and the concentrations of their metabolites measured; and muscles have been perfused and made to contract, and the total tissue concentrations of metabolites were measured at work and at rest. Evidence from incubated mitochondria assumes that the respiratory states 3 and 4 correspond to the active and resting states of muscle, respectively, although it is more likely that the active state corresponds to a respiratory rate between states 3 and 4 (Hansford, 1977). Such respiratory rates can be referred to as fractions of state 3 respiration. The assumption is also made that the mitochondria do not lose their contents during incubation. The last assumption is more likely to hold for the relatively impermeable

insect flight muscle mitochondria. Data from the perfused heart or hind-limb must be manipulated using assumptions about the distribution of the metabolites before the results can be interpreted as representative of mitochondrial concentrations.

a) Changes in the acetyl CoA/CoA concentration ratio.

Rowan & Newsholme (1979) measured the concentrations of various metabolites in locust flight muscle when the locust was at rest and after three minutes of flight. The tissue concentration ratio of acetyl CoA/CoA decreased after three minutes of flight. Using the approach explained on p.45, intramitochondrial concentrations can be calculated under two boundary conditions; the calculated concentration ratio also decreased during flight, when either boundary conditions is considered. (The assumption must be made that the distribution of acetyl CoA and CoA between cytoplasm and mitochondria does not change during the rest to flight transition.) Hansford (1974), working with incubated blowfly flight mitochondria, showed a decrease in the concentration ratio of acetyl CoA/CoA in the state 4 to state 3 transition.

In the isolated, perfused rat heart, calculated intramitochondrial concentration ratios of acetyl CoA/CoA decreased when the work load increased (Neely et al., 1972) and vice versa (Hiltunen & Hassinen, 1977).

Thus it can be seen that in rat heart and insect flight muscle, the concentration ratio of acetyl CoA/CoA decreases when flux through the reaction increases.

b) Change in the ATP/ADP concentration ratio

As mentioned on p. 1 of this chapter, the concentrations of ATP and ADP do not change appreciably during contraction. Rowan (1975) reports a 33% decrease of the ATP/ADP concentration ratio in locusts upon flight, compared with an increase of pyruvate oxidation of many fold (as shown by

the increase in oxygen consumption). In the perfused hindquarter of the rat, Hagg et al. (1976) showed that during exercise, the ATP/ADP concentration ratio decreased by only approximately 30% while the PDH activity increased two-fold. Thus it appears that the concentration ratio of ATP/ADP changes during contraction in a direction consistent with the theory of control of PDH. However, both these studies were concerned with tissue concentrations of metabolites, and these may not reflect mitochondrial changes.

For example, one problem in the measurement of free ADP in muscle tissue is that a large amount of ADP is bound to actin and to myosin (Barany & Barany, 1972; Marston & Tregear, 1974) and this is released during the acid extraction of the tissue prior to measurement of the metabolite concentration. It is not possible to measure a low concentration of free ADP since it is masked by the large amount of bound ADP. It has been suggested that the regulation of PDH by the concentration ratio of ATP/ADP may be mediated by the differing $Mg^{2+}$-chelating properties of ATP, ADP and AMP and changes in the concentrations of these nucleotides would alter the concentration of free $Mg^{2+}$ (Blair, 1970; Hucho et al., 1972; Hucho, 1974). Estimates of the concentration of free $Mg^{2+}$ in various tissues show that it is much lower in vivo than the $K_m$ of PDH phosphatase for $Mg^{2+}$ (Veloso et al., 1973).

c) Changes in the concentration ratio of $NAD^+$/NADH.

There is very little evidence on the changes of the $NAD^+$/NADH concentration ratio in the mitochondria during contraction. Chance and Williams (1956) proposed that, in mammalian mitochondria, state 4 to state 3 transitions are associated with increased oxidation of NADH. This is supported by evidence from incubated rat heart mitochondria in the work of Hansford (1977).

In the transition from state 3 to state 4, the concentration ratio of $NAD^+$/NADH was higher in state 3 and fractions of state 3 incubations than in state 4 incubations (by about 10-fold). However, in insect flight muscle, there are conflicting reports about the direction of change of the $NAD^+$/NADH concentration ratio. Rowan (1975) showed an increase in this ratio calculated from metabolite levels in locust flight muscle from 5.8 (at rest) to 8.1 (after 3 minutes flight). However, Hansford (1975) showed a decrease in the $NAD^+$/NADH concentration ratio in blowfly flight muscle from 10 (at rest) to 3.8 (after 2 minutes flight). In addition, Hansford showed, with incubated mitochondria from blowfly flight muscle, that state 4 to state 3 transitions are associated with a decrease in the concentration ratio of $NAD^+$/NADH (Hansford, 1975).

Unlike the adenine nucleotides, there is no mechanism in mammalian mitochondria for the direct transfer of nicotinamide nucleotides across the inner mitochondrial membrane. Transfer of reducing equivalents is achieved by the intercompartmental shuttling of substrates which are oxidised in one compartment and reduced in the other (e.g. the malate-aspartate shuttle) (Chappell, 1968; Williamson et al., 1973). The $NAD^+$/NADH free concentration ratio is closely linked to the ATP/ADP concentration ratio in mitochondria via oxidative phosphorylation and the electron transport chain. In the cytoplasm, the $NAD^+$/NADH free concentration ratio is also closely linked to the ATP/ADP concentration ratio via the equilibrium reactions catalysed by glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase. (See Krebs, 1973, for a review of the relationships between the adenine and nicotinamide nucleotides).

In the mitochondria, the direction in which the $NAD^+$/NADH concentration ratio changes depends upon the extent of the stimulation of the respiratory chain by changes in the ATP/ADP concentration ratio compared with the

extent of the change in the rate of the supply of NADH i.e. the extent of stimulation of the TCA cycle. If the extent of the stimulation of the TCA cycle is the greater, then the $NAD^+$/NADH concentration ratio will decrease; if the extent of the stimulation of the respiratory chain is the greater, then the $NAD^+$/NADH concentration ratio will increase.

Thus, to summarise, in isolated, respiring mitochondria the direction of change of the $NAD^+$/NADH concentration ratio appears to be consistent with the theory of regulation of PDH by this ratio in mammals, but not in insects.

d) The free concentrations of $Ca^{2+}$ and $Mg^{2+}$

It has been suggested that the concentration of free $Mg^{2+}$ is closely related to the adenine nucleotide pool, and even that it functions as a feedback signal from this pool (Blair, 1970). Blair has included in the formula for "energy charge" (a concept originally introduced by Atkinson, in 1968) the concentrations of the other variables of the pool, i.e. in addition to the total concentrations of ATP, ADP and AMP, he includes the following concentrations: total $Mg^{2+}$, free $Mg^{2+}$, $Mg.ATP^{2-}$, $Mg.ADP^{-}$, free $ATP^{3-}$, free $ADP^{2-}$ and free $AMP^{-}$. Thus, while the total concentration of $Mg^{2+}$ in rat heart is about 10 mM, the free concentration of $Mg^{2+}$ is only about 1 mM (Veloso *et al.*, 1973). This is below the $K_m$ of $PDH_b$-phosphatase and therefore changes in the free concentration of $Mg^{2+}$ could affect the activity of the phosphatase. *In vivo*, the energy charge may vary from 0.8 to 0.5, but this decrease is sufficient to increase the free concentration of $Mg^{2+}$ by approximately three-fold, which may cause considerable activation (70%) of the PDH activity (Hucho, 1974).

The kinase also requires $Mg^{2+}$, but its $K_m$ for $Mg^{2+}$ is much lower than is that of the phosphatase (20 $\mu$m as opposed to 3 mM), and at 1 mM free $Mg^{2+}$, the kinase will always have sufficient $Mg^{2+}$ to be fully active.

$Ca^{2+}$ is also complexed by adenine nucleotides, and thus a similar relation between the concentration of free $Ca^{2+}$ and the energy charge may exist. However, $Ca^{2+}$ is also chelated by many other compounds in mitochondria (e.g. phosphates, phospholipids, citrate), so the system may be regarded as a complex buffer of ionisable $Ca^{2+}$ (see Randle et al., 1974). The changes in the concentrations of these binding substances may also regulate the mitochondrial concentration of free $Ca^{2+}$. Finally, fluxes of $Ca^{2+}$ across the inner membrane are another factor likely to vary total concentrations of $Ca^{2+}$ and therefore free concentrations of $Ca^{2+}$ in the mitochondria (Bygrave, 1978; Harris, 1977).

Randle et al.(1974) suggest that the effect of $Ca^{2+}$ on the phosphatase is caused by a reduction in its $K_m$ for its substrate, $PDH_b$. Thus, at high concentrations of $PDH_b$ (low activity of PDH), $Ca^{2+}$ may have no effect.

During contraction, the following factors may cause changes in the concentration of free $Ca^{2+}$ in the mitochondria: the flux of $Ca^{2+}$ across the mitochondrial membrane, changes in citrate levels, and the change in energy charge. The energy charge decreases in mitochondria during contraction (see above) and this would tend to increase the concentration of free $Ca^{2+}$. Citrate levels in mitochondria decrease during contraction (Johnson & Hansford, 1975) and this would also tend to increase the intramitochondrial concentration of $Ca^{2+}$. However, the factors which regulate the flux of $Ca^{2+}$ across the mitochondrial membrane are not completely understood. There appears to be an energy dependent, uniporter $Ca^{2+}$ uptake mechanism, and a $Na^{+}$-dependent $Ca^{2+}$ efflux (Bygrave, 1978). It has been proposed that mitochondria, especially in heart muscle, may supplement the role of the sarcoplasmic reticulum in regulating the extramitochondrial concentration of $Ca^{2+}$ i.e. in the vicinity of the myofibrils, and the evidence for this is still

being evaluated (Nicholls, 1978; Affolter et al., 1976; Crompton et al., 1976a,b; Bygrave, 1975; Scarpa & Graziotti, 1973). If this is so, then presumably the mitochondria will act in a similar way to sarcoplasmic reticulum, in sequestering $Ca^{2+}$ when the muscle is at rest, and releasing it during contraction. This would tend to decrease the intramitochondrial free $Ca^{2+}$ during contraction. It is therefore not possible on the available evidence to predict the direction of change of free $Ca^{2+}$ in the mitochondria during contraction.

The $K_i$ values of PDH kinase and $PDH_b$-phosphatase are of the same order as estimates of the concentration of free $Ca^{2+}$ in the mitochondria (i.e. 0.3 to 5 $\mu$M in rat liver mitochondria, Nicholls, 1978) and so $Ca^{2+}$ may be an important regulator in vivo.

In summary, it can be assumed that mitochondrial concentration of free $Mg^{2+}$ increases during contraction (as the energy charge will decrease when the concentration ratio of ATP/ADP decreases). The direction of change of the concentration of free $Ca^{2+}$ in mitochondria during contraction cannot be predicted from the available evidence.

(v) Summary of the control of the PDH reaction

The theory that the steady state activity of PDH in mammalian muscle is regulated by the concentration ratios of acetyl CoA/CoA, ATP/ADP and $NAD^+$/NADH and the concentrations of $Mg^{2+}$ and $Ca^{2+}$, which act both on the interconverting enzymes and on the overall reaction, appears to be supported to some extent by the changes occurring during contraction in muscle.

None of the effectors appears to change by more than three fold, while the flux through the reaction may change a hundred-fold, as exemplified by the evidence of oxygen uptake data in flying insects. However, the sensitivity of the PDH reaction to changes in the levels of its effectors is

greatly increased by virtue of the interconverting cycle (see p. 32).

It therefore seems possible that the theory of the control of PDH may explain its regulation *in vivo*. Knowledge of its control properties may assist in the assay of PDH *in vitro*, and greater confidence can be placed in the utilisation of maximum activities *in vitro* for the experimental approach outlined above.

The control of PDH in insect flight muscle is not so clearly understood. The evidence suggests that it catalyses a flux generating step, and thus external regulation can be deduced. Possible candidates for effectors are ADP, acetyl CoA and NADH, but there is little experimental evidence.

## 3) THE TCA CYCLE

### (i) The equilibrium nature of the reactions

Most of the evidence on the equilibrium nature of the reactions of the TCA cycle concerns the enzymes citrate synthase (CS) and isocitrate dehydrogenase (ICDH). Very little is known about the equilibrium nature of the other reactions. If the cycle were to be regarded as a single metabolic pathway, starting at acetyl CoA, then the regulation of the flux through the cycle would be expected to occur at the CS reaction: i.e. this should be a flux generating step for the cycle. However, the TCA cycle could be considered to start at reactions which precede CS (i.e. PDH). In addition, the cycle may consist of more than one pathway since other reactions feed into and out of the cycle at several points (see pp. 5-10).

The first four reactions of the TCA cycle will be considered in this section, including the reaction catalysed by 2-oxoglutarate dehydrogenase (OGDH). The latter occurs at a point where residues of amino acids feed into the cycle during oxidation i.e. at 2-oxoglutarate. As pointed out on p.10, the 2-oxoglutarate-oxaloacetate span may be involved in energy production

in muscle during the utilisation of amino acids, and this raises a problem about concerted control of the two spans of the cycle. It was considered likely, therefore, that OGDH would be a possible site for regulation, and thus its equilibrium nature must be discussed.

The evidence on the equilibrium nature of the reactions of the cycle comes from the following methods:

(a) comparison of mass action ratio data with $K_{eq}$,

(b) comparison of $V_f$ and $V_r$ *in vivo*,

(c) comparison of $V_{max}$ *in vitro* and flux.

(see Section Two, for abbreviations and discussion).

1) Citrate synthase reaction (a) As explained earlier, in the section on PDH, the main problem with mass action ratio data of the enzymes of pyruvate oxidation and the TCA cycle is that of compartmentation of metabolites between mitochondria and cytoplasm. The level of TCA cycle intermediates can be measured in the mitochondria of isolated hepatocytes by the digitonin method of Zuurendonk & Tager (1974) as explained on p.43, but this has not yet been done for heart or skeletal muscle. Siess *et al.* (1977) have measured these metabolites in rat liver mitochondria separated by the digitonin method, and these data can be used to calculate mass action ratios for the CS reaction in liver. This is shown in Table XII. For comparison, the mass action ratio is also calculated for insect flight muscle, from the data of Rowan (1975), using the same conversion factors and assumptions as before.

The table shows a very large difference between $\Gamma$ and $K_{eq}$ ($K_{eq}/\Gamma$ of 9 or 10 orders of magnitude and $\Delta G$ values are approximately -13 $kcal.mol^{-1}$) suggesting that the reaction is far displaced from equilibrium in these tissues. In addition, like the data for the PDH reaction (see Table VIII), the mass action ratio for the reaction in insect flight muscle does not

TABLE XII

COMPARISON OF MASS ACTION RATIO DATA FOR THE CITRATE SYNTHASE REACTION WITH THE EQUILIBRIUM CONSTANT

The equilibrium constant for the reaction:

$$\text{acetyl CoA} + \text{oxaloacetate}^{2-} + H_2O \longrightarrow \text{citrate}^{3-} + \text{CoA} + H^+$$

was calculated from the $\Delta G^{o'}$ of -7500 $cal.mol^{-1}$ (Krebs & Kornberg, 1957 and Appendix V). The mass action ratios were calculated from the data of Rowan (1975) for insect flight muscle, and Siess et al.(1977) for rat liver from mitochondria separated from isolated hepatocytes by the digitonin method. For insect flight muscle concentrations, all the metabolites are assumed to be in the mitochondria (boundary condition A) (see p. 45). In insect flight muscle, mass action ratios calculated under boundary condition B are identical to those calculated under boundary condition A. The concentrations of $H_2O$ and $H^+$ were assumed to be 1M and $10^{-7}$M respectively.

| Tissue | Conditions | Mass action ratio | $K_{eq}$ | $\Delta G$ (at 25°C) $kcal.mol^{-1}$ |
|---|---|---|---|---|
| Rat liver | Mitochondria separated from rat by the digitonin method | $1.1 \times 10^{-4}$ | $3.2 \times 10^{5}$ | -12.9 |
| Insect flight muscle | at rest | $5.2 \times 10^{-5}$ | $3.2 \times 10^{5}$ | -13.3 |
| | 3 minutes flight | $2.4 \times 10^{-5}$ | | -13.8 |

differ greatly whether boundary condition A or B is considered, and the mass action ratio decreases during flight (i.e. $\Delta G$ increases numerically).

Tischler *et al.*(1977) also compared the mass action ratio of the CS reaction in liver with the equilibrium constant, measuring intramitochondrial metabolite concentrations by the digitonin method. Although their figures differ slightly from those in the present work, $K/\Gamma$ from their data had 6 orders of magnitude ( $\Delta G$ = approximately -8.2 kcal.mol$^{-1}$), and they too concluded that the reaction was far displaced from equilibrium.

(b) Only one attempt has been made to compare the rates of the forward and back reactions of the CS reaction *in vivo*: Randle and co-workers have used an isotopic approach to tackle the problem of whether the CS reaction is near to equilibrium in the perfused rat heart. (Randle *et al.*, 1970, and see Section Three). A pulse of ($^{14}$C) acetate was administered to an isolated perfused rat heart, and at various time intervals, the heart muscle was rapidly frozen, (freeze-clamped between two blocks of aluminium, pre-cooled in liquid nitrogen), extracted, de-proteinised and the metabolites of interest separated in order to measure their specific radioactivities.

The specific activity of acetyl CoA was found to be always greater than that of citrate, even after one hour from the pulse administration. Using a model based on the assumption that there is only one pool of citrate in the heart, Randle and co-workers suggested that since acetyl CoA and citrate did not reach isotopic equilibrium, CS catalyses a non-equilibrium reaction in heart, i.e. $V_f \gg V_r$.

(c) Alp *et al.* (1976) undertook a comparative survey of the activities of citrate synthase, $NAD^+$- and $NADP^+$-linked isocitrate dehydrogenase, and compared the maximum activities with the maximum flux through the TCA cycle, estimated from oxygen uptake data. (See Chapter Four). The data on CS activities suggested that this enzyme catalysed a near-equilibrium

TABLE XIII

CITRATE SYNTHASE: COMPARISON OF $K_m$ VALUES FOR ACETYL CoA AND OXALOACETATE AND THEIR CALCULATED INTRAMITOCHONDRIAL CONCENTRATIONS

The intramitochondrial concentrations have been calculated from tissue concentrations, unless otherwise stated, using the conversion factors in Appendix IV, and assuming boundary condition A. The concentrations of acetyl CoA or oxaloacetate under boundary condition B are given in brackets.

| SOURCE | CONDITIONS | $K_m$ $\mu$M | CONCENTRATION OF ACETYL CoA ( $\mu$M) |
|---|---|---|---|
| Rat heart | purified enzyme | 5[1] | |
| | perfused, not working | | 130(13)[2] |
| | perfused, working | | 250(25)[3] |
| Rat liver | | | 610[4] * |
| Beef heart | purified enzyme | 8[5] | |
| Insect flight muscle | blowfly | 4[6] | |
| | locust | 6[6] | |
| | locust, at rest | | 410[7] |
| | locust, during flight | | 210[7] |

| SOURCE | CONDITIONS | $K_m$ $\mu$M | CONCENTRATION OF OXALOACETATE ( $\mu$M) |
|---|---|---|---|
| Rat heart | purified enzyme | 5[1] | |
| | perfused, not working | | 0.36 - 10[8] †, 1[1] † |
| | perfused, working | | 260(26) |
| Beef heart | purified enzyme | 1.6[5] | |
| Insect flight muscle | blowfly | 4[6] | |
| | locust | 12[6] | |
| | locust, at rest | | 0.16[7] |

1) Moriyama & Srere (1971)
2) Randle et al. (1970)
3) Hiltunen & Hassinen (1977)
4) Siess et al. (1977)
5) Smith & Williamson (1971)
6) Alp (1973)
7) Rowan (1975)
8) Williamson et al. (1972)

*This concentration was measured in mitochondria separated from rat hepatocytes by the digitonin method.

†These are concentrations estimated by the authors.

reaction *in vivo*. This is at variance with the conclusion of Randle *et al*. (1970).

However, it is possible that the activity of CS *in vivo* is much lower than measured *in vitro*. Randle *et al*. (1970) showed that, in the perfused rat heart, while the flux through the CS reaction was 14 $\mu$mol.min$^{-1}$.g$^{-1}$(dry weight), the activity of the enzyme *in vitro* was 600 $\mu$mol.min$^{-1}$g$^{-1}$ (dry weight). There are two reasons why the activity of CS may be limited *in vivo*. Firstly, succinyl CoA and citrate inhibit the enzyme (Smith & Williamson, 1971, and see p.68 for discussion). Secondly, the concentration of oxaloacetate inside the mitochondria is very low (e.g. in liver, Shepherd & Garland, 1969; Olson & Williamson, 1971, and Wieland, 1968). In muscle, calculated levels of intramitochondrial oxaloacetate also support this theory (Rowan & Newsholme, 1979, and Table XIII). For example, in locust flight muscle, the $K_m$ of CS for oxaloacetate is 12 $\mu$M, while the intramitochondrial concentration of oxaloacetate is 0.16 $\mu$M(Rowan, 1975; Rowan & Newsholme, 1979).

Thus it seems likely that CS does catalyse a non-equilibrium reaction, but that its activity when measured *in vitro* does not provide a good assessment of its equilibrium nature, because of the limiting levels of oxaloacetate.

2) Aconitase reaction. There is very little evidence on the equilibrium nature of the aconitase reaction, though it is generally assumed that the reaction in near-equilibrium *in vivo*. Bowman (1966), Garland & Randle (1964b) and Williamson (1965) have presented data on the tissue levels of isocitrate and citrate from perfused rat hearts, and rat hearts frozen *in situ*, and these values have been used to calculate the mass action ratio of the aconitase reaction. This is shown, together with

the $K_{eq}$ of the reaction, in Table XIV. Comparison of these MARs with the $K_{eq}$ suggests that the reaction is close to equilibrium under these conditions; indeed $\Delta G$ is much less than -1 kcal.mol$^{-1}$ and in many cases, is positive. However, if the partition of citrate between mitochondria and cytoplasm is different from that of isocitrate, the MAR within the mitochondria may well depart from the values calculated from the tissue levels. However, citrate and isocitrate share the same mechanism for transport into the mitochondrion, so that differential distribution would seem unlikely.

3) Isocitrate dehydrogenase reaction. Two enzymes catalyse the dehydrogenation of isocitrate; one is $NAD^+$-linked and the other is $NADP^+$-linked. Although the $NADP^+$-linked enzyme (NADP-ICDH) has been more extensively studied, it has been generally believed that the $NAD^+$-linked enzyme (NAD-ICDH) is the primary enzyme involved in isocitrate oxidation in the TCA cycle. This was because previous evidence suggested that, of the two, NAD-ICDH was the only one which was exclusively intramitochondrial.

Recent research suggests that, at least in heart, NADP-ICDH may also be almost exclusively intramitochondrial (Fatania & Dalziel, 1979, 1979a). In this case, it would seem possible that both enzymes are involved in the TCA cycle.

a) Comparison of mass action ratio data with $K_{eq}$ for isocitrate dehydrogenase is shown in Table XV. These data suggest that NAD-ICDH is not at equilibrium in mitochondria from rat liver ( $\Delta G$ = -5 kcal.mol$^{-1}$) or from locust flight muscle ( $\Delta G$ = -2.9 to -4.9 kcal.mol$^{-1}$), at any of the theoretical concentration ratios of $NAD^+$/NADH. If glutamate dehydrogenase is at equilibrium in the mitochondria of liver, then this can be used to estimate the concentration ratio of $NADP^+$/NADPH from the $K_{eq}$ for the reaction and from concentrations of glutamate, 2-oxoglutarate and $NH_4^+$. Using the concentrations of metabolites from Zuurendonk et al. (1976), the concentration ratio of $NADP^+$/NADPH in liver mitochondria can be calculated to be 12.

TABLE XIV

COMPARISON OF MASS ACTION RATIO DATA OF

ACONITASE REACTION WITH THE EQUILIBRIUM CONSTANT

The $K_{eq}$ was calculated from the $\Delta G^{o'}$ reported in Mahler & Cordes (1977) by the method shown in Appendix V.

| Tissue | Conditions | Mass Action Ratio [Isocitrate] / [citrate] | $K_{eq}$ | $\Delta G$ at 25°C (kcal.mol$^{-1}$) |
|---|---|---|---|---|
| Rat heart | glucose perfusion | 0.14 to 0.083[1] | 0.068 | +0.4 to +0.1 |
| | | 0.091[2] | | +0.2 |
| | | 0.222[3] | | +0.7 |
| Rat heart | perfused with fatty acids or ketone bodies | 0.063 to 0.05[1] | 0.068 | -0.05 to -0.2 |
| | | 0.067[2] | | -0.008 |
| | | 0.080[3] | | +0.1 |
| Rat heart | anoxic | 0.14 to 0.083[1] | 0.068 | +0.4 to +0.1 |
| Rat heart | normal, frozen *in situ* | 0.059[1] | 0.068 | -0.084 |
| Rat heart | diabetic, frozen *in situ* | 0.053[1] | 0.068 | -0.147 |
| Rat heart | incubated mitochondria | 0.115[3] | 0.068 | +0.3 |

1) Bowman (1966)

2) Garland & Randle (1964b)

3) Williamson (1965)

TABLE XV

## COMPARISON OF MASS ACTION RATIO DATA OF $NAD^+$ LINKED ISOCITRATE DEHYDROGENASE REACTION WITH THE EQUILIBRIUM CONSTANT

The equilibrium constant for the reaction

$$\text{isocitrate}^{3-} + NAD^+ \longrightarrow \text{2-oxoglutarate}^{2-} + NADH + CO_2$$

is taken from Londesborough & Dalziel (1970).

Tissue concentrations of metabolites are converted to mitochondrial concentrations using the conversion factors in Appendix IV. The mass action ratio data were calculated from Rowan (1975) for insect flight muscle and Sies *et al*. (1977) for rat liver mitochondria separated from isolated hepatocytes by the digitonin method. For insect flight muscle concentrations, all the metabolites are assumed to be in the mitochondria (boundary condition A). Mass action ratio calculated under boundary condition B are shown in brackets. The range of values of the mass action ratios for rat liver is due to the different estimates of the mitochondrial free $NAD^+/NADH$ concentration ratio given by Siess *et al*. (1977).

| Tissue | Conditions | Mass action ratio | $K_{eq}$ | $\Delta G$ at 25°C (kcal.mol$^{-1}$) |
|---|---|---|---|---|
| Insect flight muscle | at rest | $8.0 \times 10^{-4}$ ($8.0 \times 10^{-4}$)M | 1.0 M | -2.9 (-4.2) |
| | 3 min. flight | $2.7 \times 10^{-4}$ ($2.7 \times 10^{-4}$)M | | -4.9 |
| Rat liver | mitochondria separated from hepatocytes by the digitonin method. | 1.1 to 1.6 $\times 10^{-4}$ M | 1.0 M | -5.2 to 5.4 |

This gives mass action ratios for the reaction catalysed by NADP-ICDH of $7.1 \times 10^{-4}$ (liver) and $3.9 \times 10^{-4}$ (locust flight muscle). Compared with a $K_{eq}$ of 0.9 (Londesborough & Dalziel, 1970) this would suggest that NADP-ICDH also catalyses a reaction which is far displaced from equilibrium. ( $\Delta G$ values are -4.2 kcal.mol$^{-1}$ for rat liver and -4.6 kcal.mol$^{-1}$ for insect flight muscle).

b) Comparison of the forward and reverse rates of the reaction was undertaken by Randle *et al*. (1970). The reverse rate of the reaction was measured by the incorporation of $[^{14}C]$from $[^{14}C]$bicarbonate into isocitrate, in perfused rat hearts. The flux through the reaction was also measured during perfusion with labelled acetate. Since the flux through the reaction is the difference between the reverse rate and the forward rate *in vivo*, the forward rate of the reaction could be calculated by subtraction ($J = V_f - V_r$).

Randle and his co-workers found that the reverse reaction proceeded at a rate which was 46% of the forward reaction i.e. $V_f/V_r = 2$. It can thus be concluded that the isocitrate dehydrogenase reaction as a whole is near to equilibrium in the perfused heart. However, this assumes one pool of isocitrate in the cell.

c) Maximum enzyme activity data were obtained by Alp *et al*. (1976) (see above). Except for insect muscles, the sum of the two isocitrate dehydrogenase activities is considerably greater than the flux through the cycle. In insect flight muscles, maximum activities of NAD-ICDH are similar to the flux through the cycle, and the activities of NADP-ICDH are much smaller. If both enzymes are exclusively mitochondrial, and if both are involved in isocitrate oxidation in the TCA cycle, these results suggest that the reaction as a whole is near-equilibrium in all muscles except insect

flight muscle, in which the reaction is far displaced from equilibrium. However, if the required metal ion for isocitrate dehydrogenase ($Mn^{2+}$ or $Mg^{2+}$) is at a very low concentration in the mitochondria, the activities may be lower *in vivo* than *in vitro*. It is also possible that the activities were inhibited by various possible regulators.

The evidence so far suggests that NAD-ICDH catalyses a non-equilibrium reaction in insect flight muscles. In other muscles, the evidence is inconclusive. It remains to be seen whether NADP-ICDH is exclusively intramitochondrial in these muscles. However, the role of the two enzymes remains incompletely understood.

4) 2-oxoglutarate dehydrogenase (OGDH) reaction.

(a) Despite the problem of compartmentation, it is possible to calculate the mass action ratio for the OGDH reaction, and compare it with the reported equilibrium constant. This has been done for rat liver (data from the digitonin method of separation) and insect flight muscle and is shown in Table XVI. It can be seen that there are many orders of magnitude of difference between MAR data and $K_{eq}$, even when the two boundary conditions for distribution of metabolites are considered. $\Delta G$ values are between -8.3 and -12.8 $kcal.mol^{-1}$. This suggests that the reaction is far displaced from equilibrium.

(b) No attempt has been made to measure the forward and backward reactions *in vivo* of the OGDH reaction.

(c) A systematic survey of the maximum activities of OGDH for comparison with the other enzymes of the TCA cycle, and with the flux through the TCA cycle has been carried out and is discussed in Chapter Four of this thesis. Thus, the way in which this comparison of $V_{max}$ values *in vitro* with the flux *in vivo* reflects on the equilibrium nature of the OGDH reaction will be discussed later. However, a recent paper by Read *et al*. (1977)

TABLE XVI

COMPARISON OF THE MASS ACTION RATIO DATA OF THE 2-OXOGLUTARATE DEHYDROGENASE REACTION WITH THE EQUILIBRIUM CONSTANT

The equilibrium constant for the reaction

$$2\text{-oxoglutarate}^{2-} + CoA + NAD^{+} \longrightarrow \text{succinyl } CoA^{-} + NADH + CO_2$$

is calculated from the $\Delta G^{o'}$ of -7240 $cal.mol^{-1}$ (Krebs & Kornberg, 1957, Appendix V).

The mass action ratio data were calculated from Rowan (1975) for insect flight muscle and Siess et al.(1977) for rat liver mitochondria separated from isolated hepatocytes by the digitonin method, except for succinyl CoA levels,which were taken from Hansford (1974) for insect flight muscle and Williamson et al. (1972) for rat liver. For insect flight muscle levels, all the metabolites are assumed to be in the mitochondria (boundary condition A). Mass action ratios calculated under boundary condition B are shown in brackets.

The range of values of the mass action ratios for rat liver is due to the different estimations of the mitochondrial free $NAD^{+}$/NADH concentration ratio given by Siess et al. (1977).

| TISSUE | CONDITION | MASS ACTION RATIO | $K_{eq}$ | $\Delta G$ at 25°C ($kcal.mol^{-1}$) |
|---|---|---|---|---|
| Rat liver | mitochondria separated from hepatocytes by the digitonin method | $1.3 \times 10^{-3}$ to $8.3 \times 10^{-5}$ | $2.04 \times 10^{5}$ | -11.2 to -12.8 |
| Insect flight muscle | at rest: | $4.0 \times 10^{-3}$ ($1.1 \times 10^{-1}$) | $2.04 \times 10^{5}$ | -10.5 (8.5) |
| | 3 mins. flight | $5.6 \times 10^{-2}$ ($1.6 \times 10^{-1}$) | | -8.9 (8.3) |

reported the activities of OGDH in mammalian hearts and bumblebee flight muscle and stated that OGDH appeared to be a non-equilibrium reaction on the basis of a comparison of maximum enzyme activity *in vitro* with flux through the TCA cycle *in vivo*. As this subject is investigated more fully in this thesis, further discussion is left to Chapters Four and Five.

In conclusion, therefore, it appears likely that the OGDH reaction may be far displaced from equilibrium in the cell, and this will be assumed to be the case in further discussion of this reaction.

(ii) *Identification of flux generating steps in the TCA cycle*

As described before, the identification of flux generating steps involves comparison of the concentration of the pathway substrate *in vivo* with the $K_m$ of the enzyme for that substrate. Possible candidates for flux generating steps are those reactions which are far displaced from equilibrium, for example, in TCA cycle, the reactions datalysed by CS, NAD-ICDH and OGDH.

Although the TCA cycle is normally regarded as a single metabolic pathway, this may not be the case; more than one flux generating step may occur in the TCA cycle and therefore there may be more than one metabolic pathway involved. (See definitions on p. 29-30). Other pathways feed into or out of the cycle at points other than acetyl CoA e.g. in muscle, amino acid oxidation may lead to an influx of substrate at the 2-oxoglutarate level and an efflux from oxaloacetate. Thus, this span of the cycle may be controlled by a different flux generating step than other spans of the cycle, and thus it may be considered as a separate metabolic pathway. Indeed, other authors refer to this span of the cycle as being separately controlled from the rest of the cycle (Randle *et al.*, 1970; Neely *et al.*, 1972; Hansford & Johnson, 1975). It is also interesting to note that this span of the pathway is conserved in parasitic helminths, and used, in the opposite direction, in anaerobic energy production. (Von Brand, 1973).

In most tissues, the concentration of the pathway substrate of these mitochondrial enzymes must be estimated from measured tissue levels using the approach given on p. 45 and in Appendix IV. However, insect flight muscle mitochondria are impermeable to TCA cycle intermediates (Van den Bergh & Slater, 1962) and this makes it easier to estimate mitochondrial levels of the cycle intermediates since it is possible to assume that all the measured intermediates are in the mitochondria. The assumptions must still be made that each metabolite is evenly distributed throughout the mitochondrial water, and that the volume of the intramitochondrial space is constant for a given tissue, and can be used as a correction factor. Hansford and Johnson have used incubated insect flight muscle mitochondria to study metabolite levels. (Hansford, 1974; Johnson & Hansford, 1975; and Hansford, 1975). These studies are more likely to give realistic values for the levels of intramitochondrial metabolites than are studies with incubated mitochondria from mammalian tissues.

1) Citrate synthase

The comparison of estimated intramitochondrial concentrations of acetyl CoA (the pathway substrate) and of oxaloacetate and the $K_m$ values of CS for acetyl CoA and oxaloacetate are shown in Table XIII. It can be seen that CS approaches saturation with acetyl CoA (i.e. the concentration of acetyl CoA is greater than 2 to 5 times the $K_m$) in all cases, whether boundary condition A or B is considered. It may therefore be concluded from these data that CS catalyses a flux generating step.

2) $NAD^+$-linked isocitrate dehydrogenase

The enzyme NAD-ICDH shows a sigmoid response to isocitrate and therefore the concentration of isocitrate giving half-maximal activity should properly be known as $S_{50}$. This parameter varies with the concentrations of

ADP and $Ca^{2+}$. At very low free concentrations of $Ca^{2+}$, the $S_{50}$ of NAD-ICDH for isocitrate may be increased (Denton et al., 1978).

Table XVII shows a comparison of estimated concentrations of isocitrate with values of $S_{50}$ under various conditions for the enzyme NAD-ICDH and with $K_m$ values for isocitrate for the enzyme NADP-ICDH. There is great variation in the $S_{50}$ values for isocitrate (16 to 1500 $\mu$M) and in the estimated concentrations of isocitrate (142 to 693 $\mu$M). Sies et al.(1977) showed that, in rat hepatocytes using the digitonin method, the isocitrate occurs mainly within the mitochondria. Thus, if the assumption is made that boundary condition A holds for rat heart, the enzyme NAD-ICDH may approach saturation with its substrate in rat heart, certainly in the presence of 1 mM ADP and 25 $\mu$M free $Ca^{2+}$. However, in insect flight muscle, in which NAD-ICDH is known to catalyse a non-equilibrium reaction, the evidence does not clearly support the same conclusion. However, there has been no rigorous measurement of the $S_{50}$ of insect flight muscle NAD-ICDH taking into account the binding properties of isocitrate, ADP and the activating metal ions $Mg^{2+}$, $Mn^{2+}$, and $Ca^{2+}$. In summary, there is no conclusive evidence that NAD-ICDH catalyses a flux- generating step.

The enzyme NADP-ICDH appears to approach saturation with its substrate, isocitrate, but the equilibrium nature of this reaction has not yet been established.

3) 2-oxoglutarate dehydrogenase

Estimated intramitochondrial concentrations of 2-oxoglutarate are shown together with the $K_m$ values of OGDH for 2-oxoglutarate in Table XVIII. Since the $K_m$ of OGDH from pig heart for 2-oxoglutarate has been shown to be dependent on the free concentrations of $Ca^{2+}$ and ADP, it should be known as the apparent $K_m$ (McCormack & Denton, 1979).

TABLE XVII

ISOCITRATE DEHYDROGENASE: COMPARISON OF $S_{50}$ VALUES WITH CALCULATED INTRAMITOCHONDRIAL CONCENTRATIONS OF ISOCITRATE

The intramitochondrial concentrations have been calculated from tissue concentrations, unless otherwise stated, using the conversion factors in Appendix IV, and assuming boundray condition A. The concentration of isocitrate under boundary condition B is given in brackets. Many of these values are reported as $K_m$ values, but as the enzyme shows sigmoid kinetics, these should properly be known as $S_{50}$ values.

| SOURCE | CONDITIONS | $S_{50}$ $\mu$M | CONCENTRATION OF ISOCITRATE $\mu$M |
|---|---|---|---|
| **$NAD^+$-LINKED ISOCITRATE DEHYDROGENASE** | | | |
| Rat liver | No added ADP | 1500[1] | |
| | 0.67 mM ADP | 140[1], 100[2] | |
| | Mitochondria from hepatocytes | | 142[3] |
| Pig heart | Purified enzyme | 16[2] | |
| Rat heart | Purified enzyme; 1 mM ADP, no free $Ca^{2+}$ | 227[4] | |
| | 1 mM ADP, 25 $\mu$M free $Ca^{2+}$ | 53[4] | |
| | Perfused, working | | 670(67)[5], 670(67)[6] |
| | Perfused, not working | | 670(67)[7] |
| Insect flight muscle | Crude extract | 90 to 100[8] | |
| | At rest | | 202(72)[9], 693(248)[10] |
| | During flight | | 149(53)[9], 466(166)[10] |
| **$NADP^+$-LINKED ISOCITRATE DEHYDROGENASE** | | | |
| Pig heart | Purified enzyme | 1.5[2] | |

1) Williamson et al. (1969)
2) Colman (1975)
3) Sies et al.(1977)
4) Denton et al. (1978)
5) Neely et al. (1972)
6) Hiltunen & Hassinen (1977)
7) Randle et al. (1970)
8) Zammit & Newsholme (1976) (waterbug)
9) Johnson & Hansford (1975) (blowfly)
10) Rowan (1975) (locust)

TABLE XVIII

2-OXOGLUTARATE DEHYDROGENASE : COMPARISON OF $K_m$ VALUES FOR OXOGLUTARATE WITH CALCULATED INTRAMITOCHONDRIAL CONCENTRATIONS OF 2-OXOGLUTARATE

The intramitochondrial concentrations have been calculated from tissue concentrations, unless otherwise stated, using the conversion factors in Appendix IV, and assuming boundary condition A. The concentration of 2-oxoglutarate under boundary condition B is given in brackets.

| SOURCE | CONDITIONS | app. $K_m$ $\mu$M | CONCENTRATION OF 2-OXOGLUTARATE $\mu$M |
|---|---|---|---|
| Pig heart | isolated enzyme | 110[1], 13[2,3] 200[4], 100[5] | |
| | : 1 nM free $Ca^{2+}$, no ADP | 2500[6] | |
| | : 10-33 $\mu$M $Ca^{2+}$, no ADP | 160[6] | |
| | : 10-33 $\mu$M $Ca^{2+}$, 1.5 mM ADP | 75[6] | |
| Rat heart | purified enzyme | 80[7] | |
| | crude extract | 60[8] | |
| | incubated mitochondria | 85[9] | |
| | perfused, not working | | 2530(253)[10] |
| | perfused, working | | 2330(233)[11], 5900(590)[12] 3660(366)[13] |
| Rat liver | | | 1200 to 2700[14*], 1640[15*], 3200[16*] |
| Insect flight muscle | crude extract | 70[8] | |
| | partially purified enzyme | 90[17] | |
| | at rest | | 597(213)[18] |
| | during flight | | 704(251)[18] |

1) Hirashima et al. (1967)
2) Sanadi (1967)
3) Massey (1960)
4) McMinn & Ottaway (1977)
5) Kansaki et al. (1969)
6) McCormack & Denton (1979)
7) Williamson et al. (1973)
8) Read et al. (1977) (bumblebee)
9) LaNoue et al. (1972)
10) Randle et al. (1970)
11) Williamson et al. (1976)
12) Opie & Owen (1975)
13) Hiltunen & Hassinen (1977)
14) Zuurendonk et al. (1976)
15) Siess et al. (1977)
16) Sies et al. (1977)
17) Hansford (1972)(blowfly)
18) Johnson & Hansford (1975)(blowfly)

*These concentrations were measured in mitochondria separated from rat hepatocytes by the digitonin method.

If 2-oxoglutarate is localised mainly within the mitochondria (boundary condition A), in rat heart and insect flight muscle, then the estimated intramitochondrial concentrations of 2-oxoglutarate from Table XVIII range from 597 $\mu$M (blowfly flight muscle) to 5900 $\mu$M (working, perfused rat heart). The data from rat liver, measured by the digitonin method, show that 2-oxoglutarate is between 5 and 20 times more concentrated in the mitochondria than the cytoplasm,i.e. boundary condition A holds for rat liver (Zuurendonk et al., 1976). The apparent $K_m$ values presented in Table XVIII vary from 13 $\mu$M to 200 $\mu$M (both from the pig heart purified enzyme). Despite this wide variation, comparison of these $K_m$ values with the higher estimates of 2-oxoglutarate concentration under boundary condition A suggests that OGDH catalyses a flux-generating step in rat heart and insect flight muscle.

The wide variation in reported values of the apparent $K_m$ may be explained by the findings of McCormack & Denton (1979). As mentioned above, they showed that the apparent $K_m$ of pig heart OGDH for 2-oxoglutarate is dependent on the free concentrations of $Ca^{2+}$ and ADP: it varies from 75 $\mu$M (in the presence of approximately 20 $\mu$M free $Ca^{2+}$ and 1.5 mM ADP) to 2.5 mM (in the presence of 1 nM free $Ca^{2+}$ and in the absence of ADP). Assuming that conditions are similar to the former situation in vivo, i.e. in the presence of some ADP and $\mu$M $Ca^{2+}$, then the apparent $K_m$ of OGDH could be lower than some of the reported values and the evidence for OGDH catalysing a flux-generating step is stronger.

To summarise, CS and OGDH in rat heart and insect flight muscle and NAD-ICDH in insect flight muscle appear to catalyse flux-generating steps.

(iii) Identification of externally regulated reactions

In the outline of the approach to metabolic regulation described on p.32, it was stated that, having identified the equilibrium nature of the reactions of a metabolic pathway and the flux generating steps, the next two steps of the approach are the identification of externally regulated reactions, and the investigation of the properties of the enzymes catalysing these reactions. These externally regulated reactions may be identified by observing the direction of change of the pathway substrate when the flux through the reaction is changed. However, since only the intramitochondrial concentrations of substrates are relevant, this method is subject to the same disadvantages as the measurement of mass action ratios.

If the assumption is made that the direction of change of a metabolite will be the same in the mitochondria as is observed in the tissue level of that metabolite, notwithstanding the amount of that change, then some useful information may be obtained from the following observations. The concentrations of acetyl CoA, isocitrate and 2-oxoglutarate have been measured in the isolated perfused heart (Neely et al., 1972, 1976; Williamson et al., 1976), in the flight muscles of insects (Rowan & Newsholme, 1979; Johnson & Hansford, 1975; Hansford, 1974) and in isolated insect flight muscle mitochondria (Johnson & Hansford, 1975; Hansford & Johnson, 1975, and Hansford, 1974) under conditions of an increase in flux through the TCA cycle. Hiltunen & Hassinen (1977) have measured the concentrations of acetyl CoA, isocitrate and 2-oxoglutarate in the perfused rat heart, before and after stopping a working heart preparation with KCl.It is assumed that after the $K^+$ arrest, the flux through the TCA cycle decreased. The direction of change of these metabolites reported by these authors is shown in Table XIX.

It can be seen that the reported direction of change of these metabolites differs from author to author. Assuming that there were no technical

TABLE XIX

REPORTED DIRECTION OF CHANGE OF CONCENTRATIONS OF ACETYL CoA, ISOCITRATE AND 2-OXOGLUTARATE DURING AN INCREASE IN FLUX THROUGH THE TCA CYCLE IN RAT HEART AND INSECT FLIGHT MUSCLE

In the isolated, perfused rat heart, tissue concentrations of metabolites were measured before and after an increased work load (except for the data of Hiltunen & Hassinen, 1977, where a working heart was arrested with $KCl$, in which case the change in the direction was assumed to be the converse on an increased flux). In isolated mitochondria, the change in mitochondrial concentration of the metabolite occurs in the state 4 to state 3 transition. In flown insects, the tissue concentrations of metabolites were measured from flight muscle from insects at rest and after 3 minutes flight.

| TISSUE | AUTHORS | CHANGE IN METABOLITE CONCENTRATION | | |
|---|---|---|---|---|
| | | ACETYL CoA | ISOCITRATE | 2-OXOGLUTARATE |
| Rat heart | Neely et al. (1972) | ↓ | - | ≈ |
| | Neely et al. (1976) | ↓ | ↓ | ↓ |
| | Hiltunen & Hassinen (1977) | ≈ | ↓ | ↑ |
| | Williamson et al. (1976) | ↑ | - | ≈ |
| Insect flight muscle: flown insects | | | | |
| | Rowan & Newsholme (1979) | ↓ | ↑ | ↓ |
| | Johnson & Hansford (1975) | - | ↓ | ↑ |
| | Hansford (1974) | ≈ | - | - |
| : incubated mitochondria | | | | |
| | Johnson & Hansford (1975) | - | ↓ | ↑ |
| | Hansford (1974) | ↓ | - | - |
| | Hansford & Johnson (1975) | - | ↓ | ↓ |

↓ decrease in concentration

↑ increase in concentration

≈ no change

difficulties in the measurement of these metabolites, it must be assumed that in those tissues in which the concentration of the metabolite increased, some imbalance occurred between the reactions which remove and supply the metabolite concerned. In any case, the increase of the concentration of the substrate when the flux through the reaction is increased does not concern us, as this can give no evidence on the regulation of the enzyme catalysing the reaction.

In both rat heart and insect flight muscle, there are several reports of the decrease in the concentrations of acetyl CoA, isocitrate and 2-oxoglutarate on an increased flux through the TCA cycle. This evidence suggests that the reactions catalysed by CS, ICDH and OGDH are regulated by factors other than their pathway substrates i.e. that they are externally regulated. Indeed, if these reactions are flux generating steps, then they must, by definition, be externally regulated.

(iv) Properties of enzymes catalysing externally regulated reactions and flux generating steps

Having identified possible flux generating steps as those catalysed by CS and OGDH, and having established that the reaction catalysed by NAD-ICDH, if it is not a flux generating step, appears at least to be externally regulated, the properties of the enzymes catalysing these reactions must be investigated *in vitro*, in order to formulate a theory of the control of the TCA cycle. A brief outline of the properties of CS, ICDH and OGDH *in vitro* will be given.

1) Citrate synthase

There are two groups of citrate synthases; bacteria possess citrate synthases of high molecular weight, which appear to be tetrameric, and higher animals possess low molecular weight dimeric citrate synthases. These two groups show different kinetic properties. Some bacterial citrate syn-

thases are inhibited by NADH and AMP relieves this inhibition. Citrate synthases from eukaryotic tissue, however, are a hundred-fold less sensitive to NADH inhibition.

Originally, it was believed that ATP was a physiological inhibitor of CS. There were many reports of ATP inhibition of CS (e.g. with the enzyme from rat liver mitochondria, Garland,(1968) or from rat heart, Smith & Williamson (1971)). However, this theory became untenable when it was realised that the $Mg.ATP^{2-}$ complex was much less inhibitory than ATP (Kosicki & Lee, 1966) and that calculation showed that the amount of $Mg^{2+}$ available for nucleotide binding was in excess of the ATP content (Veloso *et al.*, 1973), thereby showing that, *in vivo*, ATP was present only as the $Mg.ATP^{2-}$ species. There is also evidence to support this view from the work of n.m.r. (nuclear magnetic resonance) on adenine nucleotide concentrations in intact, living muscle (Hoult *et al.*, 1974; Garlick *et al.*, 1977).

In addition, Weitzmann and Hewson have investigated the regulatory effects of 2-oxoglutarate, NADH and ATP on CS *in situ* in yeast cells made permeable with toluene (Weitzman & Hewson, 1973). They showed that there was no inhibition by ATP *in situ*, although it did occur *in vitro*. Srere in 1967 had suggested that the intracellular concentrations of enzymes may be several orders of magnitude higher than the concentration in assay conditions, and speculated (Srere, 1971) that CS inhibition by ATP may not be evident if the enzyme were present at concentrations approximating those *in vivo*.

Smith & Williamson (1971), in studies on purified beef heart CS, showed inhibition by succinyl CoA and propionyl CoA (competitive with respect to acetyl CoA) and citrate (competitive with respect to oxaloacetate). Succinyl CoA and citrate show $K_i$ values well within the range of reported mitochondrial levels so that they may be important regulators *in vivo* (Smith & Williamson, 1971).

LaNoue and co-workers proposed on the basis of their studies on rat heart mitochondria that oxaloacetate availability and inhibition by succinyl CoA are the factors which regulate CS in this tissue, and not inhibition by ATP (LaNoue et al., 1973). The idea that oxaloacetate availability may control the entry of acetyl CoA into the TCA cycle was advanced many years ago (Lehninger, 1946). This will be discussed later, under the evidence for the theory of the control of the TCA cycle.

2) Isocitrate dehydrogenase (ICDH)

Isocitrate dehydrogenase activity is carried out by two enzymes, $NAD^+$- and $NADP^+$-linked ICDH. (NAD-ICDH and NADP-ICDH). As discussed on p.59, it seems likely that both enzymes may be involved in the oxidation of isocitrate in the TCA cycle. However, there are very few known effectors of NADP-ICDH: 2-oxoglutarate may be an inhibitor of rat heart NADP-ICDH (Hatfield, M., personal communication).

Experiments show that ADP is a specific activator of NAD-ICDH in vitro (Chen & Plaut, 1963; Goebell & Klingenberg, 1964). In addition, the enzyme from muscle has been shown to be inhibited by $Ca^{2+}$ (Zammit & Newsholme, 1976). However, the levels of $Ca^{2+}$ used by these workers are probably higher than the physiological range. (See Denton et al., 1978). Denton and co-workers, on the other hand, showed activation of NAD-ICDH by $Ca^{2+}$ in the concentration range 1 nM to 10 $\mu$M (Denton et al., 1978). ATP has been shown to inhibit the activity of NAD-ICDH (Plaut & Aogaichi, 1968) but the activation by ADP is thought to be more significant (see review by Plaut, 1970). NAD-ICDH activity is also inhibited by NADH (Chen & Plaut, 1963).

3) 2-oxoglutarate dehydrogenase (OGDH)

OGDH is a multi-enzyme complex, catalysing a similar reaction sequence to that of PDH, and consisting of three separate enzymes similar to those of PDH. These three enzymes are the keto-acid decarboxylase (in this

case, oxoglutarate decarboxylase, corresponding to pyruvate decarboxylase in PDH), dihydrolipoyl transsuccinylase (corresponding to dihydrolipoyl transacetylase in PDH) and dihydrolipoyl dehydrogenase. The overall reaction is as follows:

$$\text{2-oxoglutarate} + \text{CoA} + \text{NAD}^+ \longrightarrow \text{succinyl CoA} + \text{CO}_2 + \text{NADH}$$

Unlike PDH, however, there appears to be no evidence of an interconverting phosphorylation cycle for OGDH from rat heart (Reed et al., 1972) or from insect flight muscle (Hansford, 1972b).

Early work established that the enzyme was subject to product inhibition i.e. it is inhibited by succinyl CoA (competitive with CoA) and by NADH (competitive with $NAD^+$) in intact rat heart mitochondria and in the isolated perfused rat heart (Garland, 1964). Smith et al.(1974), in further studies on the kinetics of purified pig heart OGDH, showed that the inhibition by succinyl CoA was independent of the $NAD^+$/NADH concentration ratio, and showed that no inhibitory effects were observed with either GTP or ATP. It was assumed that high ATP/ADP concentration ratios would indirectly inhibit the OGDH reaction by giving rise to high succinyl CoA concentration ratios via the succinyl CoA thiokinase reaction. However, in a recent report, McCormack & Denton (1979) show that ADP activates the enzyme from pig heart (half maximal activation at 100 $\mu$M ADP) and that ATP inhibits it.

In blowfly flight muscle, Hansford reports that ATP inhibits a partially purified OGDH in vitro, and that ADP and AMP reverse this inhibition (Hansford, 1972b). In addition, ADP is required for the measurement of maximum activity in vitro of OGDH from insect flight muscles (Read et al., 1977).

( v) Formulation of a theory of control of the TCA cycle and presentation of evidence to support it

From the evidence presented above, the first four reactions of

the TCA cycle appear to consist of a flux generating step (CS), a near-equilibrium reaction (aconitase), and then two non-equilibrium reactions, which may also be flux generating steps (NAD-ICDH and OGDH).

The following factors have been proposed as regulators of these reactions:

1) The concentration ratio of $NAD^+$/NADH, affecting the NAD-ICDH and OGDH reactions

2) The concentration ratio of ATP/ADP, affecting the NAD-ICDH and OGDH reactions

3) The concentration of $Ca^{2+}$, affecting NAD-ICDH reaction and the OGDH reaction

4) The concentrations of succinyl CoA, citrate and oxaloacetate, affecting the CS reaction.

The regulation of the TCA cycle will involve the control of these externally regulated reactions. Thus, in order to look for evidence for the theory of the control of the TCA cycle by these effectors, the direction of change of the concentrations of these factors during muscle contraction must be investigated. Some of this evidence has been reviewed earlier in this section.

a) The concentration ratio of ATP/ADP

The evidence for changes in this concentration ratio was discussed on p.49, and it was shown that, in the whole tissue, it decreased during contraction. It was assumed that the intramitochondrial concentration ratio also decreased during contraction.

b) The concentration ratio of $NAD^+$/NADH

This ratio was shown to increase in isolated, incubated mammalian mitochondria, in the transition from state 4 to state 3 respiration, and to increase in some insect flight muscles and decrease in others (see pp.50-52). If the active state of muscle corresponds to state 3 in respiring isolated

mitochondria (or a fraction of state 3, see Hansford,1977) then changes observed in isolated mitochondria may be taken to reflect those occurring *in vivo*.

c) The concentration of free $Ca^{2+}$

No conclusion could be reached about the direction of change of the free concentration of $Ca^{2+}$ during contraction, as the data was inconclusive (see pp.52-54).

d) The concentrations of succinyl CoA, citrate and oxaloacetate

Hansford (1974) found a rise in the concentration of succinyl CoA in incubated mitochondria from blowfly flight muscle upon addition of an uncoupling agent, and also in the state 4 to state 3 transition. Thus in controlled respiration (or the resting state) a lower level of succinyl CoA would be expected; this is not consistent with the suggested role of succinyl CoA in the modulation of citrate synthase activity proposed by LaNoue *et al*. (1972).

Citrate levels in insect flight muscle mitochondria do fall, however, in the state 4 to state 3 transition (Johnson & Hansford, 1975) and this may reflect the situation *in vivo*, where, it is suggested, a lowered intramitochondrial citrate deinhibits CS during contraction.

Oxaloacetate availability may control the activity of CS as suggested above. The concentration of oxaloacetate will be dependent upon the $NAD^+$/NADH concentration ratio via the reaction catalysed by malate dehydrogenase which, from the evidence of the activity of the enzyme *in vitro*, is believed to catalyse a near-equilibrium reaction (Pette & Luh, 1962).

$$\text{malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH$$

In mammalian mitochondria, where the concentration ratio of $NAD^+$/NADH is believed to increase during contraction, the concentration of oxalo-

acetate would be expected to increase. Indeed, there is evidence to show that mitochondrial oxaloacetate increases with an increased work load, in perfused rat hearts (Opie & Owen, 1975).

From the evidence, therefore, it seems likely that the concentration ratios of ATP/ADP, $NAD^+$/NADH and the concentrations of citrate and oxaloacetate may be the physiological regulators of the first three non-equilibrium enzymes of the cycle.

A problem arises in the consideration of amino acid oxidation in resting muscle (see pp.6-7, 21-25). It is known that six amino acids are known to be oxidised by muscle: leucine, isoleucine, valine, aspartate, glutamate and alanine. Of these, aspartate, glutamate, valine and isoleucine enter the TCA cycle at some point in the 2-oxoglutarate-oxaloacetate span; aspartate gives rise to oxaloacetate, glutamate gives rise to 2-oxoglutarate and valine and isoleucine give rise to succinyl CoA. It has been suggested that the enzyme phosphoenolpyruvate carboxykinase may remove oxaloacetate from the cycle during amino acid oxidation (Goldstein & Newsholme, 1976). However, increased concentrations of 2-oxoglutarate and succinyl CoA may have different effects on the coordination of control between the two spans of the TCA cycle (see Fig. 7).

If the reaction catalysed by OGDH is not a flux-generating step (although the evidence presented in this Chapter suggests that it is) then an increase in 2-oxoglutarate concentration may make it so. In either case, the activity of OGDH will not be greatly increased. But if 2-oxoglutarate inhibits the ICDH reaction (there is preliminary evidence that this is the case for NADP-ICDH) then an increased concentration of 2-oxoglutarate will inhibit the flux through the ICDH reaction. This will increase the concentration of isocitrate, which will also increase the concentration of citrate,

**Figure 7** REGULATION OF THE TRICARBOXYLIC ACID CYCLE DURING AMINO ACID OXIDATION.

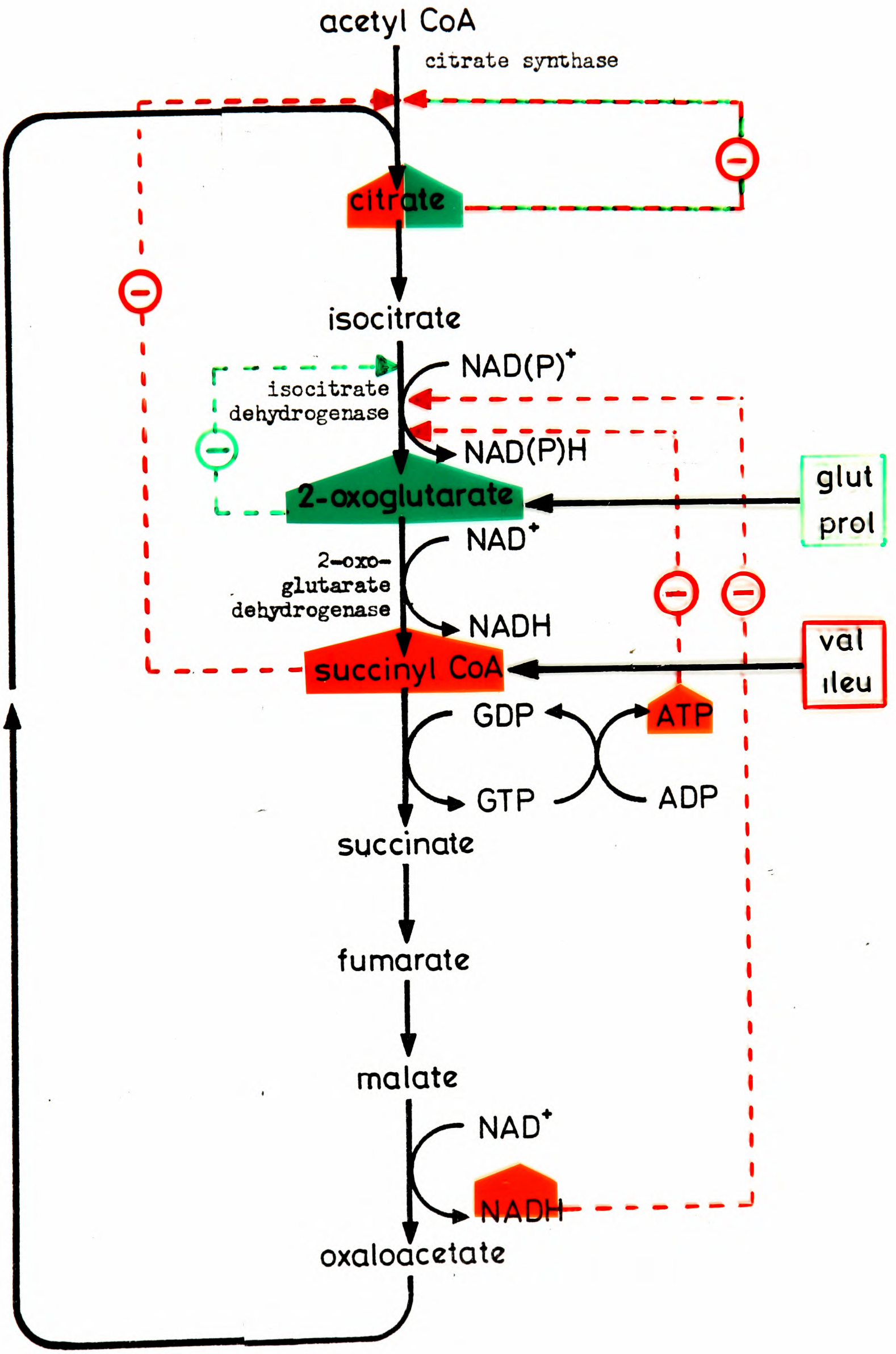
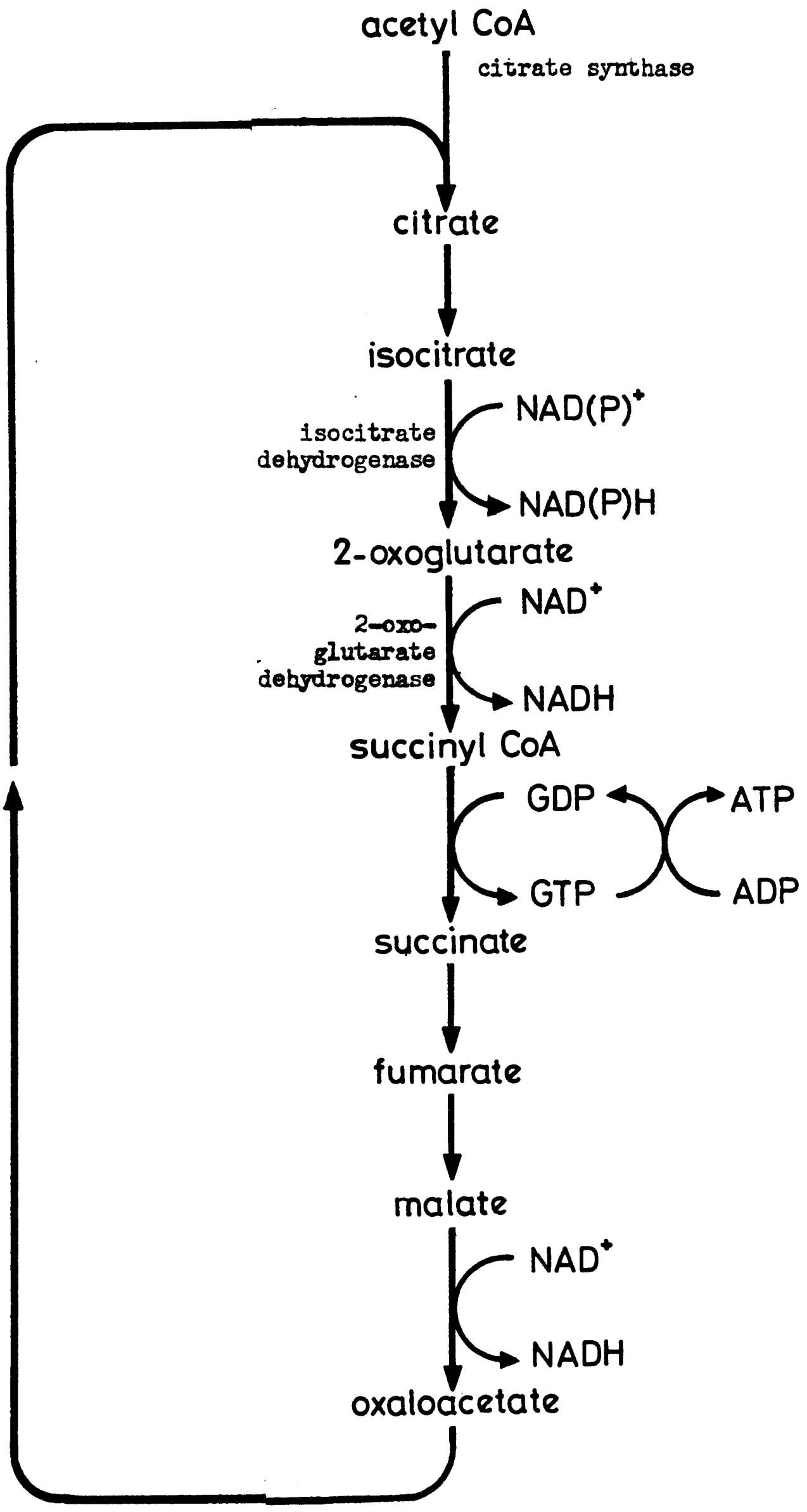


**Figure 7** REGULATION OF THE TRICARBOXYLIC ACID CYCLE DURING AMINO ACID OXIDATION.



via the near-equilibrium reaction catalysed by aconitase. This increased concentration of citrate will inhibit the activity of CS and hence decrease the flux through this reaction, as it is a flux generating step. In effect, the appearance of a fuel source at the level of 2-oxoglutarate decreases the flux through the acetyl CoA-2-oxoglutarate span of the cycle, and therefore slows down the utilisation of other fuels such as carbohydrate. (The flux through PDH will also be decreased, as a result of the inhibition of PDH by increased acetyl CoA concentrations). It is possible that other amino acids may be utilised by muscle for energy production, perhaps during high protein diets, and this sort of regulation may occur if these amino acids feed in at 2-oxoglutarate.

The increased concentrations of succinyl CoA caused by the oxidation of valine and isoleucine may give rise to increased ATP production, if the reaction catalysed by succinyl CoA-synthetase is not a flux generating step. An increased ATP/ADP ratio would inhibit PDH, ICDH and OGDH and decrease the flux through the span of the cycle from acetyl CoA to succinyl CoA. Indeed, assuming that there are no further flux generating steps in the TCA cycle, an increased concentration of succinyl CoA will increase the flux through the succinyl CoA-oxaloacetate span, which would increase the concentration of NADH. This would also have the effect of inhibiting the first span of the cycle via NADH inhibition of NAD-ICDH and OGDH (see Fig. 7).

Thus there are several possible ways in which concerted control of the TCA cycle may occur in cases where increased concentrations of TCA cycle intermediates occur, e.g. in amino acid oxidation.

4) USE OF ENZYME ACTIVITIES AS INDICATIONS OF FLUX

In this chapter, evidence has been reviewed to reveal how little quantitative information there is on the aerobic metabolic pathways crucial

to provision of energy for contraction in muscle, i.e. pyruvate oxidation and the series of reactions known as the TCA cycle. An experimental approach to the regulation of metabolism, which is based on the knowledge of the equilibrium nature of the reactions in a pathway, and how the theories of control are established has been explained above. This approach has been applied to pyruvate oxidation and the TCA cycle (Section Four).

This approach to metabolic regulation leads to two important points which are crucial in the designing of experiments in this field. Firstly, knowledge of the equilibrium nature of the reactions of a pathway suggests suitable candidates for 'indicators of flux' (see p. 37) i.e. non-equilibrium reactions and especially flux generating steps.

Secondly, from the understanding of the control properties of an enzyme, knowledge of the conditions for the assay of maximal enzyme activity *in vitro* can be obtained. If the theory of control appears to explain fully the behaviour of the enzyme *in vivo*, and if this theory is backed by the evidence, this knowledge of the control properties confers confidence in the use of maximum enzyme activity data for the indication of flux through the metabolic pathway.

1) Candidates for indicators of flux through pyruvate oxidation and the TCA cycle

It appears that there are four candidates for flux-generating steps in pyruvate oxidation and the TCA cycle: PDH, CS, OGDH and in insect flight muscle, NAD-ICDH. Now, while theoretically it can be suggested that enzymes catalysing flux generating steps are the only ones which can give useful information on the maximum flux through a pathway, it is known that this may not always be the case. CS is a case in point. As the activity of CS is limited *in vivo* by the concentration of the co-substrate oxaloacetate (which is present at saturating concentrations *in vitro*, in the assay of the enzyme) the maximum catalytic activity of CS *in vitro* is much greater than the flux through the enzyme *in vivo* (see p.58).

However,it appears that PDH and OGDH may be candidates for the role of 'indicators of flux'. That is, their maximum catalytic activity *in vitro* may provide an indication of the flux through their respective pathways *in vitro*. An investigation of whether the maximum activities of these enzymes can be used in this way has been carried out in this thesis, and is discussed further in Chapter 4.

2) *Evaluation of the theory of control of pyruvate oxidation and the TCA cycle*

From the evidence presented above, it appears that the theory of control of PDH adequately explains the behaviour of the enzyme *in vivo*. The synergistic effects of the multiple effectors, combined with the added sensitivity conferred by the interconversion cycle, should cause a sufficient increase in the activity of PDH in the initiation of contraction to account for the increased flux. However, in insects, this flux may be thirty to one hundred fold greater during flight than that at rest, and it is difficult to see how insect PDH may be controlled to this extent, unless an interconversion cycle (or other amplification mechanism to increase the sensitivity to effector concentration, see p. 32) exists. However, there is no evidence for an interconversion cycle (see p.70).

The theory of control of the TCA cycle, or rather the control of CS, NAD-ICDH and OGDH, seems to suffer from the same drawback. From the evidence presented above, none of the effectors appear to give rise to changes in enzyme activity of the order of a hundred fold. However, very little is known about the changes in the free concentration of these effectors and of the divalent cations, and these might change considerably within the mitochondria.

There are several possible explanations for this shortcoming in the theory. The most obvious one is that the theory is incomplete, i.e. there

is another aspect of control which has not yet been discovered. As explained on p. 31, only a small relative increase in activity (i.e. for a small change in effector) can be achieved by simple reversible binding of an allosteric effector. It is possible that an effector normally at very low concentrations in the cell, e.g. free $Ca^{2+}$, cAMP, may increase by a large enough amount to increase the activity of the enzymes by many fold even by a simple reversible binding mechanism; such an effector may activate (or inhibit) CS, NAD-ICDH or OGDH. There is already some evidence that $Ca^{2+}$ may activate NAD-ICDH (Denton, 1978) and OGDH (McCormack & Denton, 1979).

Alternatively, a mechanism that increases the sensitivity of the control mechanisms (e.g. a substrate cycle or an interconversion cycle) may exist. The possibility of substrate cycling at the level of CS, by the involvement of the enzyme ATP-citrate lyase, is discussed in Chapter 2. Some sort of interconversion cycle may also exist for these enzymes. It might be expected that the activity of OGDH may be controlled via an interconversion cycle, by analogy with PDH. However, while the evidence suggests that a phosphorylation cycle does not exist (see p.70), it is possible that alternative forms of covalent modification may occur, e.g. an acetylation interconversion cycle(see Chapter Five).

Another possible explanation for the failure of the theory to explain the behaviour may be that the evidence for changes in the effector concentrations is misleading. For example, changes in free ADP concentration, and hence the energy charge, may be obscured in muscle by the large amount of ADP bound to actin, as present measurements cannot distinguish between free and bound(see p.50). In addition, there is little quantitative data on the concentrations of free $Mg^{2+}$ and free $Ca^{2+}$ in mitochondria.

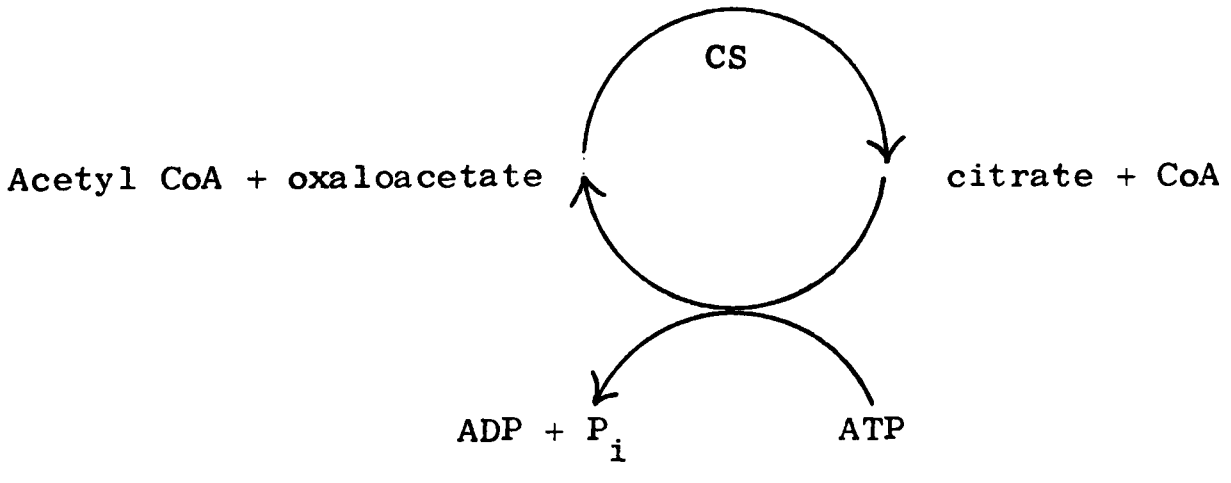
It seems therefore that of the two possible candidates for indicators of flux, PDH and OGDH, more confidence can be placed in the assay of

the maximum activity of PDH in mammals, as the control properties provide a theory of control which stands up tc the experimental tests *in vivo*. For PDH in insects, and for OGDH, the crucial test for indication of flux must be the comparative survey of maximum activities *in vitro*, and their comparison with the flux *in vivo*.

# CHAPTER TWO

## NADH OXIDATION: A PROBLEM WITH ASSAYS INVOLVING CREATINE PHOSPHATE

In the previous Chapter, the importance of the tricarboxylic acid (TCA) cycle in muscle was discussed and an approach to metabolic regulation was outlined in which maximum catalytic activities of the enzymes of metabolic pathways could give information about the rates of utilisation of fuels. This approach depends on the identification of the two types of enzyme reactions: near equilibrium and non-equilibrium. In the TCA cycle, many studies have focussed on citrate synthase, the first enzyme of the cycle. (For further discussion, see section four of Chapter One). The maximum activity of citrate synthase when measured *in vitro* is very high when compared with the estimated flux through the cycle. This might suggest that citrate synthase catalyses a near-equilibrium reaction. However, there are two circumstances which could affect the flux through the citrate synthase reaction *in vivo*. Firstly, if oxaloacetate were limiting *in vivo*, then citrate synthase activity would be limited by its availability and the flux through the citrate synthase reaction would be much lower than its activity *in vitro*. There is evidence that this may be the case (see Chapter One). Secondly, if there were a high rate of a separate, reverse reaction (from citrate to acetyl CoA) the overall flux in the direction of citrate formation would be reduced. The reverse reaction would be catalysed by the enzyme ATP-citrate lyase. Indeed, if both enzymes were simultaneously active, this would produce a substrate cycle as follows:



An advantage of such a cycle would be increased sensitivity for the control of flux through the citrate synthase reaction. (See Crabtree & Newsholme, 1976, and p. 32). It seemed important, therefore, to investigate the activity of ATP-citrate lyase, especially in insect flight muscle, where the flux through the TCA cycle increases many fold in the transition from rest to flight.

The assay of ATP-citrate lyase in muscle tissue proved initially to be difficult, and hence required further investigation. This Chapter is concerned with a problem that arose during these investigations.

The assay of this enzyme was attempted by linking the reaction to that of malate dehydrogenase and following the change in absorbance of NADH at 340 nm (see Methods) in a Gilford spectrophotometer.

ADP, the product of the ATP-citrate lyase reaction, inhibits this enzyme and this interferes with the measurement of maximum enzyme activity. To reduce the inhibitory effect of ADP, an ATP regenerating system was included to remove the ADP. The use of an ATP regenerating system for the removal of product inhibition, whether by ADP or AMP, has been used by Skidmore (1967), Newsholme & Taylor (1969) and Crabtree & Newsholme (1972a).

In the assay that was used in this work the creatine phosphate/creatine phosphokinase ATP regenerating system was used. In this system the following reaction occurs in which CPK represents creatine phosphokinase:

$$\text{creatine phosphate} + \text{ADP} \xrightarrow{\text{CPK}} \text{Creatine} + \text{ATP}$$

During the assay of ATP-citrate lyase, crude homogenates of various tissues were used and it was found that rates of NADH oxidation in the control cuvette (i.e. in the absence of the substrate, citrate) were similar to those in the experimental cuvette. If the activity of ATP-citrate lyase in flight muscle was low, it was important to try to eliminate these high 'blank' activities. Indeed, in locust, (Schistocera gregaria), blowly (Callinphora erythrocephala) and waterbug (Lethocerus cordofanus) flight muscle,

ATP-citrate lyase activity was not detectable (i.e. $< 0.5$ $\mu$mol.min$^{-1}$.g$^{-1}$).

The same high blank activities were encountered in a pyruvate carboxylase assay which also included the CP/CPK regenerating system (see Methods section for assay system). It was finally established that the NADH oxidation occurred only in the presence of creatine phosphate, CPK, ATP, $Mg^{2+}$ and a crude homogenate of one of a variety of animal tissues (see Methods section for details of the assay system for NADH oxidising activity). The activities of the NADH oxidising system obtained when the individual constituents of the assay system were omitted are presented in Table I.

The high rates of NADH oxidation could not be explained by normal respiratory NADH oxidases since these should be inhibited by the 2-heptyl-4-hydroxy-quinoline-N-oxide (HHQ) present in the assay system.

As these high 'blank' activities prevented investigations of ATP-citrate lyase activity in muscle, it seemed important to discover the source of these high rates of NADH oxidation. Various experiments were carried out to see whether the NADH oxidation was due to an enzyme or enzyme system, to investigate the properties of the reaction and to look at its distribution in various different tissues and animals.

## METHODS

1. Materials

The creatine phosphate was supplied by the Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG. Small samples of different batch numbers were forwarded from Boehringer Mannheim GmbH via the Boehringer Corporation (London) Ltd., For all other materials, see Appendix III.

2. Enzyme Assays. All enzyme assays were performed at 25$^{o}$C on a Gilford recording spectrophotometer. The enzyme activity was calculated from the

TABLE I

ACTIVITY OF THE NADH OXIDISING SYSTEM IN THE ABSENCE OF VARIOUS ASSAY CONSTITUENTS

The activity of the NADH oxidising system was measured in the standard extract of rat liver. Assay conditions are described in the Methods section. CP represents creatine phosphate and CPK represents creatine phosphokinase.

| COMPONENT OMITTED | ACTIVITY $\mu mol.min^{-1}.ml^{-1}$ | % MAXIMUM ACTIVITY |
|---|---|---|
| None | 13.9 | 100.0 |
| ATP | 1.9 | 13.7 |
| CPK | 2.5 | 18.0 |
| CP | 1.1 | 7.9 |

rate of change of absorbance. One unit of activity is defined as that which converts one $\mu$mol of substrate per minute and is expressed per g.wet weight of the tissue ( $\mu mol.min^{-1}.g^{-1}$).

ATP-citrate lyase. The assay was performed as described in the introduction, and the disappearance of NADH was followed at 340 nm in the spectrophotometer. Assay conditions were 75 mM-TRIS, 1 mM-CoA, 0.19 mM-NADH, 5 mM-$MgCl_2$, 2.5 mM-ATP, 20 mM-citrate, 5 mM- creatine phosphate, 160 $\mu g.ml^{-1}$ CPK and 7 units of malate dehydrogenase, at a final pH of 8.5 and in a total volume of 2 ml.

NADH oxidising activity. Assay conditions were 50 mM-imidazole, 10 mM-$MgCl_2$, 2.5 mM-ATP, 0.17 mM-NADH, 2.5 $\mu g.\ ml^{-1}$ HHQ (in ethanolic solution), 5 mM creatine phosphate and 160 $\mu g.ml^{-1}$ CPK, in a final volume of 2.0 ml and at a final pH of 7.1. The disappearance of NADH was followed in the spectrophotometer at 340 nm. For the comparative survey of different tissues, the extraction medium consisted of 50 mM-imidazole, 10 mM-$MgCl_2$ and 1 mM-EDTA adjusted to pH 7.1. Tissues were homogenised in glass homogenisers with 5 to 10 volumes of extraction medium at $0^{o}C$. For all other investigations into the nature of the activity, a standard preparation of rat liver supernatant was used (see "Standard extract of rat liver").

Citrate synthase (CS). This assay was performed according to the method of Srere (1969). In the following reaction

$$\text{Acetyl CoA + oxaloacetate} \xrightarrow{CS} \text{CoA + citrate}$$

the reduced CoA is estimated by the use of 5,5' dithiobis (2 nitrobenzoate) (DTNB or Ellman's reagent). The course of the reaction was followed at 412 nm at which wavelength the mercaptide anion absorbs (with an absorption coefficient of $13.6 \times 10^3$ l. $mol.^{-1}cm^{-1}$).

The assay conditions were 50 mM-TRIS, 0.2 mM-DTNB, 0.1 mM-acetyl CoA, 0.2 mM-sodium oxaloacetate in a final volume of 2 ml and at a final pH of 7.4.

Lactate dehydrogenase (LDH)

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

This reaction was followed at 340 nm.

Final concentrations in the assay system were: 60 mM-TRIS, 0.17 mM-NADH, 0.3 mM-sodium pyruvate, 1 mM-potassium cyanide in a total volume of 2 ml and at pH 7.5. The potassium cyanide was in the form of a buffered reagent, described in Appendix [II].

3. Metabolite assays. All the metabolites were measured by enzymatic assays in which the reaction utilising the metabolite is linked to a reaction utilising or producing NADH or NADPH. The NADH or NADPH was measured by the change in absorbance at 340 nm in a Gilford spectrophotometer. The amount of nicotinamide nucleotide oxidised or reduced is proportional to the metabolite present. Usually, more than one metabolite assay was performed sequentially in the same cuvette.

Fructose 6-phosphate (F6P) and glucose 6-phosphate (G6P)

F6P and G6P were measured according to the method of Lang & Michal (1974). Reactions involved:

$$\text{F6P} \xrightarrow{\text{GPI}} \text{G6P} \xrightarrow[NADP^+ \quad NADPH + H^+]{\text{G6PDH}} \text{6-phosphogluconolactone}$$

Where GPI represents glucosephosphate isomerase, and G6PDH, glucose 6-phosphate dehydrogenase.

Final concentrations in the assay sytem were: 250 mM-TRA, 2.5 mM-EDTA, 12.5 mM-$MgCl_2$, 0.2 mM-NADP at pH 7.6. The final volume was 2 ml. The sample volume was 10-100 $\mu$l. Method: Glucose-6-phosphate dehydrogenase (G6PDH) was added to a final concentration of 10 $\mu g.\ ml^{-1}$ to measure G6P. Glucosephosphate isomerase (GPI) was added to a final concentration of 2 $\mu g.ml^{-1}$ to measure F6P.

Fructose-1,6-bisphosphate (FbP), dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP)

FbP, DHAP and GAP were measured according to the method of Michal & Beutlar (1974).

Reactions involved:

1. FbP $\xrightarrow{\text{aldol}}$ DHAP + GAP

2. GAP $\xrightarrow{\text{TIM}}$ DHAP

3. DHAP $\xrightarrow{\text{G3PDH}}$ glycerol-3-phosphate

$NADH + H^+$ $\rightarrow$ $NAD^+$

where aldol represents aldolase, TIM, triosephosphate isomerase, and G3PDH, glycerol-3-phosphate dehydrogenase.

Final concentrations in the assay system were: 250 mM-TRA, 2.5 mM-EDTA, 0.17 mM-NADH at pH 7.6. The final volume was 2 ml, and the sample volume was 100-200 $\mu$l.

Method: Glycerol-3-phosphate dehydrogenase was added to a final concentration of 5 $\mu g.ml^{-1}$ to measure DHAP. Triosephosphate isomerase was added to a final concentration of 0.25 $\mu g.ml^{-1}$ to measure GAP. Aldolase was added to a final concentration of 7.5 $\mu g.ml^{-1}$ to measure FbP. Two molecules of NADH are oxidised for each molecule of FbP.

Pyruvate, phosphoenol pyruvate (PEP), 2 phosphoglycerate (2PGA) and 3-phosphoglycerate (3PGA)

Pyruvate, PEP, 2PGA and 3PGA were measured according to the method of Czok & Lamprecht (1974).

Reactions involved:

1. 3PGA $\xrightarrow{\text{PGM}}$ 2PGA

2. 2PGA $\xrightarrow[Mg^{2+}]{\text{enol}}$ PEP + $H_2O$

3. PEP + ADP $\xrightarrow[K^+,\ Mg^{2+}]{\text{PK}}$ pyruvate + ATP

4. pyruvate + NADH + $H^+$ $\xrightarrow{LDH}$ lactate + $NAD^+$

where PGM represents phosphoglycerate mutase, enol., enolase, PK, pyruvate kinase and LDH, lactate dehydrogenase. Final concentrations in the assay system were: 300 mM-TRA, 3 mM-EDTA, 0.15 mM-NADH at pH 7.6, in a final volume of 2 ml.

Method: Lactate dehydrogenase was added to a final concentration of 15 $\mu$g. $ml^{-1}$ to measure pyruvate. Then ADP, $MgSO_4$, KCl and pyruvate kinase were added to 1.2 mM, 10 mM, 37 mM and 10 $\mu$g.$ml^{-1}$ respectively to measure PEP. Enolase was added to 10 $\mu$g. $ml^{-1}$ to measure 2PGA. Phosphoglycerate mutase was added to 100 $\mu$g. $ml^{-1}$ to measure 3PGA.

<u>3-phosphoglycerate (3PGA), dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP)</u>

3PGA, DHAP and GAP were also measured according to the method of Czok (1974).

Reactions involved:

1. 3PGA + ATP $\xrightarrow{PGK}$ ADP + 3-phosphoglyceroyl phosphate
2. 3-phosphoglyceroyl phosphate + NADH + $H^+$ $\xrightarrow{GAPDH}$ GAP + $P_i$ + $NAD^+$
3. GAP $\xrightarrow{TIM}$ DHAP
4. DHAP + NADH + $H^+$ $\xrightarrow{G3PDH}$ glycerol-3-phosphate + $NAD^+$

where PGK represents phosphoglycerate kinase, GAPDH, glyceraldehyde-3-phosphate dehydrogenase, TIM, triosephosphate isomerase and G3PDH, glycerol-3-phosphate dehydrogenase.

Final concentrations in the assay system were 10 mM-TRA, 1 mM-EDTA, 0.1 mM-NADH, 8 mM-$MgSO_4$, 7 mM-ATP at pH 7.6. The final volume was 2 ml and the sample volume was 100 $\mu$l.

Method: Glycerol-3-phosphate dehydrogenase was added to a final concentration of 20 $\mu$g. $ml^{-1}$ to measure DHAP. Triosephosphate isomerase was added to a final concentration of 10 $\mu$g. $ml^{-1}$ to measure GAP. Glyceraldehyde-

3-phosphate dehydrogenase was added to a final concentration of 100 $\mu g.ml^{-1}$ and phosphoglycerate kinase was added to 100 $\mu g$. ml to measure phosphoglyceroyl phosphate and 3PGA. Each molecule of 3PGA gave rise to two molecules of NADH.

It was not possible to measure 3-phosphoglyceroyl phosphate and 3PGA separately since commercial glyceraldehyde-3-phosphate dehydrogenase contains some phosphoglycerate kinase.

ATP, glucose-6-phosphate (G6P) and creatine phosphate (CP)

ATP, G6P and CP were measured according to the method of Lamprecht & Trautschold (1974).

Reactions involved:

1. CP + ADP $\xrightarrow{CPK}$ Creatine + ATP

2. ATP + glucose $\xrightarrow{HK}$ G6P + ADP

3. G6P + $NADP^+$ $\xrightarrow{G6PDH}$ 6-phosphogluconolactone + NADPH + $H^+$

where HK represents hexokinase, G6PDH, glucose-6-phosphate dehydrogenase. Final concentrations in the assay system were 50 mM-TRA, 5 mM-$MgCl_2$, 1 mM-NADP, 0.5 mM-ADP at pH 7.6. The final volume was 2.0 ml and the sample volume was 10-100 $\mu l$.

Method: Glucose-6-phosphate dehydrogenase was added to a final concentration of 7.5 $\mu g.ml^{-1}$ to measure G6P. Glucose was added to 10 mM and immediately afterwards hexokinase was added to 7.5 $\mu g.ml^{-1}$ to measure ATP. The method was modified to include one further reaction: CPK was added to a final concentration of 40 $\mu g.\ ml^{-1}$ to measure creatine phosphate.

$NAD^+$

Reaction involved:

Lactate + $NAD^+$ $\xrightarrow[pH\ 9.5]{LDH}$ pyruvate + NADH + $H^+$

The principle depends upon the equilibrium of the lactate dehydrogenase (LDH) reaction lying to the right at pH 9.5. Final concentrations in the

assay system were 400 mM-glycine, 158 mM-hydrazine sulphate, 2 mM-EDTA (these constituents were present in a buffer adjusted to pH 9.5 just before use) and 5 mM-lactate. The total volume was 0.5 ml.

Method: Lactate dehydrogenase was added to a final concentration of 20 $\mu$g. $ml^{-1}$ to measure $NAD^+$.

4. Cell fractionation of rat liver. Mitochondrial and cytoplasmic fractions were prepared by the method of Werkheiser & Bartley (1957) except that the 7 g of rat liver were extracted in 35 ml of extraction buffer (0.25 M-sucrose, 1 mM-EDTA at pH 7.8).

5. Standard extract of rat liver. For the investigation into the properties of the NADH oxidising system, a standard preparation of rat liver supernatant was made. The rat liver homogenate as described above was centrifuged at 105,000 g for one hour to obtain a clear extract with no microsomes or mitochondria and this extract was kept frozen at -20°C in aliquots of 0.5 ml which were removed and thawed as required. These were only used for measurements of activity within the first month, as 50% of the activity was lost over three months, but other experiments such as testing creatine phosphate purity were still possible with a low activity extract.

## RESULTS OF STUDIES ON THE NADH OXIDATION

For these studies, a standard extract of rat liver was used (see above for preparation) as this was an easier system in which to obtain a mitochondrial free extract, and it gave good reproducible activity.

1. Establishing the enzymic nature of the reaction

To establish whether the observed oxidation was due to an enzyme, the following tests were carried out. The stadard extract of rat liver supernatant was boiled for three minutes and the NADH oxidising activity was lost, The activity of this extract was measured at various temperatures and a $Q_{10}$

was calculated (see Table II). A $Q_{10}$ of 2.2 was obtained which is consistent with an enzyme catalysed reaction (Lehinger, 1975, p. 196).

2. Properties of the NADH oxidising system

(i) Components of the system

Various components were omitted from the assay system, and the activity of the NADH oxidising system was measured (see Table I). These experiments led to the following conclusions.

(a) When NADH and NADPH were tested, activity was only obtained in the presence of NADH.

(b) Only 14% of the maximal activity was shown in the absence of ATP. However, ATP could be replaced by ADP in the presence of CPK. Presumably under these conditions ATP is generated from CP in the cuvette.

(c) Only 8% of the activity was shown in the absence of creatine phosphate. (further details are given below).

(d) With the standard extract, only 18% of the activity was shown in the absence of CPK. Activities of the NADH oxidising system were also measured in a variety of different tissues, in the presence and absence of CPK. (see Table VII). These values showed that the requirements for CPK varied from tissue to tissue. In all cases, CPK seemed to increase both the linearity of the response to substrate and the rate of the reaction.

It was possible that the CPK contained some contaminant, either enzymic or non-enzymic, which was exerting this effect. To test whether a non-enzymic contaminant was responsible, the CPK was boiled. The effect on the NADH oxidising system was destroyed, showing that if any contaminant in the CPK was implicated, it must also be of an enzymic nature.

It was postulated that the CPK was acting with the creatine phosphate either as an ATP-regenerating system or as an ADP-removing system. In the absence of CPK, activities of the standard extract were measured at various

TABLE II

EFFECT OF TEMPERATURE ON THE NADH OXIDISING ACTIVITY OF THE STANDARD EXTRACT OF RAT LIVER

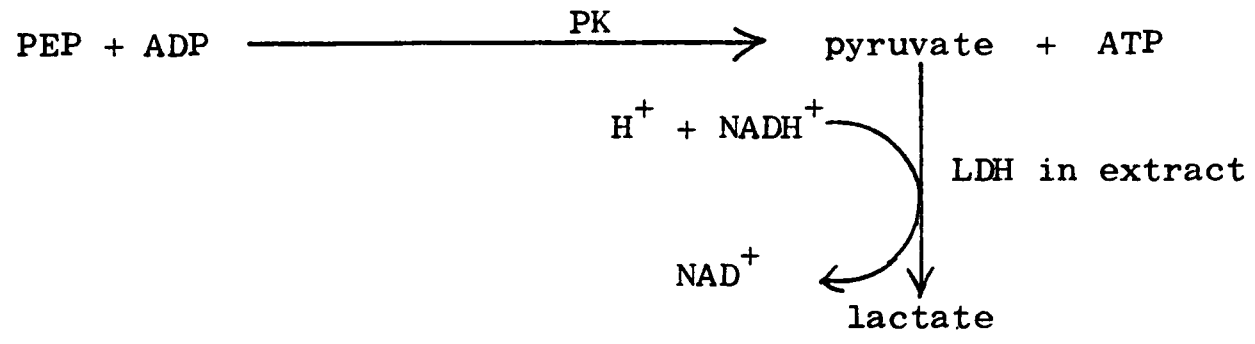
The assay system of the NADH oxidising activity and the preparation of the standard extract of rat liver are described in the Methods section.

| TEMPERATURE $^{o}C$ | NADH OXIDISING ACTIVITY ($\mu mol.min.^{-1}.g^{-1}$) |
|---|---|
| 25.0 | 6.9 |
| 35.5 | 12.5 |
| 40.6 | 16.1 |
| 44.5 | 17.8 |

concentrations of ATP (see Table III). There is no increase in activity; in fact, there may be a decrease. This suggests that the CPK is not acting as an ATP-regenerating system.

However, in the absence of CPK and in the presence of added ADP, the activity of the standard extract is reduced (see Table IV). Addition of CPK restores the original activity. Since CPK is known to convert ADP to ATP in the presence of creatine phosphate, the stimulatory effect of this enzyme on the NADH oxidising activity could be due to the reduction of the ADP concentration in the cuvette. Furthermore, this explanation could account for the slight decrease in activity at high ATP concentrations, since ADP is a contaminant of the ATP used in this work (see Table IV).

In order to test this proposed function of CPK, an alternative ADP removing system might have been used; i.e. PEP and pyruvate kinase. However, in the standard preparation of rat liver supernatant, lactate dehydrogenase (LDH) would be present and this would oxidise NADH when pyruvate was produced by the ADP removing system.




Thus the disappearance of NADH could not be attributed solely to the system under investigation.

(ii) Products of the reaction

In enzymatic assays involving dehydrogenases, the course of the reaction may be followed at 340 nm in a spectrophotometer. Any decrease in optical density is interpreted as oxidation of the nicotinamide nucleotide involved. However, it is possible that chemical degradation of the NADH to

TABLE III

EFFECT OF INCREASED ATP CONCENTRATION ON NADH OXIDISING ACTIVITY

The experiment was performed on the standard extract of rat liver supernatant. Activities are expressed in $\mu mol.min^{-1}.ml^{-1}$, and the range of observations is given in parentheses, from three separate determinations. Creatine phosphokinase was omitted from the assay system.

| ATP CONCENTRATION (mM) | ACTIVITY ($\mu mol.min^{-1}ml^{-1}$) | % MAXIMUM ACTIVITY |
|---|---|---|
| 2.5 | 0.91 (0.85-0.97) | 100 |
| 5.0 | 0.76 (0.70 - 0.82) | 84 |
| 7.5 | 0.60 (0.54 - 0.66) | 66 |
| 10.0 | 0.54 (0.50 - 0.58) | 59 |

TABLE IV

EFFECT OF ADP CONCENTRATION ON NADH OXIDISING ACTIVITY

The assay conditions of the NADH oxidising activity are given in the Methods section. The experiment was performed on the standard extract of rat liver.

| CONCENTRATION OF ADP mM | ACTIVITY $\mu mol.min^{-1}.g^{-1}$ | % MAXIMUM ACTIVITY |
|---|---|---|
| 0 | 0.79 | 100 |
| 0.5 | 0.26 | 32 |
| 1.0 | 0.19 | 24 |
| 2.5 | 0.12 | 12 |
| 2.5* | 0.80* | 100* |

*In this experiment, 320 $\mu g$ of creatine phosphokinase were added.

$NAD^+$ could be causing a decrease in optical density (e.g. in acid conditions, see Heldt et al., 1965, 1967). These possibilities could be distinguished by demonstrating the production of $NAD^+$ during the course of the reaction.

The utilisation of NADH was followed on the Gilford spectrophotometer. When a reasonable proportion of NADH had been used, the reaction in the cuvette was stopped by the addition of perchloric acid (to a final concentration of 2.5%). The sample was neutralised with $KHCO_3$ and the $NAD^+$ concentration measured (see Methods section).

The amount of NADH utilised was almost identical to the amount of $NAD^+$ formed so that it is concluded that $NAD^+$ was a product of the reaction (Table V).

(iii) Requirement for other small molecules

It seemed possible that small molecules in the extract were acting either as cofactors in the reaction or as effectors of the "enzyme". It can be calculated that any such small molecules would be diluted approximately a thousand-fold from the rat liver to the assay system (10 $\mu$l of a 1 in 5 extraction being taken in 2 ml assay system). Thus, if the molecules were present in millimolar concentration in the liver, they would be present in micromolar concentration in the assay system and could exert an effect on the reaction.

To test this possibility, small molecules were removed from the liver extract by dialysis or gel filtration. There was no effect of either treatment on the activity of the NADH oxidising system (Table VI).

3. Distribution of the NADH oxidising activity in different tissues and animals

The activity of this enzyme was measured in various tissues and from different animals and the results are shown in Table VII together with values for phosphofructokinase (PFK) and lactate dehydrogenase (LDH). PFK has been shown to catalyse a non-equilibrium reaction in various tissues and its

TABLE V

EXPERIMENT TO TEST THE PRODUCTION OF $NAD^+$ FROM NADH BY THE NADH OXIDISING SYSTEM

The details of the experimental system are given in the text p.89 .

Values given are duplicates for experiments 1 and 2.

| EXPERIMENT | $\mu$mol NADH USED | $\mu$mol $NAD^+$ PRODUCED |
|---|---|---|
| 1 | 0.127 | 0.131 |
| | 0.126 | 0.131 |
| 2 | 0.135 | 0.153 |
| | 0.095 | 0.108 |

TABLE VI

EFFECT OF GEL FILTRATION AND DIALYSIS ON THE NADH OXIDISING ACTIVITY

The standard extract of rat liver was applied to a G25 Sephadex column, and eluted using a buffer consisting of 0.25M-sucrose. 1 mM-EDTA adjusted to pH 7.8 with TRIS. Protein was estimated by the method of Warburg & Christian (1941). A volume of liver extract wad dialysed overnight against 0.25M-sucrose, 1 mM-EDTA adjusted to pH 7.8 with TRIS.

| TREATMENT | NADH OXIDISING ACTIVITY $\mu mol.min^{-1}.ml^{-1}$ | PROTEIN CONCENTRATION $mg.ml^{-1}$ | SPECIFIC ACTIVITY $\mu mol.min^{-1}.mg^{-1}$ (protein) |
|---|---|---|---|
| Sample subjected to filtration | 0.91 | 3.45 | 0.26 |
| Sample not subjected to gel filtration | 1.17 | 5.17 | 0.23 |
| Dialysed sample | 0.58 | 3.36 | 0.17 |
| Sample not subjected to dialysis | 0.91 | 4.73 | 0.19 |

TABLE VII

ACTIVITIES OF THE NADH OXIDISING SYSTEM, PHOSPHOFRUCTOKINASE AND LACTATE DEHYDROGENASE INDTISSUES FROM VERTEBRATES AND INVERTEBRATES

The assay of the NADH oxidising system in given in the Methods section. Sources of animals are given in Appendix III.

| ANIMAL | TISSUE | ENZYME ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$ fresh weight) | | | |
|---|---|---|---|---|---|
| | | NADH OXIDISING ACTIVITY | | PHOSPHOFRUCTO-KINASE | LACTATE DEHYDROGENASE |
| | | in the presence of CPK | in the absence of CPK | | |
| Cockroach (Periplaneta americana) | Flight muscle | 17.0 | | *19.0 | * 1.5 |
| Desert locust (Schistocerca gregaria) | Flight muscle | 21.1 | 2.6 | *17.0 | * 2.9 |
| Laboratory mouse (Mus musculus) | Liver | 27.4 | 23.0 | † 2.4 | |
| | Heart | 55.6 | 49.4 | | |
| | Diaphragm | 71.2 | 60.9 | | |
| | Gastrocenmius | 57.2 | 53.3 | †*28.0 | |
| | Brain | 5.5 | | ‡ 14.6 | |
| Laboratory rat (Rattus norvegicus) | Heart | 26.0 | | *10.0 | *311.0 |
| | Liver | 5.7 | 0.7 | 2.5 | |
| | Gastrocnemius | 27.7 | 14.2 | †* 47.0 | *448.0 |
| | Lung | 4.9 | 4.3 | | |
| | Adipose tissue | 3.9 | 3.9 | | |
| | Kidney | 3.6 | 4.6 | ‡ 2.8 | |
| | Brain | 6.6 | | ‡ 18.0 | |
| Chicken (Gallus gallus) | Pectoral muscle | 149.0 | 141.0 | * 105.0 | *870.0 |
| | Leg muscle | 36 | 29.4 | | |
| Pheasant (Phasanus calchicas) | Pectoral muscle | 184.0 | | * 143.0 | *542.0 |

*These values are from Crabtree & Newsholme (1972a)

‡ These values are from Sugden (1973)

† These values were from the quadriceps muscle, not from the gastrocnemius

maximal catalytic activity *in vitro* can be used as an indicator of flux through the glycolytic pathway, *in vivo*. (Crabtree & Newsholme, 1972a). The survey of the activities of the NADH oxidising enzyme was carried out to see if its variation could be related to the glycolytic capacity of the tissue, as indicated by PFK.

The NADH oxidising activity was highest in muscle, especially in chicken and pheasant pectoral and in the leg muscles from mouse, rat and chicken. There was some correlation between the NADH oxidising activities and the capacity of the tissue for glycolysis. Assuming that phosphofructokinase activity is an indicator of glycolytic capacity, a correlation coefficient was estimated for the relationship between NADH oxidising activity and glycolytic capacity (see Table VIII). A correlation coefficient of 0.96 was obtained, and this was significant at $P = 0.001$. This showed a positive correlation between NADH oxidising activity and glycolytic capacity.

4. Subcellular localisation of the NADH oxidising system

Although it seemed unlikely that respiratory NADH oxidases (intramitochondrial) were involved, since HHQ was present in the assay system, it seemed important to determine whether the NADH oxidising activity was mitochondrial or cytoplasmic. Fractionation of rat liver homogenate was undertaken (see Methods) and the main components of the cell (mitochondrial and cytoplasmic) were assayed for NADH oxidising activity. Citrate synthase (CS) and LDH were used as markers for the mitochondrial and cytoplasmic compartments respectively. Fractions containing mitochondria were subject to ultrasonic disintegration before assaying for enzyme activities. The results are shown in Table IX.

The ratio of the activity in the cytoplasmic fraction to that in the mitochondrial fraction was high for lactate dehydrogenase and NADH oxidising enzyme (14.8 for LDH and 11.0 for NADH oxidising activity) and low for CS (0.1),

TABLE VIII

CORRELATION BETWEEN NADH OXIDISING ACTIVITY AND GLYCOLYTIC CAPACITY

The data are taken from Table VII.

x = NADH oxidising activity

y = PFK activity = indicator of glycolytic flux.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| x | 17.0 | 21.1 | 24.4 | 57.2 | 5.5 | 26.0 | 5.7 | 27.7 | 3.6 | 6.6 | 149.0 | 184.0 |
| y | 19.0 | 17.0 | 2.4 | 28.0 | 14.6 | 10.0 | 2.5 | 47.0 | 2.8 | 18.0 | 105.0 | 143.0 |

Correlation coefficient $r = \frac{1}{n-1}\left[\frac{(x-\bar{x})\,(y-\bar{y})}{S_x S_y}\right]$

This can be written $r = \dfrac{xy - (x_n)(y)}{\left[x^2 - \frac{(x)^2}{n}\right]\left[y^2 - \frac{(y)^2}{n}\right]}$

From above values, r = 0.96 with 10 degrees of freedom.

This is significant at P = 0.001.

TABLE IX

CITRATE SYNTHASE AND LACTATE DEHYDROGENASE ACTIVITIES OF THE NADH OXIDISING SYSTEM IN THE CYTOPLASMIC AND MITOCHONDRIAL FRACTIONS FROM RAT LIVER

The assay conditions for the NADH oxidisng system, citrate synthase and lactate dehydrogenase are given in the Methods section. The fractionation of rat liver is described in the Methods section.

| ENZYME (OR ENZYME SYSTEM) | ENZYME ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$ fresh wt.) | | RATIO OF CYTOPLASMIC TO MITOCHONDRIAL FRACTIONS |
|---|---|---|---|
| | MITOCHONDRIAL FRACTION M | CYTOPLASMIC FRACTION C | C/M |
| NADH oxidising system | 0.02 | 0.22 | 11.0 |
| citrate synthase | 0.09 | 0.01 | 0.1 |
| lactate dehydrogenase | 0.23 | 3.40 | 14.8 |

suggesting that the activity of the NADH oxidising system is located in the cytoplasm.

Triton X-100 was added to the assay system to a concentration of 0.05% in order to disrupt the mitochondria. No increase in activity was observed, and this confirmed the cytoplasmic location of the enzyme.

## INTERIM DISCUSSION

The properties presented above suggested an NADH oxidising system with the following characteristics.

1) The activity is located in the cytoplasm.

2) The system requires creatine phosphate, ATP and NADH.

3) The activity is inhibited by ADP.

4) The inhibition by ADP may be reduced in the presence of CPK.

5) The maximal activity correlates with glycolytic capacity.

These properties were difficult to explain on the basis of any known simple enzyme system. Such an NADH oxidising system could have important significance in the operation of the glycolytic pathway, as its activity appeared to be related to that of the rate of glycolysis. It could have constituted a new glycolytic reaction or a reaction which might increase the energy production of that tissue.

However, when the dependence of the system on creatine phosphate was investigated, an interesting point was noted. Although only 8% of the activity was shown in the absence of creatine phosphate, no increase in activity was demonstrated when creatine was added to the cuvette. In the presence of creatine, ATP and CPK, creatine phosphate should be generated in the cuvette. This suggested that the system did not require creatine phosphate itself, but that there was a contaminant in the preparation of creatine phosphate used in this work.

The Biochemica Catalogue (1968 Edition) of Boehringer und Soehne GmbH Mannheim (Germany) stated that the creatine phosphate that they supplied was prepared chemically, thus suggesting a possible chemical contamination.

It was necessary to investigate further this contaminant to discover whether it could be acting as a substrate for the NADH oxidising system. Firstly, the concentration of the contaminant in creatine phosphate solutions

was measured, using an assay system for NADH oxidising activity (as described below). Secondly, the investigation of the identity of the contaminant would have to be carried out.

Many lines of investigation could have been followed to identify the contaminant. One approach to the problem would be to isolate the contaminants involved by using standard chemical procedures and identify the nature of the compounds. However, as the amount of contaminant would be small, and as isolation would require the use of a bioassay, this approach would require considerable development of biochemical and chemical separation techniques and this would require a great deal of time.

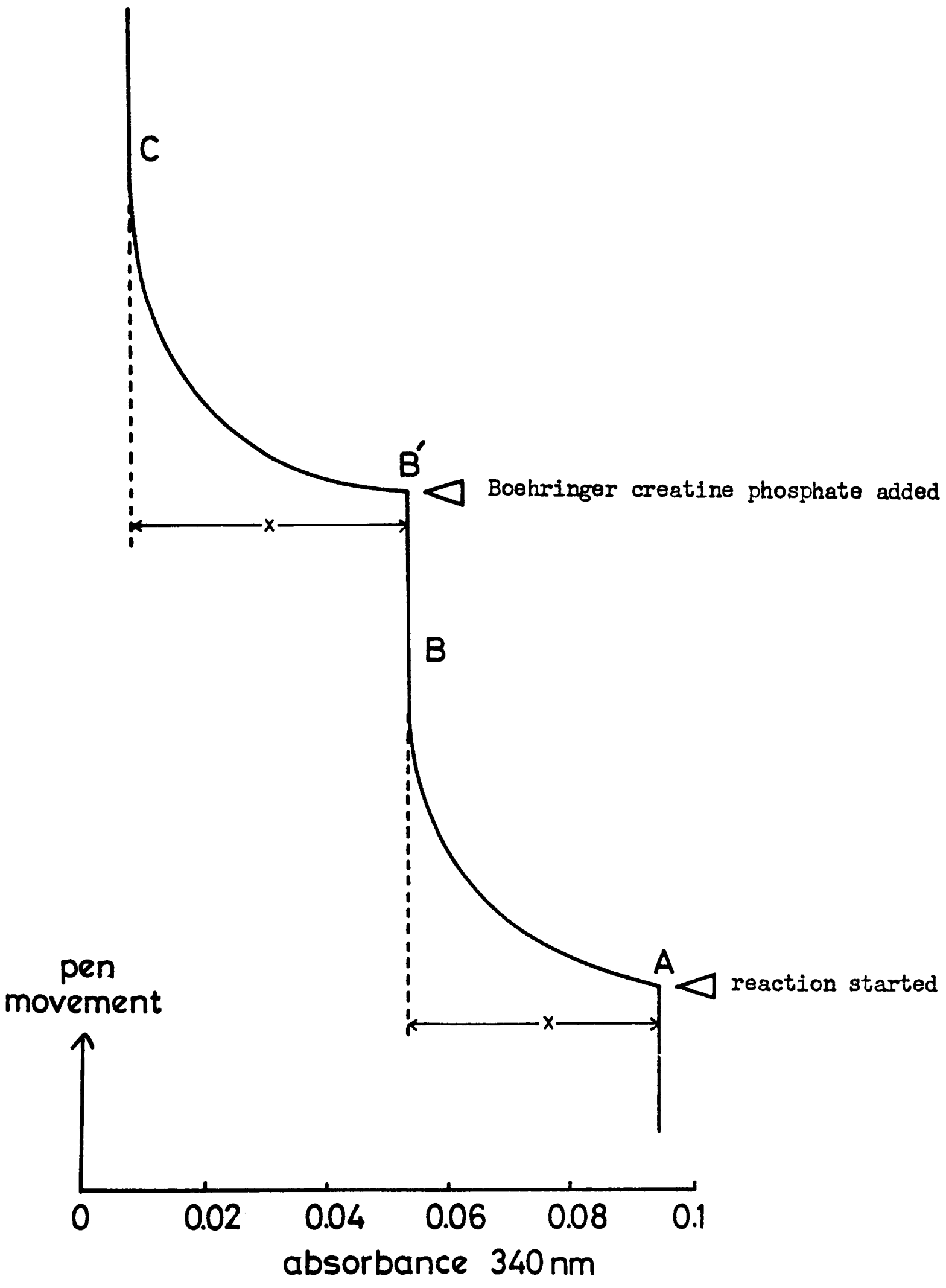
Since it appeared possible that the contaminant would be an organic phosphate produced during the chemical phosphorylation of creatine, the technique of phosphorus magnetic resonance (phosphorus n.m.r.) available in the Department of Biochemistry, Oxford, could be applied to the identification of the substance. This technique might be able to identify an organic phosphate without the need for purification. Phosphorus n.m.r. has been developed as a method of detection of phosphorus compounds (Hoult et al., 1974). It has become increasingly useful for identifying and measuring phosphorylated intermediates in biological samples e.g. blood, homogenates and even intact, isolated organs. However, a limitation of the method is that the compound must be present at a concentration of approximately 1 mM to be detected.

## RESULTS OF EXPERIMENTS ON CONTAMINATION OF CREATINE PHOSPHATE

### 1) Test System

The NADH oxidising activity assay system was set up to test possible contaminants. Quantitative experiments on the components of the system were performed by recording the absorbance change after the reaction has stopped i.e. when the component has been used up (see Figure 8). If the reaction

**Figure 8** DIAGRAM OF SPECTROPHOTOMETER TRACE USED TO ESTABLISH THE SOURCE OF CONTAMINANT.



is started at A, with extract, then the NADH oxidation no longer occurs at B, after x absorbance units. None of the possible contaminants of ATP (AMP, ADP, CTP, GTP, CDP, GDP) gave rise to any absorbance change after B, and the only component capable of restarting the reaction at B' was Boehringer creatine phosphate.

If creatine phosphate was added at B' to the same final concentration (i.e. 5 mM) as was previously present, then the reaction stopped again after x absorbance units at C. In a typical experiment, x = 0.3 absorbance units, which corresponds to a utilisation of 0.1 $\mu$mol of NADH (equivalents) in a total volume of 2 ml. Thus whereas 10 $\mu$mol of creatine phosphate were added, only 0.1 $\mu$mol of NADH were utilised. Consequently, it was deduced that a contaminant was present in creatine phosphate at a level of 1%. The presence of a small percentage of contaminant in the creatine phosphate was confirmed by another line of evidence. Creatine phosphate can be generated in the cuvette in the presence of creatine, ATP and CPK. However, when these components were used to replace creatine phosphate, no NADH oxidising activity was observed.

Obvious contaminants of creatine phosphate include creatine and inorganic phosphate (formed by hydrolysis) and creatinine (formed by non-enzymic cyclisation). None of these compounds were effective in restarting the reaction, nor would they substitute for creatine phosphate in the assay system.

2) Investigation into the degree of contamination of creatine phosphate

The samples of creatine phosphate supplied by Boehringer were designated by a batch number (60, 70, 72 etc.) and each batch was investigated for the level of contamination. In Table X values are given for levels of contamination of various batches of creatine phosphate obtained from Boehringer Corporation. Alternative sources of commercial creatine phosphate were

TABLE X

CONCENTRATIONS OF CREATINE PHOSPHATE AND ITS CONTAMINANT IN DIFFERENT SAMPLES OF CREATINE PHOSPHATE

The concentration of creatine phosphate in aqueous solutions of different samples from the Boehringer Corporation Ltd., was measured as described in the Methods section. The contaminant (C) was measured in the same solutions by the assay system described in the text, p.94 . In the assay of the contaminant of batches 72, 90, 91, 92 and 93, the NADH oxidising assay system (plus creatine phosphate) was pre-incubated with lactate dehydrogenase before addition of extract. The range of three separate determinations is given in parentheses.

| SAMPLES OF BOEHRINGER CREATINE PHOSPHATE Batch no. | CONCENTRATION OF CREATINE PHOSPHATE mM | CONCENTRATION OF CONTAMINANT (C) mM | % CONTAMINATION (PRE-INCUBATED) | % CONTAMINATION (NOT PRE-INCUBATED) |
|---|---|---|---|---|
| 63 | 106 (102-110) | 1.03 (0.99-1.07) | - | 0.97 (0.93-1.01) |
| 72 | 392 (383-401) | 2.64 (2.61-2.67) | 0.67 (0.64-0.70) | - |
| 80 | 370 (364-376) | 1.51 (1.47-1.55) | - | 0.41 (0.34-0.48) |
| 90 | 105 (100-110) | 0.640(0.635-0.645) | 0.61(0.58-0.64) | - |
| 91 | 106 ( 97-115) | 0.770(0.765-0.775) | 0.73 (0.72-0.74) | - |
| 92 | 123 (104-142) | 0.41 (0.39-0.43) | 0.33 (0.26-0.40) | - |
| 93 | 98 (98-98.2) | 0.55 (0.53-0.57) | 0.56 (0.54-0.58) | - |

Sigma Chemical Company and Calbiochem. Creatine phosphate obtained from these latter firms contained no detectable contamination (i.e. they gave rise to no change in absorbance in the assay system described above).

To investigate the source of the variation of the levels of contaminant, observations were made on the stability of the contaminant under laboratory conditions. It was observed that during freezing and thawing of aqueous solutions of creatine phosphate, in the course of this investigation, the contaminant was stable but the creatine phosphate was degraded (Table XI). Consequently, the contamination, expressed as a percentage of the creatine phosphate, increased. Similarly while creatine phosphate was unstable in solution at room temperature, the contaminant was stable (Table XI).

3) N.m.r. Experiment

In order to be detected by phosphorus n.m.r., a phosphorylated compound must be present at approximately 1 mM. The contaminant of creatine phosphate appeared to be present at between 0.8 and 1.0% of the creatine phosphate. Thus a solution of 1M creatine phosphate will contain between 8 and 10 mM contaminant. If this contaminant is a single phosphorylated compound, it should be detected at this concentration. A sample of 1M-creatine phosphate in buffer was put in the n.m.r. spectrometer but no peaks other than those of creatine phosphate and inorganic phosphate were seen. Thus either the contaminant is not phosphorylated or it is present at a concentration of less than 0.8-1.0% of the creatine phosphate.

TABLE XI

LEVELS OF CREATINE PHOSPHATE AND CONTAMINANT

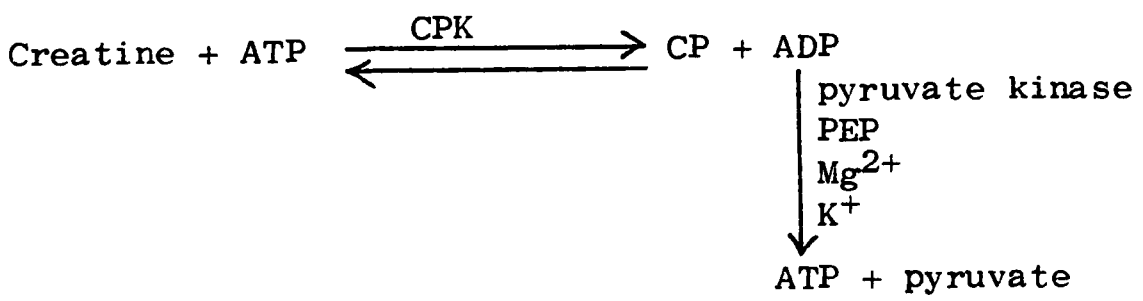
IN A SOLUTION OF CREATINE PHOSPHATE

From days 1-29 the solution of creatine phosphate was frozen and thawed. From days 29-31 the solution of creatine phosphate was left at 20°C. The concentration of contaminant was estimated using the metabolite assay described in the text on p.94 . Creatine phosphate was measured as described in the Methods section.

| DAY | CONCENTRATION OF CONTAMINANT (mM) | CONCENTRATION OF CREATINE PHOSPHATE (mM) | APPARENT % CONTAMINATION |
|---|---|---|---|
| 1 | 0.87 | 101 | 0.86 |
| 3 | 0.86 | 94 | 0.91 |
| 29 | 0.84 | 89 | 0.94 |
| 31 | 0.84 | 35 | 2.40 |

## RESULTS AND DISCUSSION ON THE NATURE OF THE CONTAMINANT

At this stage of the investigation, the Boehringer Corporation (London) Ltd. were contacted and they supplied the information that their creatine phosphate was made enzymatically from creatine and ATP, using PEP and pyruvate kinase to shift the equilibrium in favour of creatine phosphate formation.




(It was previously believed from the information given in their catalogue that the creatine phosphate was made chemically.) They also provided information from Boehringer Mannheim GmbH that certain glycolytic intermediates were present in the creatine phosphate preparation to the extent of 0.1% (Table XIII).

In order to see whether any of the contaminants present gave rise to NADH oxidation in the system under investigation, each of these compounds was added to the assay system and the activity of the standard preparation (i.e. rate of NADH oxidation) was measured (Table XII). Thus it can be seen that the following contaminants, pyruvate, PEP, 2PGA and 3PGA, give rise to NADH oxidation under these conditions. The high activity due to pyruvate was presumably due to the lactate dehydrogenase in the extract.

The levels of the various glycolytic intermediates present in the creatine phosphate were measured (Table XIII). In order to measure the concentration of the contaminant, (abbreviated as C in Table X) the assay system for the NADH oxidising enzyme was used, and allowed to run out of substrate, as described previously. However, in some cases, (i.e. batches

TABLE XII

NADH OXIDISING ACTIVITY OF EXTRACT WITH GLYCOLYTIC INTERMEDIATES SUBSTITUTED FOR CREATINE PHOSPHATE

The metabolites were added to the NADH oxidising activity assay system, described in the Methods section. The extract used was the standard extract of rat liver.

| SUBSTANCE ADDED | FINAL CONCENTRATION IN ASSAY SYSTEM (mM) | NADH OXIDISING ACTIVITY ($\mu$mol .min$^{-1}$.ml$^{-1}$) |
|---|---|---|
| creatine phosphate | 5 | 5.1 |
| ATP | 1 | 0 |
| G6P | 1 | 0 |
| PEP | 1 | 1.9 |
| 2PGA | 1 | 1.8 |
| 3PGA | 1 | 3.6 |
| pyruvate | 1 | 21.4 |

TABLE XIII

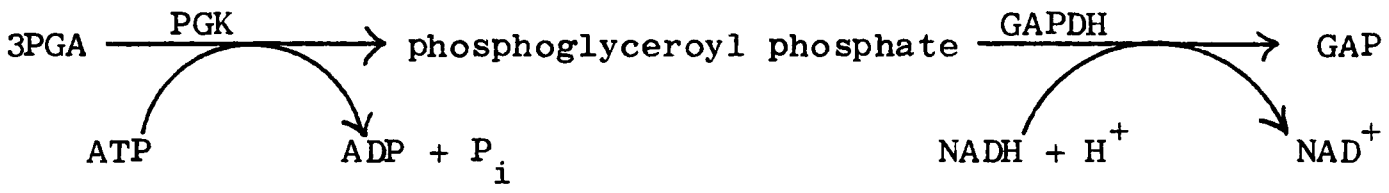
MEASUREMENT OF LEVELS OF CONTAMINATION OF CREATINE PHOSPHATE BY GLYCOLYTIC INTERMEDIATES

The glycolytic intermediates and creatine phosphate were measured as described in the Methods section. The figures below express the percentage contamination of creatine phosphate of the intermediates. Figures in brackets are those supplied by Boehringer Mannheim GmbH (letter dated 16.10.75)

| CONTAMINANT | % CONTAMINATION OF CREATINE PHOSPHATE | | |
|---|---|---|---|
| | Boehringer Batch no. 72 | 80 | 90 |
| ATP | 0 | 0 | 0 (0.0037) |
| G6P | 0.009 | 0.020 | 0.020 (0.0057) |
| FDP | 0.006 | 0.006 | 0.006 |
| Pyruvate | 0.190(0.046) | 0.060 | 0.059 (0.021) |
| PEP | 0.067(0.050) | 0.040 | 0.052 (0.024) |
| 2PGA | 0.020(0.016) | 0.020 | 0.006 (0.007) |
| 3PGA | 0.177(0.114) | 0.174 | 0.225 (0.110) |
| DHAP | 0.006 | 0.006 | 0.006 |
| GAP | 0.006 | 0.006 | 0.006 |

72, 90, 91, 92 and 93) the assay system was pre-incubated with 10 $\mu$g lactate dehydrogenase in order to remove the pyruvate present, as this is known to be one of the contaminants (see below). Thus, in these batches the concentration measured is that of the remainder of the contaminants.

As can be seen from Table XIII, contamination by 3PGA is greater than by the other intermediates and this can give rise to NADH oxidation as shown in Table XII. If, as is likely, the glycolytic enzymes phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are present in the standard extract, then the following reactions could occur.

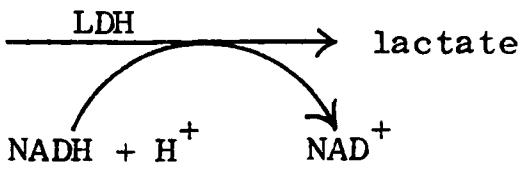



The presence of ATP and NADH in the assay system would shift the equilibrium position of these reactions in the direction shown.

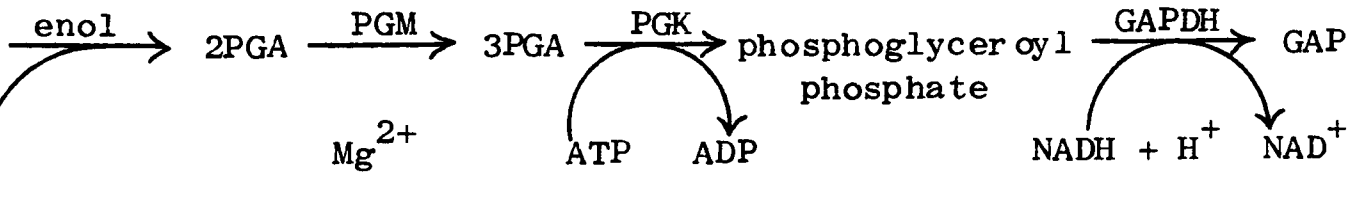
The other contaminants 2PGA, PEP and pyruvate will also cause oxidation of NADH (Table XII). In the presence of the glycolytic enzymes phosphoglycerat mutase and enolase, 2PGA will be converted to 3PGA and, in the presence of $Mg^{2+}$, PEP will be converted to 2PGA.

Thus the following reactions could explain the properties so far establishe

(1)




(2)




In addition further reactions are possible from glyceraldehyde-3-phosphate. In the presence of triosephosphate isomerase and glycerol-3-phosphate dehydrogenase, a further molecule of NADH can be oxidised for each molecule of the

glycolytic intermediate present.

(3) GAP $\xrightarrow{\text{TIM}}$ DHAP $\xrightarrow[NADH + H^+ \quad NAD^+]{\text{G3PDH}}$ glycerol-3-phosphate

Thus, the maximum oxidation of NADH that can occur within this scheme is given by all the glycolytic intermediates that can be converted to glycerol-3-phosphate. In this way, the amounts of the contaminants will sum and each molecule of the intermediates involved in reaction (2) will oxidise two molecules of NADH to $NAD^+$. The "apparent contamination", as measured by the NADH oxidation system by these intermediates (PEP, 2PGA and 3PGA) will be double the actual contamination as measured individually by direct enzymatic assays.

However, pyruvate cannot be converted to glycerol-3-phosphate, so that the effect of this compound is achieved via the LDH reaction. Thus although it is a contaminant which must be taken into account, the extent of its contamination is not doubled as with the other glycolytic compounds. To overcome this problem, pyruvate is removed prior to the investigation of the percentage contamination due to the other intermediates. This is done by pre-incubating with LDH (see above).

The concentration of PEP, 2PGA and 3PGA can be added together ( $\Sigma$ ) and then multiplied by two to give a value X, which represents the NADH oxidation assay. Values of X are presented with values of C, the apparent contamination, in Table XIV. For the creatine phosphate samples of batch no. 72 and 90, the apparent contamination was measured after pre-incubation with LDH. In batch no. 80, however, the apparent contamination (C) was measured without pre-incubation with LDH. In this case, the value of the percentage contamination of pyruvate was added to ($2 \times \Sigma$) to give the value X.

TABLE XIV

APPARENT PERCENTAGE CONTAMINATION OF CREATINE PHOSPHATE AND PERCENTAGE CONCENTRATIONS OF PYRUVATE, PEP, 2PGA AND 3PGA IN SAMPLES OF BOEHRINGER CREATINE PHOSPHATE

The apparent percentage contamination (C) of creatine phosphate, measured via the NADH-oxidising system, is taken from Table X. The value X is the sum of all the percentage concentrations of the contributing metabolites. The value $\Sigma$ is the sum of the percentage contaminations of PEP, 2PGA and 3PGA. In the samples of creatine phosphate referred to as batch nos. 72 and 90, $X = 2 \times \Sigma$. In batch no. 80, $X$ = (% concentration of pyruvate) + $(2 \times \Sigma)$. This is because C was measured in this sample without pre-incubation with LDH (see text). The range of observations is given in parentheses, and the number of observations was 3.

| CONTAMINANT | % CONTAMINATION (OR APPARENT CONTAMINATION) OF CREATINE PHOSPHATE | | |
|---|---|---|---|
| Batch no. | 72 | 80 | 90 |
| PEP | 0.07 (0.06-0.07) | 0.04 (0.03-0.05) | 0.05 (0.05-0.06) |
| 2PGA | 0.02 (0.019-0.022) | 0.02 (0.019-0.021) | 0.01 (0.005-0.008) |
| 3PGA | 0.18 (0.17-0.19) | 0.17 (0.13-0.18) | 0.23 (0.21-0.24) |
| $\Sigma$ | 0.26 (0.25-0.28) | 0.23 (0.18-0.25) | 0.28 (0.26-0.30) |
| $2 \times \Sigma$ | 0.53 (0.50-0.56) | 0.47 (0.36-0.50) | 0.57 (0.51-0.61) |
| pyruvate | - | 0.06 (0.05-0.06) | - |
| X | 0.53 (0.50-0.56) | 0.53 (0.41-0.56) | 0.57 (0.51-0.61) |
| C | 0.67 (0.62-0.70) | 0.41 (0.34-0.48) | 0.61 (0.58-0.61) |

It can be seen that these estimates of X are similar to those of C and account for the degree of contamination shown. As the values for apparent contamination for batches 72 and 90 are those from experiments where pre-incubation with LDH has removed pyruvate, the actual NADH oxidation taking place will be greater due to the contamination by pyruvate and participation of LDH in the extract.

Thus an experimental system containing creatine phosphate and crude tissue extracts will exhibit NADH oxidation due to reactions (1), (2) and (3). As the creatine phosphate was in this case being used as part of an ATP regenerating system, the ATP/ADP concentration ratio was high. The NADH/$NAD^+$ concentration ratio was also high and under these conditions the reactions (1), (2) and (3) favour NADH oxidation (i.e. reversal of the normal glycolytic reactions).

If the results of these investigations are correct and the contamination represents primarily phosphorylated intermediates, the question arises as to why they were not detected by phosphorus n.m.r. There may be two answers. Firstly, as several contaminants were present, no one phosphorylated intermediate was present at a high enough level to be detectable in the n.m.r. sample of creatine phosphate. Secondly, the stoichiometry of NADH to contaminant molecule was not 1:1 and thus the apparent contamination suggested a higher concentration of the contaminants than was present. Thus the phosphorylated intermediates though present in the 1M-creatine phosphate sample were all below the level of detection.

## CONCLUSION

The problem discussed in this Chapter shows how contamination of biochemical compounds can raise complications in experimental systems. It is

important, therefore, to check on the purity of substances used and to be aware of the contribution that even small amounts of contamination may make to an experimental system. It is also important to contact the biochemical company supplying the product as soon as possible when this sort of complication arises, as the information which the company can supply may help solve the problem. In addition, the company may benefit from observations made on side reactions involving their product. In the case discussed here, Boehringer acknowledged the problem (see Boehringer Information Service Issue 7b, August 1977) and are now improving the purity of their creatine phosphate.

Having established the nature of the contamination of Boehringer creatine phosphate, it was considered desirable to utilise creatine phosphate from other sources i.e. Sigma Chemical Company (see Appendix III). By using this creatine phosphate in the ATP regenerating system, the assay for ATP-citrate lyase was again attempted. There was very little change in absorbance in the control cuvette, i.e. in the absence of citrate, showing that the problem of high 'blank' activities had been eliminated. It was then demonstrated that the activities of ATP-citrate lyase were indeed low, i.e. in locust, blowfly and waterbug flight muscle, ATP-citrate lyase activities were still undetectable, i.e. $< 0.05\ \mu mol.\ min^{-1}.g^{-1}$.

Thus it was assumed that in insect flight muscle generally, ATP-citrate lyase activities were not high enough to suggest that a substrate-cycle at the level of citrate synthase might control the flux through this enzyme reaction. It was deduced that the major effectors of the flux through the citrate synthase reactions were oxaloacetate, citrate and succinyl CoA concentration, as proposed in Chapter One.

# CHAPTER THREE:

# DEVELOPMENT OF ASSAYS FOR PYRUVATE DEHYDROGENASE AND 2-OXOGLUTARATE DEHYDROGENASE

## INTRODUCTION

In Chapter One, the equilibrium nature of the enzymes of pyruvate oxidation and the TCA cycle was discussed. The enzymes pyruvate dehydrogenase (PDH) and 2-oxoglutarate (OGDH) were shown to catalyse non-equilibrium reactions. Furthermore, comparison of $K_m$ values for their pathway substrate with the estimated levels of these substrates *in vivo* suggested that both reactions may be flux generating (see p.75). If this is so, then these two enzymes would be responsible for the generation of flux through two very important pathways in oxidative metabolism, i.e.pyruvate oxidation and the pathway known as the TCA cycle (see Chapter One for discussion).

Since these enzymes catalyse non-equilibrium reactions, their maximum catalytic activities *in vitro* may provide an indication of the maximum flux through these pathways. Although the TCA cycle is the most important route for energy production under aerobic conditions, no one enzyme activity has been found which gives a quantitative index of the maximum rate of operation of the cycle. The enzymes so far investigated (i.e. citrate synthase, $NAD^+$-linked and $NADP^+$-linked isocitrate dehydrogenase) have proved to be unsatisfactory. (See next Chapter for fuller discussion.) Consequently, it was important to ascertain if the maximum activities of OGDH and/or PDH could provide an indication of flux through the TCA cycle.

It must be stressed that, as aerobic metabolism is so important in muscle (see Chapter One), it would be very valuable to have a quantitative index of the extent of aerobic metabolism in different muscles. A survey

of the maximum activities *in vitro* of PDH and OGDH from muscles in which some knowledge of the flux through pyruvate oxidation and the TCA cycle is known was therefore carried out. In this way, it would be possible to test whether such activities could be used as indicators of flux through pyruvate oxidation and the TCA cycle, as suggested in Chapter One, i.e. by comparison of $V_{max}$ values with maximum flux.

PDH and OGDH are both multi-enzyme complexes, located within the mitochondrion, and are possibly membrane-bound (see Chapter One and later discussion). They are both keto-acid dehydrogenases and catalyse a similar reaction sequence. The reaction sequence of the PDH complex in particular is very complicated, involving as it does an interconversion cycle. It might be expected, therefore, that problems would be encountered in the assay of these enzymes, especially since this was to be done using extracts of different muscles from a wide variety of animals. Indeed, during the development of the assays for these enzymes, many problems were encountered and had to be overcome before the maximum activities could be compared with the flux through the pathways. This Chapter discusses the development of the enzyme assays and the modifications required to overcome the problems encountered. The Chapter is divided into two sections; the development of the assay system for PDH and the development of the assay system for OGDH.

## SECTION 1: THE DEVELOPMENT OF THE ASSAY SYSTEM FOR PYRUVATE DEHYDROGENASE

### 1) MATERIALS

Sources of the chemicals and animals used are given in Appendix III.

### 2) METHODS

#### a) Preparation of extracts

Animals were killed and the muscle (or other tissue) was removed as rapidly as possible after death. Birds were anaesthetised with carbon dioxide and killed by cervical fracture; rats and mice were stunned and killed by cervical fracture; amphibians were pithed; *Xenopus* was first anaesthetised with Sandoz MS-222; invertebrates were anaesthetised at 4°C by putting them in the refrigerator for 30 minutes and killed by cutting off the head or by severing the nerve cord from the brain or cerebral ganglia. The muscle was kept on ice for less than an hour until required for assay, when it was chopped as finely as possible. The muscle was homogenised in ground glass homogenisers with 10 volumes of extraction medium at 0°C. Two extraction media were used. For vertebrate tissues, the extraction medium consisted of 100 mM-TRIS-HCl, 1 mM-EDTA, 25 mM-$MgCl_2$, 1 mM-$CaCl_2$, 5 mM-mercaptoethanol (MSH) and 10 mM-sodium pyruvate. The final pH was 7.8.

For insects and molluscs, the extraction medium consisted of 100 mM-TRIS-HCl, 1 mM-EDTA, 5 mM-MSH, 2 mM-$MgCl_2$ and 10 mM-sodium pyruvate. The final pH was 7.8.

#### b) Extraction of the active fraction of PDH

Only a proportion of total PDH is active in most tissues, the rest is present in the inactive, phosphorylated form (see Chapter One, p.46). In order to measure the proportion in the active form the following procedure was used.

It was found that stress increased the active proportion of PDH, especially in the case of stunning rats. In these experiments, therefore, the rats were killed by interperitoneal injection of Barbital. The muscles were rapidly excised and freeze clamped (i.e. clamped with a pair of aluminium tongs cooled in liquid nitrogen) and then powdered in a percussion mortar at -70°C. The powder was kept in liquid nitrogen until extraction. Ceramic mortars and pestles were cooled on solid carbon dioxide and a weighed amount of muscle powder was added to each mortar.

Extraction medium, consisting of 100 mM-TRIS-HCl, 5 mM-MSH, 5 mM-EDTA at pH 7.8, was pipetted on to the powder to a dilution of 1 in 10 (weight per volume). The mortars were transferred to ice and the frozen mixture was ground as it melted. The homogenates were decanted into tubes, subjected to ultrasonic disintegration in an M.S.E. sonicator (for 60 seconds at an amplitude of 6) to complete the homogenisation and disrupt the mitochondria, and were then kept on ice for up to ten minutes. The activity of PDH in the extracts was measured as described below.

c) Incubation for conversion to total PDH

Pig heart $PDH_b$-phosphatase was prepared by the method of Denton et al. (1972). Extracts of muscle, phosphatase ($850\,U.ml^{-1}$)*, rat serum and a stock solution of $MgCl_2$ and $CaCl_2$ (250 mM-$MgCl_2$ plus 10 mM-$CaCl_2$) were added in the ratio of 4:4:1:1. A typical incubation consisted of 16 $\mu$l extract, 16 $\mu$l phosphatase, 4 $\mu$l of rat serum and 4 $\mu$l of the stock solution of $MgCl_2$ and $CaCl_2$. (Final concentrations of $MgCl_2$ and $CaCl_2$ were 25 mM and 1 mM respectively Maximal conversion to the active form was complete in 20 minutes.

d) Enzyme assay

PDH was assayed by following the rate of change of absorption at 460 nm in a Gilford recording spectrophotometer (model 240) at 25°C.

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* One unit of $PDH_b$ phosphatase activity is defined as the amount of enzyme which converts 1% of $PDH_b$ to $PDH_a$ per minute.

The assay is coupled to the acetylation of the dye, para-aminoazobenzene sulphonic acid (pAABS); the acetylation is catalysed by the enzyme arylamine acetyl transferase (AAAT). (See Tabor et al., 1953; Jacobson, 1961).

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{PDH}} \text{Acetyl CoA} + \text{NADH} + \text{CO}_2 + \text{H}^+$$

$$\text{Acetyl CoA} + \text{pAABS} \xrightarrow{\text{AAAT}} \text{acetyl-pAABS} + \text{CoA}$$

The molar absorption coefficient for the difference between acetyl-pAABS and pAABS is 6.5 $l.mol^{-1}cm^{-1}$ (Denton, R.M., personal communication). The assay medium consisted of 100 mM-TRIS-HCl, 1 mM-EDTA, 5 mM-2-mercapto-ethanol, 2 mM-$MgCl_2$, 0.05% Triton X-100, 10 $\mu g.ml^{-1}$ pAABS, 0.5 mM-$NAD^+$, 0.1 mM-CoA, 1 mM-sodium pyruvate, 1 mM-TPP, 50-100 mU AAAT and 10-50 $\mu$l homogenate. The total volume was 1 ml and the final pH was 7.8.

e) Preparation of the coupling enzyme, pigeon liver arylamine acetyltransferase (AAAT)

(1) Method A: Purification of the enzyme. The procedure followed was similar to that described in Jacobson (1961).

Materials. AR acetone obtained in 1 l quantities was obtained from BDH Atherstone, Warwickshire and stored at -10°C to -20°C. Details of all other materials are given in Appendix III.

1) Extraction of fresh livers

The pigeons were killed by anaesthetising in carbon dioxide, and cervical fracture. The livers were removed rapidly, placed on ice, and chopped as finely as possible. The livers were then extracted in 5 mM-TRIS-HCl, 1 mM-cysteine-HCl and 1 mM-EDTA at pH 8.0, 5 ml of extraction buffer per g of pigeon liver being used. The livers were homogenised with a Silverson tissue disintegrator, and the homogenate was centrifuged for 45 min at 13,000 g on an M.S.E. 18 centrifuge at 4°C.

2) Acetone fractionation

AR acetone (at -10°C to -20°C, see above) was added to the supernatant which was kept near to the freezing point of the mixture by immersing the beaker in a stirred bath of methanol and solid carbon dioxide. As an approximate guide to the freezing points of the acetone mixtures, Table I shows the temperature ranges of the mixtures while the acetone was added, and the equilibration time of the mixtures, as preliminary experiments had demonstrated that these factors affected when the enzyme was precipitated.

TABLE I

| Acetone fraction (%) | Temperature range (°C) | Equilibration time (hr) |
|---|---|---|
| 35 | 0 to -5 | 1.5 |
| 40 | -5 to -8 | 0.5 |
| 60 | -10 to -12 | 0.5 |

The mixtures were centrifuged at 10,000 g at 3°C for 10 min after equilibration with acetone. Precipitates were collected at 35%, 40% and 60% acetone (assuming additive volumes i.e. that 35 ml acetone plus 65 ml homogenate was equivalent to 35% acetone). The activities of AAAT during the acetone fractionation are shown in Table II.

One quarter of the precipitate from the 60% acetone mixture was taken up in 2 ml extraction buffer and dialysed overnight against two changes of two l. of the same buffer. Activities of AAAT before and after dialysis are shown in Table III. After dialysis, the crude extract was stored frozen in aliquots of 200 $\mu$l. The activity of pyruvate dehydrogenase (PDH) was measured in this extract (see Methods Section) and was found to be as high as 10% of the AAAT activity. As the latter enzyme is to be used as the coupling enzyme in the assay of PDH in extracts of muscle, this would give an over-

TABLE II

ACTIVITIES OF ARYLAMINE ACETYLTRANSFERASE DURING ACETONE FRACTIONATION

Details of the acetone fractionation are given in Table I, and in the text. The activity of arylamine acetyltransferase (AAAT) was assayed as described in the Methods section. An estimate of the protein concentration was obtained by the absorbance at 280 nm ($A_{280}$). A unit of activity is defined as that which converts 1 $\mu$mol of substrate to the acetyl form per minute. In the Table S/N represents supernatant, P/T represents precipitate and N/D represents not detectable.

| FRACTION (VOLUME IN ML IN BRACKETS) | ACTIVITY OF A.A.A.T. U. $ml^{-1}$ | TOTAL UNITS U. | PROTEIN $mg.ml^{-1}$ | SPECIFIC ACTIVITY OF AAAT $U.mg^{-1}$ |
|---|---|---|---|---|
| S/N from homogenate (380) | 0.63 | 241 | 125 | 0.005 |
| S/N from 35% acetone mix (510) | 0.47 | 239 | 52 | 0.009 |
| S/N from 40% acetone mix (520) | 0.56 | 292 | 73 | 0.009 |
| S/N from 60% mix (500) | N/D | N/D | - | - |
| P/T from 60% mix a) (5.4) | 7.4 | 40 | - | - |
| b) (17.0) | 10.3 | 176 | - | - |

TABLE III

ACTIVITIES OF ARYLAMINE ACETYLTRANSFERASE OF PRECIPITATE FROM 60% ACETONE MIXTURE BEFORE AND AFTER DIALYSIS

Details of acetone fractionation and dialysis are given in the text.

| TREATMENT | VOLUME OF SAMPLE ml | ACTIVITY OF AAAT $U.ml^{-1}$ | TOTAL UNITS OF ACTIVITY OF AAAT U. |
|---|---|---|---|
| P/T taken up in buffer, before dialysis | 2.0 | 8.6 | 17.0 |
| After dialysis | 5.4 | 7.4 | 40.0 |

estimate of the PDH of the extract under investigation. Therefore it seemed important to purify further the AAAT. The precipitate from the 60% acetone mixture was subjected to further procedures to purify the enzyme.

3) Separation on a DEAE column

The precipitate from the 60% acetone mixture was dissolved in 17 ml buffer consisting of 10 mM-sodium phosphate buffer, 1 mM-cysteine-HCl and 1 mM-EDTA at pH 7.8. This sample was put on an 8 x 30 DEAE column, which had been washed with 0.5M sodium hydroxide and equilibrated with 10 mM-sodium phosphate buffer, 1 mM cysteine-HCl and 1 mM EDTA at pH 7.8 (the elution buffer). After addition of the sample to the column, the elution buffer was passed through at a rate of 0.5 ml per minute and fractions of 10 ml were collected.

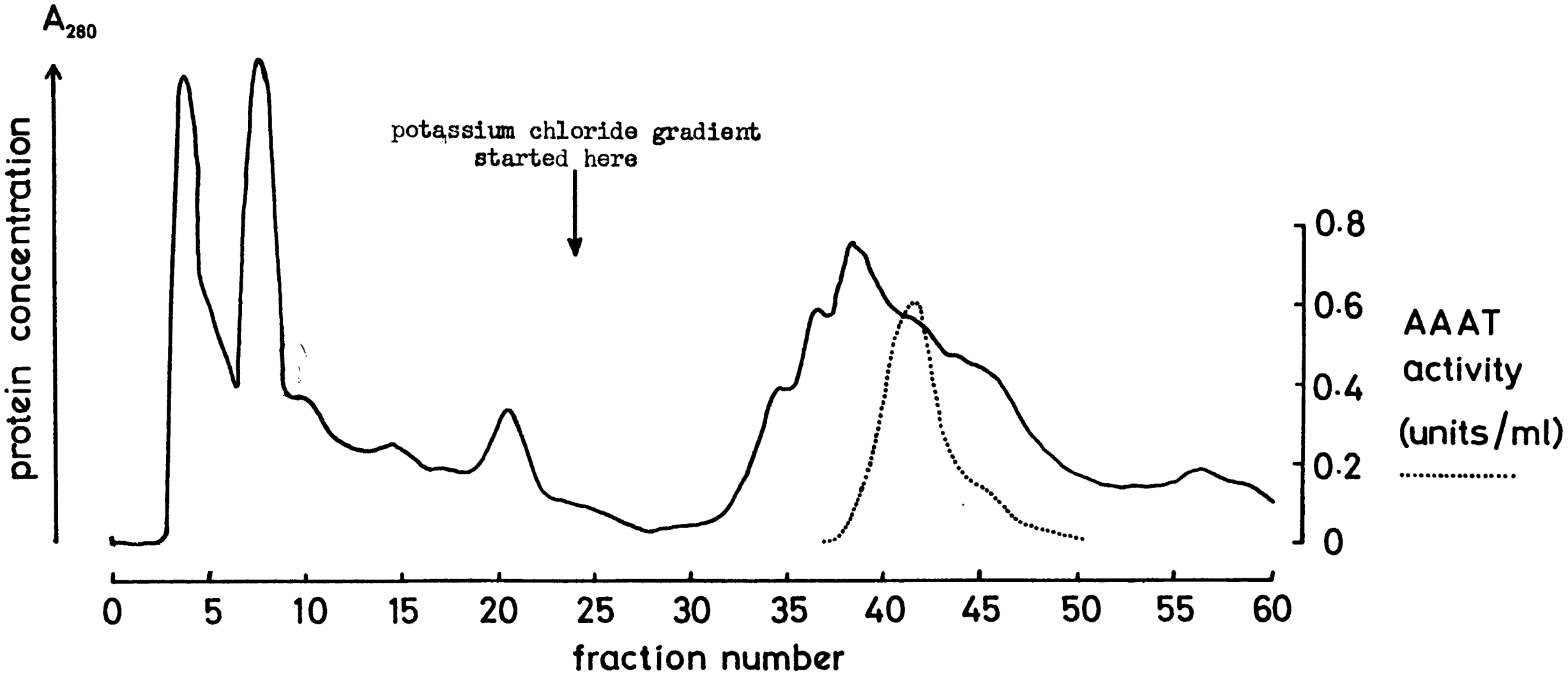
When no further protein peaks were observed on the trace from the Unicord recorder, a gradient of KCl was started. The reservoir contained 500 ml of 2M-KCl, 1 mM-sodium phosphate buffer at pH 7.8 and the mixing chamber contained 500 ml of 1 mM-sodium phosphate buffer. Both containers were the same size and shape.

The column fractionation and collection of samples were performed at 3°C. Figure 9 shows the elution profile of a typical experiment with profiles of protein concentration and activity of AAAT. Fractions of similar activities were pooled and subjected to ammonium sulphate fractionation.

4) Ammonium sulphate fractionation

Ammonium sulphate was added to 50% and 70%. The mixtures were stirred at 3°C for 1 hour and then centrifuged at 10,000 g for 10 min in an M.S.E. 18 centrifuge. Precipitates were taken up in the elution buffer, and assayed for AAAT.

**Figure 9** ELUTION PROFILE OF PROTEIN AND ARYLAMINE ACETYLTRANSFERASE ACTIVITY FROM DEAE-CELLULOSE COLUMN.



Results and discussion

The highest specific activity was recovered after dialysis of the precipitate from the 60% acetone mixture (7.4 $U.ml^{-1}$). However this contained contaminating PDH activity.

Separation of the sample on the DEAE column removed PDH activity from the central peak of AAAT activity (PDH activity was found to be 1% of the AAAT activity). However, yields of the AAAT activity were low. Only 40% of the original activity was recovered in 40 ml of eluate, and 50% was recovered in 600 ml of eluate. The highest activity of AAAT was found to be 0.6 U.ml which was found in one fraction of 10 ml at the top of the peak. This fraction also contained the highest specific activity of AAAT: 0.45 $U.mg^{-1}$ (protein).

The ammonium sulphate precipitation recovered only 20% of the activity from 40 ml of the peak from the column eluate, which is 8% of total original activity. In a typical run, the highest specific activity was 1.4 $U.ml^{-1}$ (or 0.4 $U.mg^{-1}$ protein). This was recovered in 2.8 ml from a 70% ammonium sulphate precipitate. Thus only 4 units were recovered from an original 64 units. Thus the ammonium sulphate precipitation did not improve on the specific activity of the samples from the DEAE column.

It seemed that the yield and the specific activity of the AAAT preparation had been sacrificed at the expense of removing the PDH activity and the turbidity of the samples from the original acetone fractionation. The method of Tabor et al. (1953) included the making of an acetone powder of the livers before extracting them in buffer prior to acetone fractionation. It was felt that this step might remove the PDH activity and this was incorporated into method B.

Method B : Acetone fractionation

Materials AR diethyl ether was obtained from Fisons Scientific Apparatus Ltd., Bishop Meadow Rd., Loughborough, and pretreated to remove peroxides. (The ether was allowed to stand over AR sodium hydroxide pellets for several hours in a fume cupboard, and then filtered through a column of alumina, Woelm basic $Al_2O_3$, cationotropic from Camlab,Ltd., Nuffield Rd, Cambridge The ether was also precooled to $-10^{o}C$ to $-20^{o}C$. Details of all other materials are given above and in Appendix III.

1) Preparation of the acetone powder. (The whole process must take place in a fume cupboard, preferably in a cold room.) Six pigeon livers were removed as described above, and minced finely. The livers were then dropped into 1 l of AR acetone at $-10^{o}C$ in an Atomixer, and homogenised for 2 min. The mixture was filtered through a Buchner funnel, through 2 Whatman no. 1 filter papers. The vacuum was supplied via a water pump. (If a motor pump is used, a trap for the acetone must be used, and great care must be taken to keep the pump physically far away from the trap and the flask containing the filtrate.)

The cake formed from filtering is dropped back into 1 l of fresh acetone at $-10^{o}C$. The homogenisation and filtration is repeated. The filtered cake is then washed with precooled, pretreated diethyl ether (see above). The cake should turn brown in colour. Air is continually drawn through the cake for 3 to 5 min until the cake becomes dry (the colour turns to a pale beige.) The cake is dropped into an evaporating dish, and crumbled with fingers till the powder acquires the consistency of talcum powder. The powder is then kept in a vacuum desiccator overnight and then stored in the deep freeze. (The activity of AAAT is stable in this form for up to 6 months.)

2) Preparation of enzyme from acetone powder by acetone fractionation

The acetone powder (8 g) is added to 80 ml of 0.1 mM EDTA at 0°C. The mixture is homogenised by the Polytron PT 10 (large probe) for 5 min at position 6, while the mixture is kept on ice. The homogenate is spun at 13,000 rpm in a MSE 18 centrifuge for 20 min at 0°C. The supernatant is assayed for activity of AAAT. The acetone fractionation is carried out in the cold room. Acetone (AR, previously cooled to -10°C to -20°C, see above) is added dropwise to the supernatant, which is continually stirred. The mixture is kept as near to its freezing point as possible i.e. in ice till acetone has been added to 20%, then in a freezing mixture of ice and ammonium chloride. When 40% acetone has been added (e.g. 30 ml acetone + 45 ml supernatant), the mixture is allowed to equilibrate for 15 min. and then spun at 13,000 rpm in a MSE 18 centrifuge for 20 min at -10°C.

The supernatant from this spin should be clear, and when assayed for AAAT, contain all the activity. 25 ml acetone are then added dropwise, as before, to the stirred mixture. A sample of this mixture (10-50 $\mu$l) is taken and spun on the microcentrifuge. The supernatant is assayed for AAAT activity. If there is activity left in the supernatant, a further 5 ml of acetone are added, and a sample taken for assay as before. This process is repeated until the activity has disappeared from the supernatant. (The activity should all be precipitated by 60% acetone, but the complications of evaporation and of the solubility of acetone in water i.e. that the volumes are not additive, necessitate the testing of the disappearance of activity in this way.)

The mixture is then spun at 13,000 rpm for 15 min at -10°C on an MSE 18 centrifuge. The supernatant is discarded, and the pellet is taken up in 10 mM-potassium phosphate buffer, pH 7.5, which should be made up freshly. A precooled test-tube or 'cold-finger' (a test-tube filled with crushed ice)

should be used to gently resuspend the pellet in as small a volume of buffer as possible. The sample is dried under vacuum over concentrated sulphuric acid, for several hours at 4°C, and then dispensed into 200 $\mu$l aliquots. These are stored in the deep freeze and only 10% of the activity is lost within four weeks. No aliquot should be refrozen for re-use. This preparation should make 2 ml of an extract containing AAAT at an activity of 5 $U.ml^{-1}$. No PDH activity was detectable in this extract.

## 3) RESULTS AND DISCUSSION

### a) Problems associated with the preparation of extracts for the assay of PDH

(i) Homogenisation. Many vertebrate skeletal muscles (especially those from fish and amphibians) proved difficult to homogenise by hand. Motor driven homogenisers such as the Silverson mixer/emulsifier, the Sorvall homogeniser and the M.S.E. overhead drive pestle for glass homogenisers were used, but these methods caused frothing of the extracts. The activity of PDH was decreased by frothing. (This was also the case with OGDH activity).

However, the Polytron PT10 was found to be very effective for the homogenisation of vertebrate tissue, especially if the container fitted closely around the probe, and if the tissue had been finely chopped. For retention of activity, it was important to keep the homogenate cold (i.e. at 0°C) at all stages during preparation.

Whelk radular muscle was also difficult to homogenise and it was found necessary to use ground-glass hand homogenisers (0.1 ml) with loose ground glass pestles (0.065 mm clearance) but using tighter pestles (0.050 mm clearance) as homogenisation progressed. It was not possible to use the Polytron for this tissue, since only small amounts of tissue were available, so that only small volumes of extraction medium could be used. Since it was necessary to cover the blades of the Polytron probe with fluid to avoid

damage to the machine, these small volumes could not be used with this machine.

(ii) Loss of enzyme activity upon extraction. Wieland (1975) reported an inactivation of pyruvate dehydrogenase in extracts from rat liver and suggested that rat serum, present at a concentration of 5% of the extraction buffer, would stabilise the activity. To test this report, extracts of rat heart were made in extraction buffer with and without 5% rat serum and were incubated at $0^{o}C$, and the activity of the extracts was then further incubated for 30 minutes, but at $25^{o}C$. The results are shown in Table IV. It was shown that at $0^{o}C$, no loss of PDH activity occurs in either extract for up to 77 minutes. However, in the incubation performed at $25^{o}C$, PDH activity is lost in the absence of the rat serum, but is stable in its presence. It was therefore considered unnecessary to add rat serum to extraction buffers provided that the extract could be kept on ice and assayed within one hour. However, in some cases, it was necessary to incubate the extracts at $25^{o}C$ (see later) and, in these cases, rat serum was added to a final concentration of 5% in the extraction buffer.

In addition to the loss of total activity, it is also necessary to consider the inactivation of the PDH by phosphorylation, at least in those tissues where the phosphorylation cycle is known to exist. This is discussed in the next section.

(iii) Extraction of total PDH activity. The activity of PDH is regulated by a phosphorylation-dephosphorylation interconversion cycle, involving PDH kinase and $PDH_b$-phosphatase (see Chapter 1, p.46). It has been found that, in muscle and other tissues, only a proportion of the total PDH is normally present in the active form, $PDH_a$ (Wieland et al., 1971; Hagg et al., 1976; Hennig et al., 1975), the rest being present as $PDH_b$, the inactive form.

TABLE IV

STABILITY OF PYRUVATE DEHYDROGENASE ACTIVITY IN EXTRACTS OF RAT HEART

The basic extraction buffer consisted of 50 mM-TRIS-HCl, 1 mM-EDTA, 5 mM-$MgCl_2$, 5 mM-2-mercaptoethanol and 10 mM-sodium pyruvate at a final pH of 7.0. Extracts were made with and without rat serum, which was added to a final concentration of 5% (v/v).

| TEMPERATURE OF INCUBATION ($^{o}C$) | TIME OF INCUBATION (min) | ACTIVITY OF PYRUVATE DEHYDROGENASE $\mu mol.min^{-1}.g^{-1}$ (fresh weight) | |
|---|---|---|---|
| | | No serum added | 5% serum added |
| 0 | 0 | 4.7 | 6.8 |
| | 7 | 6.0 | 6.1 |
| | 33 | 6.4 | 6.0 |
| | 77 | 5.9 | 6.4 |
| 25 | 0 | 5.9 | 6.4 |
| | 30 | 3.8 | 6.1 |

The proportion of PDH present in the active form depends upon the relative activities of the kinase and the phosphatase. The activators and inhibitors of the kinase and the phosphatase are listed in Table XI, Chapter One.

In this work, extraction was carried out under conditions that should activate the phosphatase and inhibit the kinase (i.e. in the presence of $Ca^{2+}$, $Mg^{2+}$ and pyruvate) and so that PDH should be converted fully to the active form. These are the conditions under which total conversion to the active form is found to occur in rat heart (Whitehouse et al., 1974). However, it was necessary to show that these conditions activated the PDH in at least some of the animals chosen for study in the present work.

The activating effect of $Mg^{2+}$ plus $Ca^{2+}$ was tested by comparing the effects of incubation with these agents with the activation produced by the presence of excess of the activating enzyme, i.e. the phosphatase. Three muscles were selected: rat heart, (since the phosphatase is known to function with PDH from this muscle), pigeon pectoral muscle, and trout red muscle. The latter two tissues were chosen to see whether the conditions, already established for rat heart, were sufficient for bird and fish muscle.

The results are shown in Table V. It can be seen that normal extraction conditions activated the PDH to an extent that could not be improved upon by incubation with excess of the phosphatase, in extracts from all three animals. Thus total activation of pyruvate dehydrogenase has already been achieved under normal extraction conditions. Presumably, endogenous phosphatase from these tissues was active under these conditions.

The activity of the preparations of $PDH_b$-phosphatase was first tested on extracts of rat heart made in buffer containing 5 mM-EDTA and 1 mM-EGTA. These extracts, made from the hearts of nembutalised rats, gave a mean value for PDH activity of 1.1 $\mu$mol.$min^{-1}$.$g^{-1}$(fresh weight). On incubating with

TABLE V

ACTIVITY OF PYRUVATE DEHYDROGENASE IN EXTRACTS FROM VARIOUS TISSUES UNDER NORMAL EXTRACTION CONDITIONS AND AFTER ACTIVATION WITH PHOSPHATASE

The normal extraction buffer contained 25 mM-$MgCl_2$, 1 mM-$CaCl_2$ and 10 mM-sodium pyruvate. Incubation were carried out for 10 minutes at 25°C. Pig heart $PDH_b$-phosphatase was prepared by the method of Denton et al. (1972). Incubations with phosphatase were carried out as described in the Methods section. The Figures in brackets show the quantity $S_w$ as defined in Dean & Dixon (1951). This quantity is derived from the range, and is an estimate of the standard deviation for small numbers of observations. The number of determinations was between 4 and 6.

| TISSUE | ACTIVITY OF PYRUVATE DEHYDROGENASE ($\mu$mol.min$^{-1}$.g$^{-1}$ fresh weight) | |
|---|---|---|
| | Incubation with no additions | Incubation with phosphatase |
| Rat heart | 7.10 (0.76) | 7.50 (0.47) |
| Trout red muscle | 3.10 (1.00) | 3.37 (0.22) |
| Pigeon pectoral muscle | 4.92 (1.05) | 4.10 (0.84) |

phosphatase (as described in the Methods section) the mean pyruvate dehydrogenase activity in the extracts rose to 5.7 $\mu$mol. $min^{-1}.g^{-1}$ (fresh weight). Thus it could be seen that the pig heart phosphatase was activating the rat heart pyruvate dehydrogenase. (This value of 20% for the active fraction of pyruvate dehydrogenase of normal, fed rat hearts *in situ* agrees well with the data of Kerbey *et al*. (1976) and Whitehouse & Randle (1973).)

In trout red muscle, the active fraction of PDH was approximately 90% of the total activity. However, there were many technical difficulties involved in rapidly killing the trout, and in removing red muscle, uncontaminated by white muscle, for freeze clamping. It seems likely that there would have been time for the activation of PDH before freeze clamping. In pigeon pectoral muscle, the active fraction of PDH was 57% of the total PDH. It is interesting to note that pig heart phosphatase is capable of activating fish and bird PDH.

b) Problems associated with the assay of PDH

(i) Release of activity from the mitochondrion

The exact location of the PDH complex within the mitochondrion is not known. According to Addink *et al*. (1972) PDH is located within the matrix of rat heart mitochondria. However, Randle *et al*. (1974) suggest that a substantial part of the PDH may be bound to the inner mitochondrial membrane. Indeed Allman *et al*. (1966) showed that both the keto acid dehydrogenase complexes (PDH and OGDH) were bound to a membranous fraction on the mitochondria and were released by slightly different preparative techniques than the other TCA cycle enzymes. Similarly in insects, it is suggested that keto-acid dehydrogenases are located on the inner membrane of mitochondria (Candy & Kilby, 1975, p.55; Sacktor, 1976).

As the mitochondria are impermeable to the assay components it is necessary to break up the mitochondria, either by ultrasonic disintegration

or by treatment with detergents. The use of detergents in liberating enzyme activity is discussed by Morton (1955). Under certain conditions, detergents can form stable micelles and hence cause physical dispersion of water-insoluble material. This process is known as "solubilisation". Non-ionic detergents (e.g. Lubrol W and Triton X-100) have advantages over the naturally occurring ionic detergents in that they are not as reactive with proteins, but disperse lipoproteins very effectively. Thus although the exact mechanism of action is not known, it is suggested that they may either dissociate a lipid-protein complex or destroy a particulate membrane surrounding the enzyme-containing sub-cellular particles. Nakajin et al. (1979) also suggested that these detergents act on membrane bound enzymes by facilitating the access of substrate to the enzymes. Triton X-100 and Lubrol W have been used previously in the assay of mitochondrial enzymes (Scrutton & Fatebene, 1975).

It was considered possible that high concentrations of Triton X-100 may inhibit the enzyme e.g. by removing essential phospholipids in an enzyme or by dissociating a multi-enzyme complex, such as the keto-acid dehydrogenases. It was therefore necessary to test for optimal concentrations of Triton X-100 Pre-incubation of the extract with 0.05% Triton X-100 caused a loss in the PDH activity, whereas the same concentration of Triton in the assay system did not. The experiment was therefore conducted by adding Triton X-100 to the assay system. The results are reported in Table VI; they show that maximal activity is obtained at approximately 0.05% Triton X-100. It is interesting to note in this context that Nakajin et al. (1979) also obtain an optimal concentration of 0.05% Triton X-100 for the activation of cholesterol side chain cleavage activity of cytochrome P-450. (This enzyme is also known as cholesterol 7$\alpha$-monooxygenase).

TABLE VI

EFFECT OF TRITON X-100 ON THE ACTIVITY OF PYRUVATE DEHYDROGENASE IN EXTRACTS OF LOCUST FLIGHT MUSCLE

Details of the assay system for pyruvate dehydrogenase are given in the Methods section. Values are means of three determinations. The figure in brackets is the quantity $S_w$ defined in Dean & Dixon (1951). It is an estimate of the standard deviation obtained from the range of observations.

| % TRITON IN CUVETTE (v/v) | ACTIVITY OF PDH $\mu mol.min^{-1}.g^{-1}$ (fresh weight) | % MAXIMUM ACTIVITY |
|---|---|---|
| 0 | 3.1 (1.2) | 30 |
| 0.01 | 1.4 (0.3) | 13 |
| 0.02 | 9.1 (3.5) | 87 |
| 0.035 | 10.1 (0.9) | 96 |
| 0.05 | 8.7 (1.1) | 83 |
| 0.07 | 10.5 (1.0) | 100 |
| 0.1 | 9.4 (0.9) | 90 |
| 0.2 | 10.5 (1.7) | 100 |

(ii) Concentrations of substrates, cofactors and effectors

In order to ensure that the maximum catalytic activity of pyruvate dehydrogenase is being expressed, the enzyme must approach saturation with its substrates and cofactors, i.e. the concentration of the substrate must be large in comparison with the $K_m$ of the enzyme for that substrate. Since many different animals would be used in the comparative survey, the possibility arises of variation in the $K_m$ values of the enzyme from one animal to another. To eliminate these difficulties, a detailed analysis on the properties of the enzyme from each tissue and each animal should be carried out. However, the amount of work required would restrict the accumulation of data to a very few animals. This detracts from the whole purpose of the comparative survey.

In order to test whether the concentrations of metabolites used in the assay system were high enough to permit the measurement of the maximum activity, a comparison was made between a range of reported values of $K_m$ for the different substrates of PDH and the concentrations of these constituents of the assay system. The data are presented in Table VII and it can be seen that, in all cases, the substrate is present at a concentration greater than 5 times the largest reported value of its $K_m$.

The maximum rate (V) is related to the measured rate ($v_i$) in the following expression:

$$S = \frac{v_i \times K_m}{V - v_i}$$ (Bergmeyer, 1978)

where S is the concentration of substrate and $K_m$ is the Michaelis-Menton constant for that substrate or cofactor. (Cofactors behave as substrates from the point of view of reaction kinetics). This can be rearranged as follows:

TABLE VII

COMPARISON OF REPORTED $K_m$ VALUES OF PYRUVATE DEHYDROGENASE FOR ITS SUBSTRATES WITH THE CONCENTRATIONS OF THESE COMPONENTS IN THE ASSAY SYSTEM

The error in the estimate of $V_{inf}$ from the concentration of the assay component shown, assuming that the $K_m$ for that component is the largest value shown, is calculated from $1-v_i/V$ as described in the text, p.118.

| SUBSTRATE OR COFACTOR | $K_m$ $\mu M$ | CONCENTRATION IN ASSAY SYSTEM $\mu M$ | % ERROR IN ESTIMATE OF $V_{inf}$ |
|---|---|---|---|
| pyruvate | 36[1], 19[2], 15[3] | 1000 | 3.5 |
| CoA | 2[1], 21[3] | 100 | 17.0 |
| $NAD^+$ | 53[1], 79[3] | 500 | 13.6 |
| $Mg^{2+}$ | 5[1] | 2000 | 0.2 |

1. Denton et al. (1975) pig heart
2. Kansaki et al. (1969) pig heart
3. Hamada et al. (1975) pig heart

$$\frac{v_i}{V} = \frac{S}{K_m + S}$$

Substitution of the values from Table VII into this equation gives a value for the expression $v_i/V$ or the measured rate as a fraction of the maximum rate. The expression $1 - v_i/V$ gives the deviation from maximum, or the error in the estimation of the maximum activity. This fraction (expressed as a percentage) has been calculated for each component of the assay system and is shown in Table VII.

The highest deviation from maximal, as shown in Table VII, is 17%. This is within the range of the experimental variation encountered in the activities of pyruvate dehydrogenase measured from muscles of different individuals of the same species. It is therefore assumed that for the purposes of the present work the maximal activity is being measured. It is also assumed that, for the enzyme from the muscles from all the species investigated, no substrate or cofactor inhibition occurs.

Although the extraction buffer was designed to convert all the PDH present to the active form (see above) it is also necessary to ensure that the PDH remains in this form during the assay. Since CoA, $NAD^+$, TPP and pyruvate all inhibit the kinase, the presence of these compounds in the assay system ensures the inactivation of the kinase during the PDH assay.

It has been reported for mitochondria isolated from blowfly flight muscle that ADP and $P_i$ are required for pyruvate oxidation (Hansford, 1972a). Whether a phosphorylation interconversion cycle exists in insects is not known. (See Chapter One, p. 46). In the present work, extracts of bumble bee flight muscle which were incubated with 10 mM-$MgCl_2$ showed no increase in the activity of pyruvate dehydrogenase. However, Hansford also suggests that ADP may be an activator of insect flight muscle pyruvate oxidation (Hansford, 1972b). Hence, several extracts of insect flight muscle and muscles from other invertebrates were assayed for pyruvate dehydrogenase activity in the presence and

absence of 2 mM-ADP. The results are shown in Table VIII. In the Coleopterans (African dung beetle, and rosechafer), the giant waterbug and the blowfly, ADP was required for maximum activity. Indeed, preincubation with ADP in the cuvette was required before any activity of pyruvate dehydrogenase was expressed. This may be indirect evidence of the existence of an interconversion cycle in these insects.

(iii) The use of a coupling enzyme

Arylamine actyltransferase (E.C.2.3.1.5) is used in the assay of pyruvate dehydrogenase to couple the production of acetyl CoA by pyruvate dehydrogenase to the acetylation of the dye, p-amino azo benzene sulphonic acid (p-AABS). Some of the problems involved in the use of coupled enzyme assays are discussed in Bergmeyer (1978), pp. 69-75, and are briefly discussed here.

Let the following equation represent a coupled enzyme reaction:

$$S \xrightarrow{E_{prim}} P_1 \xrightarrow{E_{ind}} P_2$$

where $E_{prim}$ represents the primary enzyme of unknown activity, and $E_{ind}$ represents the indicator enzyme (or coupling enzyme). For the correct estimation of the activity of $E_{prim}$ the following conditions must be observed.

1) The concentration of the intermediate product $P_1$ must be kept as low as possible to keep the lag phase to a minimum (i.e. the time taken to reach a steady state)

2) The primary reaction must always be rate limiting. The rate constant of the indicator reaction, $k_{ind}$, must be very much greater than that of the primary reaction, $k_{prim}$. In enzyme reactions, k corresponds to the expression V (in zero order reactions) and $V/K_m$ (in first order reactions). Thus $k_{ind} = \frac{V_{ind}}{K_m}$. (Other relationships hold for two substrate reactions: these are discussed in Bergmeyer, 1978).

TABLE VIII

ACTIVITIES OF PYRUVATE DEHYDROGENASE IN DIFFERENT EXTRACTS

IN THE PRESENCE AND ABSENCE OF ADP

The assay system for pyruvate dehydrogenase is described in the Methods section. The number of determinations is given in parentheses, and values lay within 20% of the mean.

| ANIMAL | TISSUE | ACTIVITIES OF PDH ($\mu$mol.min$^{-1}$.g$^{-1}$) | |
|---|---|---|---|
| | | no added ADP | 2 mM-ADP |
| Locust (*Schistocerca gregaria*) | flight muscle | 9.2 (3) | 9.0 (3) |
| Rosechafer (*Pachnoda ephippiata*) | flight muscle | 0.9 (3) | 2.0 (3) |
| Housefly (*Musca domestica*) | flight muscle | 15.4 (3) | 15.0 (3) |
| Fleshfly (*Sarcophaga barbata*) | flight muscle | 14.0 (2) | 13.2 (2) |
| Blowfly (*Calliphora vomitoria*) | flight muscle | 0.1 (3) | 13.6 (3) |
| African dung beetle (*Heliocopris sp.*) | flight muscle | 0.1 (2) | 0.4 (2) |
| Giant waterbug (*Lethocerus cordofanus*) | flight muscle | 0.1 (2) | 0.4 (3) |
| Whelk (*Buccinum undatum*) | radula retractor | 0.4 (4) | 0.3 (4) |

In general, a limiting value of the rate constants of $k_{ind} \geqslant 100 \times k_{prim}$ is recommended where $k_{prim}$ is the rate constant of the primary reaction and $k_{ind}$ is the rate constant of the indicator reaction. Under this condition, there is only a 4% error in the estimation of the rate of the primary reaction. Another way of expressing this limiting condition is as follows (for first order reactions):

$$\frac{V_{ind}}{(K_m)_{P_1}} \geqslant \frac{100 \times V_{prim}}{(K_m)_{S_1}}$$

where $(K_m)_{S_1}$ is the $K_m$ of the enzyme catalysing the primary reaction for the initial substrate, and $(K_m)_{P_1}$ is the $K_m$ of the enzyme catalysing the indicator reaction for the intermediate product, $P_1$.

Of course, the $K_m$ of the enzyme can not be varied, so that, in order to comply with the above condition, it is necessary to increase $V_{ind}$, which may be done by adding increased amounts of coupling enzyme. It was found that 50 mU of arylamine acetyltransferase were sufficient to measure the activity of up to 50 mU of PDH. When PDH was assayed in a new tissue, increasing volumes of coupling enzymes were added until no further increase in the activity of PDH was observed.

A problem involved in the use of the coupling enzyme was the turbidity and low specific activity of some of the preparations of arylamine acetyltransferase. In some assays, in which the measured PDH was high, volumes as large as 200 $\mu$l of coupling enzymes had to be added to increase $V_{ind}$, and in these assays, the turbidity of some of the preparations of arylamine acetyltransferase caused interference with the assay of PDH. Various attempts were made to increase the purity and the yield of the enzyme by modifications to the methods of Tabor et al. (1953) and Jacobson (1961), but it was found that some of these methods reduced the specific activity of the enzyme. A

modified method was evolved which gave preparations of a high specific activity ( 5 $U.ml^{-1}$) and relatively low turbidity, and this is shown in the Methods section, together with the techniques originally attempted.

(iv) Variation in activity of PDH

One of the major difficulties presented in the study of PDH and OGDH for the purposes indicated above was the variation encountered in the activities measured. The variation was observed from animal to animal, and, surprisingly, from one assay to the next, using the same extract. Variation in enzyme activities from one animal to another caught in the wild is not surprising since enzyme activities will vary with diet, sex and age of animals. Consequently, when the variation was noted, every effort was made to provide animals of the same sex, a similar size, collect them at the same time of day, etc. Nonetheless, in the present work, precise interpretations of the flux through the TCA cycle are not made, so that some variation is acceptable. However, variation from extract to extract was not expected.

In other comparative surveys carried out on maximum enzyme activities (Alp et al., 1976; Crabtree & Newsholme, 1972a, b) it was considered sufficient to give means of four determinations (i.e. from different animals), all of which lay within 20% of the mean. This was not possible with results from the PDH assays, since the variation was sometimes greater than this. Consequently another way of presenting the data was sought.

The approach of Dean & Dixon (1951) was found useful in the analysis of the present results. They showed that the range and the median are better statistics for small numbers of observations than the mean square and the mean, and calculate a quantity known as $S_w$ as a measure of the dispersion of the sample. This quantity is derived from the range, and is an estimate of the standard deviation.

They also give a test for 'outliers' or extraneous values, which was used as a criterion for the rejection of extraneous values in the present survey. Table IX shows some of the results from the survey of maximum PDH activities, with the tabulation of the replicate values, the medians and the values of $S_w$ from the extracts of six different tissues. The same data are also given for citrate synthase for comparison, and are taken from Alp et al. (1976).

This problem of variation was mainly encountered in extracts of muscles of insects, fish and amphibia; it was also encountered in extracts of white skeletal muscle in which the activities of PDH were very low. It is possible to put forward two explanations for the variation. The first explanation emphasises the problem of experimental technique. Thus the activities of these two enzymes are low and there is a greater error inherent in measuring a low activity than in measuring a high activity i.e. as the limits of detection are approached, the experimental error increases. This can be demonstrated by reference to Table IX; the 'standard deviation' ($S_w$) forms a lower percentage of the mean (or median) in citrate synthase activities than it does in PDH activities.

A second explanation lies in the consideration of the equilibrium nature of the PDH reaction. In Chapter One, it was demonstrated that PDH catalyses a non-equilibrium reaction, and probably a flux-generating step. Hence, the maximum activity of this enzyme should govern the maximum flux that is possible through the reaction. This would be expected to vary with factors such as the sex of the animal, the time of the season, the dietary history of the animal prior to capture, the age of the animal, etc. Although every attempt was made to standardise these factors, variation due to them may nevertheless have occurred.

TABLE IX

VARIATION IN ACTIVITY OF PYRUVATE DEHYDROGENASE AND CITRATE SYNTHASE IN EXTRACTS OF MUSCLE FROM VARIOUS ANIMALS

The median (M) and $S_w$ (an estimate of the standard deviation which can be obtained from the range) values for pyruvate dehydrogenase are given for the replicate assays of a single extract, and for the different animals (M' and $S_w'$). $S_w$ is calculated according to Dean & Dixon (1951). The median value for each tissue (M') is calculated from the medians (M) of the replicates. For citrate synthase, the data are taken from Alp *et al.* (1976), but the mean is given, instead of the median. The figures in brackets show $S_w'$ as a percentage of the median (or mean). For full latin names of the animals, see Chapter Four.

| ANIMAL | TISSUE | PYRUVATE DEHYDROGENASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$) | | | | CITRATE SYNTHASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$) Variation within animals | |
|---|---|---|---|---|---|---|---|
| | | Variation within replicates | | Variation within animals | | | |
| | | M | $S_w$ | M' | $S_w'$ | Mean | $S_w'$ |
| Whelk | radular muscle | 0.195 | 0.01 | 0.20 | 0.06 (30%) | 24.8 | 3.01 (12%) |
| | | 0.18 | 0.03 | | | | |
| | | 0.21 | 0.05 | | | | |
| | | 0.31 | 0.08 | | | | |
| Cockroach | flight muscle | 16.60 | 0.59 | 13.7 | 9.69 (71%) | 185.0 | 40.12 (22%) |
| | | 15.90 | 0.59 | | | | |
| | | 12.40 | 0.18 | | | | |
| | | 15.00 | 0.89 | | | | |
| | | 6.91 | 0.75 | | | | |
| | | 7.08 | 0.13 | | | | |
| Dogfish | red muscle | 4.22 | 0.19 | 3.14 | 1.15 (37%) | 35.0 | 3.5 (10%) |
| | | 3.41 | 0.03 | | | | |
| | | 1.88 | 0.01 | | | | |
| | | 2.87 | 0.17 | | | | |

TABLE IX (cont'd.)

| ANIMAL | TISSUE | PYRUVATE DEHYDROGNEASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$) | | | | CITRATE SYNTHASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | Variation within replicates | | Variation within animals | | Variation within animals | |
| | | M | $S_w$ | M' | $S_w$' | Mean | $S_w$' |
| Frog | heart | 4.65<br>5.72<br>5.70<br>4.73 | 0.35<br>0.13<br>1.25<br>0.52 | 4.69 | 0.52 (11%) | 40.0 | 3.5 (10%) |
| Pigeon | heart | 6.96<br>5.85<br>9.31<br>4.44 | 0.44<br>0.51<br>0.79<br>0.50 | 6.43 | 2.39 (37%) | 127.0 | 7.1 (5%) |
| Pheasant | pectoral muscle | 0.94<br>0.68<br>0.52<br>0.94<br>0.79 | 0.11<br>0.14<br>0.04<br>0.13<br>0.06 | 0.68 | 0.11 (16%) | 14.4 | 1.7 (12%) |

Since the activity of the enzymes that catalyse near-equilibrium reactions is considerably greater than the flux, the enzyme will be able to accommodate changes in flux via changes in its substrate concentration (Crabtree, 1976b; Crabtree & Newsholme, 1978). Hence the maximum capacity (i.e. $V_{max}$) of the enzyme may be changed by factors such as sex, season, previous dietary history etc. to a lesser extent than that of the 'flux-generating step' enzyme.

## SECTION 2: THE DEVELOPMENT OF THE ASSAY SYSTEM FOR 2-OXOGLUTARATE DEHYDROGENASE

### 1) MATERIALS

(See Appendix III).

### 2) METHODS

#### a) Preparation of extracts

The animals were killed and the muscle (or other tissue) was excised and placed on ice, as described on p.104. The extraction medium consisted of 50 mM-TRA, 2 M-glycerol, 80 mM-2-mercaptoethanol, 2 mM-$MgCl_2$, 1 mM-EDTA, and 0.1 mM-2-oxoglutarate. In some extracts 2 mM-ADP was also added. The final pH was 7.4.

#### b) Enzyme assay

2-Oxoglutarate dehydrogenase was assayed by following the rate of change extinction at 340 nm in a Gilford recording spectrophotometer (model 240) at 25°C. The enzyme catalyses the following reaction:

$$\text{2-oxoglutarate} + NAD^+ + CoA \longrightarrow \text{succinyl CoA} + NADH + H^+ + CO_2$$

The assay medium for 2-oxoglutarate dehydrogenase consisted of 50 mM-TRA, 7.5 mM-2-mercaptoethanol, 1 mM-KCN (added as cyanide reagent, see Appendix III), 2 mM-2-oxoglutarate, 0.4 mM-CoA, 2 mM-$NAD^+$, 0.05% (w/v) Triton X-100 and a volume of suitably diluted homogenate (10 to 50 $\mu$l). The total volume was 1 ml and the final pH was 7.4.

### 3) RESULTS AND DISCUSSION

#### a) Problems associated with the preparation of extracts

(i) Homogenisation. The same problems were encountered as were described on p.112. Indeed, the extraction of the tissue for the assay of this enzyme proved more difficult than that for PDH. There are at least two reasons for this. Firstly, it was discovered that even slight foaming of

the extract caused a decrease in the activity of 2-oxoglutarate dehydrogenase. Secondly, a high degree of homogeneity was required to reduce interference due to turbidity. This problem was more severe than in the PDH assays, since particles in the homogenate caused more absorption of light at 340 nm than at 460 nm.

(ii) Stability of the enzyme in extracts. The activity of 2-oxoglutarate dehydrogenase is unstable in crude extracts; up to 50% of the activity may be lost in one hour. It was discovered that the presence of 2 M-glycerol in the extraction buffer considerably reduced the loss in enzyme activity. Glycerol has been reported to stabilise the activity of $NAD^+$-linked isocitrate dehydrogenase (Cox & Davies, 1967; Alp et al., 1976).

In some tissues, ADP was found to activate the enzyme, and in extracts of these tissues, 2 mM-ADP was always added to the extraction buffer and this helped to stabilise the activity (see later).

(iii) Concentration effects of enzymes in extracts. As mentioned above, some proteins will denature in dilute aqueous solutions, so that enzyme activity may be lost in dilute extracts.

In the present work on OGDH, highest activities were obtained in more concentrated extracts e.g. the activity was higher if 1 g (fresh weight) of muscle was homogenised in 5 ml buffer (20% w/v) compared to 1 g of muscle being homogenised in 20 or 30 ml (5 or 2% w/v). Thus a 3% (w/v) extract of locust flight muscle gave 67% of the activity obtained with a 10% (w/v) extract of the same muscle. Somewhat surprisingly, addition of bovine serum albumin to the extraction buffer did not prevent the effect of dilution.

b) Problem associated with the assay of OGDH

(i) Release of activity from the mitochondrion. The precise location of the OGDH complex within the mitochondrion is unknown (i.e. whether it is in the inner membrane or in the matrix) but there is evidence that it occurs

TABLE X

EFFECTS OF SONICATION AND TRITON X-100 ON THE ACTIVITY OF 2-OXOGLUTARATE DEHYDROGENASE FROM DIFFERENT EXTRACTS

The extracts were sonicated for 30 seconds at amplitude 6 in a M.S.E. ultrasonic disintegrator at 0°C.

Triton X-100 was added to the assay system in the cuvette at a final concentration of 0.05%.

| ANIMAL | TISSUE | 2-OXOGLUTARATE DEHYDROGENASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$ fresh weight) | | | % MAXIMUM ACTIVITY | | |
|---|---|---|---|---|---|---|---|
| | | Triton in assay system | Sonication of extract | Triton plus Sonication | Triton in assay system | Sonication of extract | Triton plus sonication |
| Pig | heart | 3.13 | 2.58 | 4.15 | 75.4 | 62.2 | 100 |
| Rat | heart | 4.47 | 4.36 | 7.45 | 60.0 | 58.5 | 100 |
| Dogfish | heart | 2.89 | 2.69 | 3.47 | 82.3 | 77.5 | 100 |
| Waterbug | flight muscle | 11.38 | 10.73 | 15.60 | 72.9 | 68.7 | 100 |
| Blowfly | flight muscle | - | 26.26 | 40.95 | - | 64.1 | 100 |
| Locust | flight muscle | 15.0 | 21.70 | 20.80 | 69.1 | 100 | 95.9 |
| Whelk | radular muscle | - | 3.80 | 4.09 | - | 92.9 | 100 |

in the same location as the PDH complex (see above). The data of Allman et al. (1966) suggest that OGDH may also be associated with a membrane. Disruption of mitochondria by ultrasonic disintegration of the extracts or the addition of Triton X-100 in the assay system were both used to release the OGDH activity.

The results of an experiment to test these two methods for releasing activity from the mitochondria of various extracts are presented in Table X. The data show that maximal activities are obtained only when both methods are used.

(ii) Concentration of substrates, cofactors and effectors. As described on p.117, comparison of a range of reported $K_m$ values for the various substrates with the concentration of that substrate in the assay system gives some idea of whether the enzyme is approaching saturation with that participant of the reaction. These data for OGDH are presented in Table XI. The highest deviation from maximal is shown to be 10%. This is within the range of the experimental variation encountered in the activities of OGDH measured from muscles of different individuals from the same species. It is therefore assumed that, for the purposes of the present work, the maximal activity is being measured. (The experimental error is discussed above in Section 1,b(iii)).

OGDH is subject to product inhibition by NADH and succinyl CoA (Garland, 1964; Smith et al., 1974), competitively with $NAD^+$ and 2-oxoglutarate respectively. These inhibitory effects may have been responsible for the 'tailing-off' in the increase in absorbance in some of the early assays of OGDH. Non-linear progress curves for NADH production were also observed in OGDH assays by Sanadi et al. (1959). As these authors suggested that higher initial concentrations of $NAD^+$ overcome product inhibition, higher concentrations of CoA (0.4 mM) and $NAD^+$ (4 mM) were included in the assay.

TABLE XI

COMPARISON OF REPORTED $K_m$ VALUES OF 2-OXOGLUTARATE DEHYDROGENASE FOR ITS SUBSTRATES AND COFACTORS WITH THE CONCENTRATIONS OF THESE COMPONENTS IN THE ASSAY SYSTEM

| SUBSTRATE OR COFACTOR | $K_m$ VALUES $\mu M$ | CONCENTRATION IN ASSAY SYSTEM $\mu M$ | ERROR IN ESTIMATE OF $V_{inf}$ % |
|---|---|---|---|
| 2-oxoglutarate | 100[1], 220[2], 110[3], 200[4], 13[5], 70[6] | 2000 | 10 |
| CoA | 25[2], 2.7[7], 3.5[4], 0.1[5], 30[6] | 400 | 7 |
| $NAD^+$ | 50[2,4], 21[7], 4.5[5], 100[6] | 2000 | 5 |

1. Kansaki et al. (1969)
2. Hamada et al. (1975)
3. Hirashima et al. (1967)
4. McMinn & Ottoway (1977) These values are reported as $S_{50}$ values, not $K_m$
5. Massey (1960)
6. Read et al. (1977)
7. Smith & Williamson (I97I)

The 'tailing-off' effect disappeared so that the activity was linear (with respect to time) for up to three minutes. However, in order to measure accurately the initial velocity over this period of time, various modifications were required to improve the rapidity of the assay. One way of mixing cuvettes, previously used in enzyme activity measurements, was to cover the cuvette with Parafilm and invert it. However, this was too slow for the rapid measurement of enzyme activity required for the present purposes. The stirring process was modified by addition of the extract on a plastic stirrer to start the reaction.

In addition, the Gilford 240 model has an adaptation making possible the simultaneous recording of four cuvettes. While this is very useful for enzymatic assays which are linear for five minutes or more, it was decided that it was not accurate to measure the present assays in this way. The absorbance of each cuvette was therefore measured individually.

ADP is reported to relieve inhibition of OGDH activity by ATP in blowfly flight muscle (Hansford, 1972b) and to activate the enzyme in extracts of flight muscle from various insects (Read et al., 1977). Therefore ADP was added to the assay medium for the assay of OGDH in extracts of insect flight muscle to counteract the effect of ATP present in the extracts (see later). However, ADP was found to increase the activity of OGDH in extracts other than insect flight muscle. This suggested that ADP could act as an activator for OGDH in other animals. The effect of ADP upon extracts of muscles from a wide range of animals was therefore investigated.

The activities of OGDH in various animals in the presence and absence of ADP are presented in Table XII. It can be seen that the largest increases (from 20 to 30-fold) were obtained in locust flight muscle, trout heart muscle, pigeon pectoral muscle, mouse kidney, rabbit heart and pig heart. Substantial increases (more than 3-fold) were obtained with the enzyme from all mammalian tissues investigated, and in housefly and blowfly flight muscle. Unfortunately, it was not possible to include ADP in the

TABLE XII

2-OXOGLUTARATE DEHYDROGENASE ACTIVITIES IN MUSCLES (AND OTHER TISSUES) FROM INVERTEBRATES AND VERTEBRATES IN THE PRESENCE AND ABSENCE OF ADP

For the assay of 2-oxoglutarate dehydrogenase, see Methods section. The figure in brackets is the value $S_w$, an estimate of the standard deviation which is obtained from the range of observations. (Dean & Dixon, 1951). The number of observations is given as a subscript. For the specific names of the animals, see Chapter Four, Table II.

| ANIMAL | TISSUE | 2-OXOGLUTARATE DEHYDROGENASE ACTIVITIES $\mu mol.min^{-1}.g^{-1}$ (fresh weight) | |
|---|---|---|---|
| | | No added ADP | 2.5 mM ADP |
| Whelk | radular muscle | $5.3\ (0.4)_2$ | $4.7\ (0.9)_4$ |
| Hawk moth | flight muscle | $44.7\ (11.0)_2$ | $42.7\ (16)_3$ |
| Wasp | flight muscle | $44.8\ (4.0)_2$ | $71.2\ (11)_4$ |
| Dung beetle | flight muscle | $23.3\ (3.5)_4$ | $22.3\ (5.0)_6$ |
| Waterbug | flight muscle | $10.2\ (1.1)_2$ | $15.2\ (1.6)_5$ |
| Locust | flight muscle | $0.6\ (0.2)_2$ | $20.8\ (4.6)_9$ |
| Fleshfly | flight muscle | $31.7\ (11.3)_5$ | $32.8\ (11.5)_5$ |
| Housefly | flight muscle | $12.3\ (3.2)_4$ | $39.8\ (8.4)_3$ |
| Blowfly | flight muscle | $6.8\ (0.9)_2$ | $46.7\ (11.0)_6$ |
| Tsetse fly | flight muscle | $13.4\ (4.4)_3$ | $26.5\ (0.8)_3$ |
| Chafer | flight muscle | $57.5\ (26.0)_5$ | $25.6\ (2.8)_3$ |
| Dogfish | red muscle | $0.2\ (0.2)_5$ | $2.2\ (0.6)_3$ |
| | heart | $0.7\ (0.7)_2$ | $3.3\ (0.7)_3$ |
| Trout | red muscle | $0.1_3$ | $1.8\ (0.4)_3$ |
| | heart | $0.1_3$ | $3.3\ (0.9)_5$ |
| Frog | heart | $3.5\ (1.3)_5$ | $0.5\ (0.1)_2$ |
| Chicken | heart | $4.1\ (0.8)_2$ | $6.0\ (0.8)_3$ |
| Pigeon | pectoral muscle | $0.2\ (0.08)_2$ | $3.2\ (1.3)_4$ |
| Mouse | kidney | $0.4\ (0.01)_2$ | $8.1\ (1.3)_4$ |
| Rat | heart | $1.4\ (0.9)_5$ | $10.1\ (2.0)_7$ |
| | kidney | $0.6\ (0.4)_3$ | $6.9\ (0.9)_5$ |
| | diaphragm | $0.3\ (0.4)_3$ | $2.4\ (0.7)_5$ |
| | soleus muscle | $0.2\ (0.08)_3$ | $2.4\ (0.6)_5$ |
| Rabbit | heart | $0.4\ (0.2)_3$ | $8.3\ (1.4)_7$ |
| Pig | heart | $2.5\ (1.6)_3$ | $3.1\ (0.6)_7$ |

TABLE XII$_I$

ACTIVATION OF 2-OXOGLUTARATE DEHYDROGENASE BY ADP IN EXTRACTS FROM DIFFERENT ANIMALS

The assay conditions for OGDH are given in the Methods section. Sources of animals are given in Appendix III. Specific names of animals are given in Chapter Four, Table II.

| ANIMAL | TISSUE | CONCENTRATION of ADDED ADP mM | ACTIVITY OF 2-OXOGLUTARATE DEHYDROGENASE $\mu$mol.min$^{-1}$.g$^{-1}$(fresh weight) | CONCENTRATION OF ADP GIVING HALF-MAXIMAL ACTIVATION mM |
|---|---|---|---|---|
| Locust | flight muscle | 0 | 0.2 | |
| | | 0.04 | 2.2 | |
| | | 0.08 | 4.3 | 0.41 |
| | | 0.16 | 8.1 | |
| | | 0.40 | 17.8 | |
| | | 0.80 | 20.3 | |
| Wasp | flight muscle | 0 | 59.2 | |
| | | 0.1 | 80.3 | |
| | | 0.4 | 63.7 | - |
| | | 1.0 | 69.0 | |
| | | 2.5 | 82.5 | |
| Trout | heart muscle | 0 | 0.1 | |
| | | 0.1 | 0.7 | |
| | | 0.2 | 1.3 | |
| | | 0.3 | 1.6 | |
| | | 0.5 | 2.2 | 0.32 |
| | | 1.0 | 2.7 | |
| | | 1.25 | 3.0 | |
| | | 2.5 | 3.3 | |
| | | 5.0 | 3.0 | |

TABLE XIII

ACTIVATION OF 2-OXOGLUTARATE DEHYDROGENASE ACTIVITY BY ADP IN EXTRACTS FROM DIFFERENT ANIMALS (cont'd.)

| ANIMAL | TISSUE | CONCENTRATION OF ADDED ADP mM | ACTIVITY OF OGDH $\mu mol.min^{-1}.g^{-1}$ (fresh wt) | CONCENTRATION OF ADP GIVING HALF-MAXIMAL ACTIVATION mM |
|---|---|---|---|---|
| Pigeon | pectoral muscle | 0 | 0.2 | |
| | | 0.1 | 0.5 | |
| | | 0.2 | 1.5 | |
| | | 0.3 | 1.3 | 1.65 |
| | | 0.4 | 1.5 | |
| | | 0.5 | 2.4 | |
| | | 1.0 | 3.2 | |
| | | 1.25 | 5.1 | |
| | | 5.0 | 9.6 | |
| Rabbit | heart muscle | 0 | 0.3 | |
| | | 0.1 | 1.3 | |
| | | 0.2 | 3.3 | |
| | | 0.3 | 3.4 | 0.41 |
| | | 0.4 | 4.0 | |
| | | 0.5 | 5.2 | |
| | | 1.0 | 6.5 | |
| | | 1.25 | 7.2 | |
| | | 2.5 | 8.8 | |
| | | 5.0 | 9.2 | |
| Rat | heart muscle | 0 | 7.2 | |
| | | 0.1 | 7.3 | 0.30 |
| | | 1.25 | 9.8 | |
| | | 2.5 | 12.4 | |
| | | 5.0 | 11.4 | |

assay system for the enzyme for all the muscles, since inhibition of OGDH by ADP was observed in extracts of frog heart and chafer flight muscle.

ATP concentration in muscle is approximately 5 mM (Beis & Newsholme, 1975). In the assay system of OGDH, 10 $\mu$l of a 10% extract are added to a final volume of 1.0 ml, which represents a total of a thousand-fold dilution of extract. Thus, assuming no ATP had been broken down by endogenous ATPases in the extract, the concentration of ATP in the cuvette might be 5 $\mu$M. Unfortunately, it was not possible to dialyse the extract to remove ATP, since the enzyme lost activity during the period of dialysis. Similarly, the use of a Sephadex column to remove small molecules diluted the extract which resulted in a loss of activity.

The activation of OGDH activity by ADP in extracts from six animals was investigated further, and the results are presented in Table XIII. The concentration of ADP giving half maximal activation varied from 0.3 to 1.65 mM. The effect is seen in insects, fish, birds and mammals, and may therefore be regarded as fairly widespread within the animal kingdom.

(iii) Variation in activity

As in the assays of PDH activity, great variation was encountered in the enzyme activities of OGDH from animal to animal. Table XIV shows some of the results of the preliminary survey of maximum OGDH activities, with values from replicates, $S_w$ values and medians from the extracts of muscle from six different animals. The same data are also given for CS for comparison, and are taken from Alp et al. (1976). The reasons for the variation in the maximum activities of OGDH could be the same as those suggested for the variation in PDH activity (see p.122) as OGDH also catalyses a non-equilibrium reaction, and possibly a flux-generating step (see Chapter One), and the activities are lower than, for example, CS activities.

TABLE XIV

VARIATION IN ACTIVITY OF 2-OXOGLUTARATE DEHYDROGENASE AND CITRATE SYNTHASE FROM EXTRACTS OF MUSCLE FROM VARIOUS ANIMALS

The median (M) and $S_w$ (an estimate of the standard deviation which can be obtained from the range) values for 2-oxoglutarate dehydrogenase are given for the replicate assays of a single extracts, and for the different animals (M' and $S_w'$). In the latter case, the median value (M') is calculated from the madians (M) of the replicates. $S_W$ is calculated according to Dean & Dixon (1951). For citrate synthase, the data are taken from Alp et al. (1976), but the mean is given instead of the median. The figures in brackets show $S_w'$ as a percentage of the median (or mean).

| ANIMAL | TISSUE | 2-OXOGLUTARATE DEHYDROGENASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$) | | | | CITRATE SYNTHASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$) VARIATION WITHIN ANIMALS | |
|---|---|---|---|---|---|---|---|
| | | VARIATION WITHIN REPLICATES | | VARIATION WITHIN ANIMALS | | | |
| | | MEDIAN M | $S_w$ | MEDIAN M' | $S_W'$ | MEAN | $S_w'$ |
| Whelk | radular muscle | 3.43 | 0.68 | | | | |
| | | 4.09 | 0.26 | 4.71 | 0.93 | 24.8 | 3.0(12%) |
| | | 5.32 | 0.21 | | | | |
| | | 5.32 | 0.21 | | | | |
| Honey bee | flight muscle | 50.52 | 5.92 | | | | |
| | | 69.19 | 3.37 | | | 346.0 | 1.8(0.5%) |
| | | 49.65 | 7.39 | 49.65 | 8.03 | | |
| | | 49.6 | 4.4 | | | | |
| | | 63.7 | 3.86 | | | | |
| Dogfish | heart | 2.46 | 0.18 | | | | |
| | | 3.59 | 0.34 | 3.29 | 0.67 | 46.0 | 11.8 (26%) |
| | | 3.29 | 0.21 | | | | |
| Frog | heart | 1.71 | 0.17 | | | | |
| | | 3.06 | 0.49 | | | | |
| | | 3.87 | 0.54 | 3.52 | 1.27 | 40.0 | 3.5 (9%) |
| | | 3.52 | 0.22 | | | | |
| | | 4.67 | 0.99 | | | | |

TABLE XIV (cont'd.)

| ANIMAL | TISSUE | 2-OXOGLUTARATE DEHYDROGENASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$) | | | | CITRATE SYNTHASE ACTIVITIES ($\mu$mol.min$^{-1}$.g$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | VARIATION WITHIN REPLICATES | | VARIATION WITHIN ANIMALS | | VARIATION WITHIN ANIMALS | |
| | | MEDIAN M | $S_w$ | MEDIAN M' | $S_w$' | MEAN | $S_w$' |
| Pigeon | Pectoral muscle | 2.4<br>3.12<br>5.38<br>3.30 | 0.35<br>0.73<br>0.30<br>0.36 | 3.21 | 1.28 | 115.0 | 26.2 (23%) |
| Rat | heart | 4.42<br>4.68<br>6.81<br>10.30<br>5.57<br>10.79<br>9.89 | 0.26<br>0.73<br>0.57<br>0.84<br>0.50<br>1.04<br>0.08 | 6.81 | 2.36 | 96.0 | 8.3 (8.6%) |

# CHAPTER FOUR

# A COMPARATIVE SURVEY OF MAXIMUM ACTIVITIES OF PYRUVATE DEHYDROGENASE AND 2-OXOGLUTARATE DEHYDROGENASE IN MUSCLE

## INTRODUCTION

The specialisation of muscles gives rise to a wide variety of muscle types which have different physiological functions and different metabolic characteristics suited to their physiological performance. A discussion of the metabolism and physiology of these types of muscle was given in Chapter One, pp. 16-23, and it was stressed that, although there is no doubt that the TCA cycle plays a very important role in energy production in some muscles, there was no simple means of estimating the quantitative significance of the TCA cycle. This is particularly the case for estimations of the flux in different muscles from animals across the animal kingdom.

The use of the $V_{max}$ values (maximum catalytic activities *in vitro*) of certain 'rate-indicator' enzymes has been described in Chapter One. The prime requisites for candidates for such enzymes are that, firstly, they should catalyse non-equilibrium reactions, and secondly, that they should catalyse flux-generating steps. However, the $V_{max}$ of such enzymes, even those catalysing flux-generating steps, may be considerably greater than the maximum flux ($J_{max}$) through the pathway (e.g. CS, see pp.56-58). Hence it is always necessary to compare the $V_{max}$ of such enzymes with the measured maximum rate of operation of the pathway i.e. $J_{max}$. If $V_{max}$ is similar to $J_{max}$, the enzyme could be used as a 'rate-indicator'.

The enzymes that have so far been tested for this role in the TCA cycle are CS and ICDH (both NAD-ICDH and NADP-ICDH), (see Alp *et al.*, 1976). It was shown in Chapter One that CS catalysed a flux-generating step, as did

NAD-ICDH in insect flight muscle. However, when the $V_{max}$ values of these enzymes were compared with the $J_{max}$ through the TCA cycle, they were found to exceed the $J_{max}$. It appears likely that NADP-ICDH is also involved in isocitrate oxidation in the TCA cycle (see pp.58-59) and therefore the $V_{max}$ of both enzymes should be added to be compared with the $J_{max}$; the $V_{max}$ of total ICDH was found to exceed considerably the $J_{max}$ (see Chapter One, p.60 and this Chapter, Table II).

The enzyme OGDH was considered as the next candidate for the role of 'rate-indicator' for the TCA cycle. In addition, the enzyme PDH was included in considerations of the TCA cycle since entry of all carbohydrate fuels to the TCA cycle occurs via this enzyme. It has been established that both these enzymes catalyse non-equilibrium reactions and they are probably flux-generating (see Chapter One). It remained necessary to compare the $V_{max}$ of these enzymes with the $J_{max}$ through the TCA cycle, in those muscles in which the $J_{max}$ can be measured. These muscles are those for which oxygen uptake data during maximum exercise are available. The justifications for the use of oxygen uptake data to estimate flux are discussed in Crabtree & Newsholme (1972a, 1975).

Since this comparison provided evidence that the activities of these enzymes are useful indicators of flux, it was possible to carry out a comparative survey of the maximum capacity of the TCA cycle in muscles throughout the animal kingdom.

## METHODS

The sources of animals and chemicals are given in Appendix III. A full description of the extraction and assay conditions of PDH and OGDH is given in Chapter Three. In addition, homogenates were also prepared for the assay

of CS, NAD-ICDH and NADP-ICDH as described in Alp et al. (1976).

## RESULTS

### 1) Comparison of maximum activities of PDH and OGDH with maximum flux through the TCA cycle

Oxygen uptake data are available for the following insects during flight: cockroach, bumble-bee, honey-bee, silver Y moth, locust and blowfly. They are also available for the following muscles during maximum or near-maximum exercise: trout red muscle, pigeon pectoral muscle, rat heart, rat skeletal muscle and human thigh muscle, under conditions where it can be assumed that the increase in oxygen uptake over the resting value is due mainly to the muscle under consideration. (Further explanations are given in Appendix VI and in Crabtree & Newsholme, 1972a, 1975). These data can be converted to values for $J_{max}$ through the TCA cycle at 25°C using assumptions and conversion factors explained in Appendix VI.

The $V_{max}$ values of OGDH and PDH in nine muscles are presented together with the estimates of $J_{max}$ through the TCA cycle in Table I. It can be seen that there is reasonable agreement between $V_{max}$ and $J_{max}$ values for OGDH: the ratio $V_{max}/J_{max}$ is approximately unity for all the muscles investigated. For PDH activity in vertebrates, there is also good agreement between the values of $V_{max}$ and $J_{max}$. Unfortunately, in insects, the $V_{max}$ values of PDH are lower than those of $J_{max}$, considerably so in the case of the silver Y moth.

These results lead to the suggestion that the activity of OGDH provides a reasonable quantitative index of the maximum flux through the TCA cycle. This appears to be the only enzyme that can fulfill this role.

It has been shown recently that, for a working perfused rat heart preparation, whose work load may be similar to that in vivo, pyruvate (1 mM)

TABLE I

COMPARISON OF ESTIMATES OF THE FLUX THROUGH THE TCA CYCLE FROM OXYGEN UPTAKE DATA WITH MAXIMUM ACTIVITIES OF OGDH AND PDH

The oxygen uptake data were collated from the papers shown, and were converted to estimates of maximum flux ($J_{max}$) through the TCA cycle at 25°C using the conversion factors shown in Appendices VI and VII. Maximum activities ($V_{max}$) of OGDH and PDH are taken from the survey of maximum activities, full details of which are shown in Table II. The specific names of the animals are given in Table II.

| ANIMAL | TISSUE | $J_{max}$ THROUGH TCA CYCLE at 25°C ( $\mu$mol.min$^{-1}$.g$^{-1}$) | $V_{max}$ OF ENZYMES at 25°C ($\mu$mol.min$^{-1}$.g$^{-1}$) | | RATIO OF $V_{max}/J_{max}$ | |
|---|---|---|---|---|---|---|
| | | | OGDH | PDH | OGDH | PDH |
| Honey-bee | flight muscle | 53.0[1] | 49.7 | - | 0.9 | - |
| bumble-bee (queen) | flight muscle | 46.1[2] | 45.9 | 10.8 | 1.0 | 0.2 |
| blowfly | flight muscle | 66.3[3] | 46.7 | 14.3 | 0.7 | 0.2 |
| cockroach | flight muscle | 22.3[4] | 48.6 | 15.1 | 2.2 | 0.7 |
| silver Y moth | flight muscle | 24.8[5] | 25.9 | 0.8 | 1.0 | 0.03 |
| desert locust | flight muscle | 20.7[6] | 20.8 | 10.5 | 1.0 | 0.5 |
| trout | red muscle | 2.7[7] | 1.8 | 3.2 | 0.7 | 1.2 |
| pigeon | pectoral muscle | 4.3[8] | 3.2 | 4.9 | 0.7 | 1.1 |
| rat | heart | 6.3[9] | 6.8 | 6.9 | 1.1 | 1.1 |
| | soleus | 2.2[10] | 2.4 | 1.2 | 1.1 | 0.6 |
| man | quadriceps | 1.0[11] | 1.2 | - | 1.2 | - |

1. Jongbloed & Wiersma (1934)
2. Kammer & Heinrich (1974)
3. Davis & Fraenkel (1940)
4. Polacek & Kubista (1960)
5. Zebe (1954)
6. Krogh & Weis-Fogh (1951)
7. Webb (1971)
8. Butler et al. (1977)
9. Taegtmeyer et al. (1979)
10. Rennie & Holloszy (1977)
11. Asmussen et al. (1939); Costill & Fox (1969)

and lactate (10 mM) can support 90% of the maximal oxygen consumption, and that the performance of the heart perfused with these substrates does not decline for approximately two hours (Taegtmeyer et al., 1979). Thus, in the perfused rat heart at least, the $V_{max}$ of PDH provides an indication of the $J_{max}$ through pyruvate oxidation, as calculated from the oxygen uptake of heart. In addition, the agreement of $V_{max}$ of PDH with $J_{max}$ through the TCA cycle in all four vertebrate muscles investigated suggests that the $V_{max}$ of PDH can indicate $J_{max}$ through the TCA cycle: this suggests that these muscles can support the maximum rate of the TCA cycle from carbohydrate fuels alone, and this provides further evidence that the $V_{max}$ of PDH is an indication of $J_{max}$ through pyruvate oxidation.

Thus, it will be assumed that the $V_{max}$ of PDH is primarily an indicator of $J_{max}$ through pyruvate oxidation, but that it may also be an indicator of $J_{max}$ through the TCA cycle in muscles in which this can be supported by carbohydrate fuels (see later discussion and Chapter Five).

2) Comparative survey of maximum activities of PDH and OGDH in muscles from vertebrates and invertebrates

The problem of variation in the activities of PDH and OGDH discussed in Chapter Three, entailed careful and more rigorous investigations into the $V_{max}$ of these enzymes from each animal. This process took more time than expected and prevented a more exhaustive survey of animal muscles. The $V_{max}$ values of PDH and OGDH are presented for three molluscs, nineteen insects, two fish, two amphibia, three birds and seven mammals, together with the $V_{max}$ values of CS and total ICDH (i.e. NAD-ICDH plus NADP-ICDH) in Table II. For these thirty-six species, the $V_{max}$ values of PDH and OGDH show considerable variation. The $V_{max}$ of PDH ranges from 0.1 $\mu$mol.min$^{-1}$.g$^{-1}$ (in giant water-bug flight muscle) to 17.3 $\mu$mol.min$^{-1}$.g$^{-1}$ (in the blowfly flight muscle,

TABLE II

MAXIMUM ACTIVITIES OF PDH AND OGDH FROM MUSCLES OF VERTEBRATES AND INVERTEBRATES

Maximum activities of citrate synthase (CS) and isocitrate dehydrogenase (total ICDH, i.e. NAD-ICDH plus NADP-ICDH) are shown for comparison. These data are from Alp et al. (1976). PDH and OGDH were assayed as described in the Methods section of Chapter Three. Enzyme activities of PDH and OGDH are presented as medians, with the number of observations below this in parentheses. The figure adjacent to the medians, also in parentheses, is the value of $S_w$ (see Dean & Dixon, 1951). This is an estimate of the standard deviation, which is obtained from the range.

| ANIMAL | TISSUE | ENZYME ACTIVITIES $\mu$mol.min$^{-1}$.g$^{-1}$ at 25°C | | | | | |
|---|---|---|---|---|---|---|---|
| | | PDH | | OGDH | | CS | TOTAL ICDH |
| Mollusca | | | | | | | |
| Common whelk (Buccinum undatum) | radula retractor | 0.2 (5) | (0.1) | 4.7 (4) | (0.9) | 24.8 | 28.6 |
| common limpet (Patella vulgata) | radula retractor | 0.4 (3) | (0.2) | 1.7 (4) | (0.6) | 26.8 | 109.5 |
| periwinkle (Littorina littorea) | radula retractor | 0.4 (1) | | 2.1 (1) | | - | - |
| Insecta: Lepidoptera | | | | | | | |
| Yellow underwing moth (Noctua pronuba) | flight muscle | 9.0 (3) | (2,0) | 39.6 (9) | (9.5) | 296.0 | 179.0 |
| silver Y moth (Plusia gamma) | flight muscle | 0.8 (2) | (0.5) | 25.9 (2) | (1.2) | 352.0 | 129.0 |
| poplar hawk moth (Laßthoe populi) | flight muscle | 3.7 (3) | (0.2) | 42.7 (3) | (15.2) | 430.0 | 109.0 |
| Hymenoptera | | | | | | | |
| common wasp (Vespula vulgaris) | flight muscle | 16.7 (3) | (3.0) | 71.2 (4) | (11.0) | 332 | 116 |
| German wasp (Vespula germanica) | flight muscle | 6.3 (3) | (1.5) | 76.5 (3) | (9.5) | - | - |

TABLE II (cont'd)

| ANIMAL | TISSUE | ENZYME ACTIVITIES $\mu$mol. $min^{-1}.g^{-1}$ at 25°C | | | | | |
|---|---|---|---|---|---|---|---|
| | | PDH | | OGDH | | CS | TOTAL ICDH |
| honey bee (Apis mellifera) | flight muscle | - | | 49.7 (5) | (8.0) | 346 | 98 |
| bumble bee (Bombus terrestris) | flight muscle | | | | | | |
| queen | | 10.8 (2) | (0.5) | 45.9 (4) | (6.7) | 325 | 107 |
| worker | | 13.6 (3) | (4.3) | 47.0 (5) | (7.3) | 382 | 153 |
| male | | 27.2 (4) | (4.8) | 67.6 (5) | (14.3) | 349 | 116 |
| Coleoptera | | | | | | | |
| rosechafer (Pachnoda ephippiata) | flight muscle | 2.6 (3) | (0.9) | 36.8 (5) | (7.1) | 561 | 83 |
| summer chafer (Amphimallon solstialis) | flight muscle | - | | 57.5 (5) | (26.1) | 243* | 183* |
| African dung beetle (Heliocopris sp) | flight muscle | 0.4 (1) | | 23.3 (7) | (3.5) | 294 | 72 |
| great diving beetle (Dysticus marginalis) | flight muscle | - | | 12.0 (3) | (3.8) | 26.8† | 3.8† |
| Hemiptera | | | | | | | |
| giant water bug (Lethocerus cordofanus) | flight muscle | 0.1 (1) | | 15.2 (5) | (1.6) | 244 | 104 |
| Dictyoptera | | | | | | | |
| cockroach (Periplaneta americana) | flight muscle | 15.1 (5) | (1.8) | 48.6 (7) | (8.9) | 185 | 77 |
| | leg muscle | - | | 6.8 (3) | (0.2) | - | - |

TABLE II (cont'd.)

| ANIMAL | TISSUE | ENZYME ACTIVITIES $\mu mol.min^{-1}.g^{-1}$ at 25°C | | | | | |
|---|---|---|---|---|---|---|---|
| | | PDH | | OGDH / | | CS | TOTAL ICDH |
| Orthoptera | | | | | | | |
| desert locust (Schistocerca gregaria) | flight muscle | 10.5 (6) | (1.4) | 20.8 (9) | (4.6) | 242 | 62 |
| African locust (Locusta migratoria) | flight muscle | 5.1 (4) | (0.5) | 44.2 (5) | (4.2) | - | - |
| Diptera | | | | | | | |
| blowfly (Calliphora vomitoria) | flight muscle | 14.3 (3) | (1.4) | 46.7 (6) | (11.0) | 418 | 180 |
| fleshfly (Sarcophaga barbata) | flight muscle | 14.6 (3) | (0.2) | 32.8 (12) | (9.3) | 316 | 145 |
| housefly (Musca domestica) | flight muscle | 17.3 | (1.4) | 38.1 | (10.7) | 345† | 103.2† |
| tsetse fly (Glossina morsitans) | flight muscle | 0.9 (7) | (0.4) | 26.4 (3) | (0.8) | 74 | 29 |
| Pisces | | | | | | | |
| Dogfish (Scylliorhinus canicula) | heart | 2.5 (4) | (0.6) | 3.3 (3) | (0.7) | 46 | 53.4 |
| | red muscle | 3.2 (4) | (1.1) | 2.2 (3) | (0.6) | 35 | 31.4 |
| | white muscle | <0.1 (3) | | < 0.1 (3) | | 1.7 | 0.6 |
| trout (Salmo gairdneri) | heart | 3.6 (4) | (1.5) | 3.8 (5) | (0.9) | 38 | 101 |
| | red muscle | 3.2 (4) | (2.5) | 1.8 (3) | (0.4) | 50 | 125 |
| | white muscle | 0.3 (4) | (0.1) | < 0.1 (3) | | 5.2 | 9.2 |

TABLE II (cont'd.)

| ANIMAL | TISSUE | ENZYME ACTIVITIES $\mu mol.min^{-1}.g^{-1}$ at 25°C | | | | | |
|---|---|---|---|---|---|---|---|
| | | PDH | | OGDH | | CS | TOTAL ICDH |
| Amphibia: | | | | | | | |
| frog (Rana temporaria) | heart | 4.8 (3) | (0.8) | 3.5 (5) | (1.3) | 40 | 75 |
| | sartorius | 0.4 (5) | (0.2) | 0.1 (6) | (0.2) | 10 | 14 |
| | gastrocnemius | 0.7 (3) | (0.3) | < 0.1 (3) | | | |
| African clawed toad (Xenopus laevis) | heart | 3.2 (4) | (1.2) | 3.3 (2) | (1.0) | | |
| | sartorius | 0.6 (3) | (0.3) | 1.2 (2) | (0.1) | 7.0 | 5.5 |
| | gastrocnemius | 1.0 (2) | (0.6) | 1.0 (2) | (0.5) | 12.0 | 12.0 |
| Aves: | | | | | | | |
| chicken (Gallus gallus) | heart | 5.0 (4) | (0.3) | 6.0 (3) | (0.8) | 51 | 59.7 |
| | pectoral muscle | 0.2 (4) | (0.1) | <0.1 (4) | | 6.8 | 3.3 |
| pigeon (Columba livia) | heart | 6.8 (3) | (2.2) | 4.3 (4) | (1.2) | 127 | 74 |
| | pectoral muscle | 4.9 (4) | (1.5) | 3.2 (4) | (1.3) | 115 | 54.8 |
| pheasant (Phasianus colchicus) | heart | 8.9 (3) | (0.7) | 4.7 (2) | (1.0) | 99.0 | 48.7 |
| | pectoral muscle | 0.9 (4) | (0.4) | 1.5 (2) | (1.0) | 14.4 | 3.5 |
| Mammalia: | | | | | | | |
| laboratory mouse (Mus musculus) Balb/C | heart | 12.0 (3) | (0.2) | 8.9 (5) | (1.0) | 146 | 106 |

TABLE II (cont'd,)

| ANIMAL | TISSUE | ENZYME ACTIVITIES $\mu mol.min^{-1}.g^{-1}$ at 25°C | | | | | |
|---|---|---|---|---|---|---|---|
| | | PDH | | OGDH | | CS | TOTAL ICDH |
| laboratory rat (Rattus norvegicus) | heart | 6.9 (8) | (0.7) | 6.8 (7) | (2.4) | 96 | 77 |
| | soleus | 1.2 (3) | (0.5) | 2.4 (4) | (0.2) | - | - |
| | diaphragm | 1.9 (3) | (0.9) | 2.4 (5) | (0.8) | - | - |
| guinea pig | heart | 2.9 (1) | | 7.1 (1) | | | |
| rabbit (Oryctolagus caniculus) | heart | 6.4 (3) | (2.6) | 9.0 (4) | (2.0) | 69 | 34.2 |
| | adductor longus | 0.4 (3) | (0.1) | 0.7 (2) | (0.2) | | |
| | semitendinosus | 0.5 (3) | (0.4) | 0.9 (4) | (0.5) | | |
| sheep (Ovis aries) | heart | 4.7 (3) | (0.9) | 4.8 (6) | (0.8) | 58.0 | 21.6 |
| pig (Sus scrofa) | heart | 4.3 (7) | (0.7) | 3.8 (6) | (0.4) | 50.0 | 13.6 |
| man (Homo sapiens) | thigh muscle ≠ | 1.1 (4) | (0.7) | | | | |

* These data were from the flight muscle of the cockchafer, which is very closely related to the summer chafer (see text).

≠ These values were measured by Mrs. T. Williams in Norway, on thigh muscle biopsies from human volunteers.

† These values were measured by the author. The assay conditions for CS, NAD-ICDH and NADP-ICDH are the same as those in Alp et al. (1976)

Calliphora vomitoria). The $V_{max}$ of OGDH ranges from undetectable (i.e. < 0.1 $\mu$mol. $min^{-1}.g^{-1}$ in various vertebrate white muscles) to 76.5 $\mu$mol.$min^{-1}.g^{-1}$ (in the common wasp flight muscle). The $V_{max}$ of PDH or OGDH for any individual muscle was always lower than the $V_{max}$ of CS or total ICDH. For example, in the rosechafer, $V_{max}$ values were 2.6, 36.8, 561.0 and 83.0 $\mu$mol.$min^{-1}.g^{-1}$ for PDH, OGDH, CS and total ICDH respectively. In the rat heart, $V_{max}$ values were 6.9, 6.8, 96.0 and 77.0 $\mu$mol.$min^{-1}.g^{-1}$ for PDH, OGDH, CS and total ICDH respectively.

## DISCUSSION

The enzyme OGDH has been shown to catalyse a non-equilibrium reaction and probably a flux-generating step in muscle. The $V_{max}$ values of this enzyme are similar to the $J_{max}$ of the TCA cycle in a number of muscles from different animals across the animal kingdom. Hence, the $V_{max}$ of OGDH can be used as a quantitative index of the TCA cycle. The results of the comparative survey should therefore give a quantitative indication of the maximum rate of operation of the TCA cycle in a wide variety of muscles across the animal kingdom.

The enzyme PDH, chosen as an 'honorary' enzyme of the TCA cycle, due to the importance of carbohydrate as a source of fuel for muscular contraction, had also been shown to catalyse a non-equilibrium reaction and a flux-generating step. The $V_{max}$ of PDH was shown to be similar to the $J_{max}$ of the TCA cycle in vertebrate muscles (see earlier discussion). For vertebrate muscles, therefore, it was of interest to compare the $V_{max}$ of PDH with that of OGDH.

In all muscles the $V_{max}$ of PDH should provide a quantitative index of flux from carbohydrate into the TCA cycle, i.e.of pyruvate oxidation, and this can be compared with the contribution of other fuels to the TCA cycle. It must be emphasised at this early stage, however, that these values give an

indication only of the maximum possible flux through the pathways, and not the flux through the pathway at any given time. For example, it is known that the proportion of active PDH ($PDH_a$) in hearts frozen <u>in situ</u> from normal fed rats is only 20% of its $V_{max}$ (see Chapter Three, p.115). In addition, it must be stressed that the $V_{max}$ of PDH gives only an indication of the maximum flux from carbohydrate into the TCA cycle, and that other fuels such as lipids or ketone bodies may contribute to the flux through the cycle (see Fig. 3 , Chapter One).

In this Chapter, the index of flux through the TCA cycle will be compared with other estimates of flux through carbohydrate and fat utilising pathways which feed into the TCA cycle. The implications of this comparison for the utilisation by muscles of fat and carbohydrate as fuels will be discussed. A brief comparison will be made between the aerobic capacity (i.e. index of the TCA cycle) of insect flight muscle and vertebrate white muscle. Further discussion on the physiological specialisation of muscles, for example, aerobic versus anaerobic energy production and the differences between the $V_{max}$ values of OGDH and PDH will be postponed to Chapter Five.

1) <u>Comparison of estimates of flux through the TCA cycle from the $V_{max}$ values of 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase, hexokinase, phosphofructokinase, diglyceride lipase, triglyceride lipase and carnitine palmitoyltransferase</u>

The activities of other enzymes (see below) have been used to indicate flux through the fuel-utilising pathways which feed into the TCA cycle, e.g. from fat and carbohydrate fuels. Therefore some indication of the contribution of these fuels to the TCA cycle may be gained by a comparison of these activities with the $V_{max}$ values of PDH and OGDH. The maximum rate of the TCA cycle which could be supported by different fuels can be shown by

expressing the $V_{max}$ of these 'rate-indicator' enzymes in terms of TCA cycle equivalents i.e. $C_3/C_2$ fragment oxidation.

The enzymes whose $V_{max}$ values will be used are hexokinase (for the maximum flux from glucose oxidation, see Crabtree & Newsholme, 1972a), phosphofructokinase (for the maximum flux from carbohydrate oxidation in insects, see below), diglyceride and triglyceride lipase (for the maximum flux from lipid oxidation) and carnitine palmitoyltransferase (for the maximum flux from fatty acid oxidation). The data are taken from Crabtree & Newsholme (1972 a, b).

Phosphofructokinase (PFK) has been used as an indicator of total flux through glycolysis from both endogenous glycogen stores and exogenous glucose (see Crabtree & Newsholme, 1972a; Crabtree & Newsholme, 1975). In insects, it can be assumed that there is very little flux from carbohydrate to lactate. This is primarily because $V_{max}$ values of lactate dehydrogenase are low in insect flight muscles (see Crabtree & Newsholme, 1972a and Table IV, this Chapter), and as this enzyme is believed to catalyse a near-equilibrium reaction, the flux through the reaction should be even lower. Thus, the $V_{max}$ values of PFK may give an indication of the maximum flux of carbohydrate into the TCA cycle in insect flight muscle. These $V_{max}$ values have been multiplied by two (to give the TCA cycle equivalent flux in $\mu$mol $C_3/C_2.min^{-1}.g^{-1}$) and these values are presented in Table III.

The $V_{max}$ values of hexokinase (HK) (to represent flux from glucose or trehalose oxidation) have been converted to TCA cycle equivalent flux in the same way, and are presented for muscles from invertebrates and vertebrates in Table III.

During the oxidation of glycerides (or acylglycerols) glycerol is produced, and this raises a problem in the interpretation of the expression of

TABLE III

RATES OF FUEL UTILISATION BY MUSCLES

The maximum activities of PDH and OGDH are taken from Table II. The TCA cycle equivalent flux is calculated from the maximum activities of enzymes in the following way: $V_{max}$ values of hexokinase (HK) and phosphofructokinase (PFK) are multiplied by 2 to be expressed at $\mu$mol $C_3/C_2$ fragment. $min^{-1}.g^{-1}$; $V_{max}$ values of diglyceride lipase (DGL) are multiplied by 16 (as 16 mol of acetyl CoA are produced from 1 mol of dipalmitoyl glycerol); $V_{max}$ values of triglyceride lipase (TGL) are multiplied by 24 (as 24 mol of acetyl CoA are produced from 1 mol of tripalmitoyl glycerol); $V_{max}$ values of carnitine palmitoyl transferase (CPT) are multiplied by 4, since 1 mol of palmitate supplies 8 mol of acetyl CoA, and because the $V_{max}$ is the sum of two enzyme activities - the enzyme occurs at two points in the pathway of fatty acid oxidation, on either side of the mitochondrial barrier to fatty acyl CoA (see Greville & Tubbs, 1968). The assumptions are made that the fatty acid components of the acylglycerols (glycerides) will not differ greatly in chain length from $C_{16}$ i.e. palmitate, and that glycerol is not oxidised (see text). Longer chain length glycerides may give rise to greater flux through the TCA cycle. The superscripts indicate the actual species or muscle used in the assay: $^{a}$, Amphimallon solstitialis; $^{m}$, Melolontha melolontha; $^{s}$, soleus muscle; $^{q}$, quadriceps muscle.

| ANIMAL: MUSCLE | MAXIMUM ENZYME ACTIVITIES ($\mu$mol.$min^{-1}$.$g^{-1}$) | | TCA CYCLE EQUIVALENT FLUX ($\mu$mol $C_3/C_2$.$min^{-1}$.$g^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|
| | OGDH | PDH | HK | PFK | DGL | TGL | CPT |
| whelk: radular retractor | 4.7 | 0.2 | 7.4 | - | - | - | - |
| limpet: radular retractor | 1.7 | 0.4 | 6.2 | - | - | - | - |
| African locust: flight muscle | 44.2 | 5.1 | 16.0 | 26.0 | 9.6 | 1.7 | 14.4 |
| desert locust: flight muscle | 20.8 | 10.5 | 23.0 | 35.0 | 11.2 | 1.4 | 11.2 |
| cockroach: flight muscle | 48.6 | 15.1 | 36.0 | 38.0 | 1.6 | 0.5 | 0.4 |
| giant waterbug: flight muscle | 15.2 | 0.1 | 9.0 | 12.0 | 14.4 | 1.7 | 14.0 |
| chafer: flight muscle | $57.5^{a}$ | - | $4.6^{m}$ | $26.0^{m}$ | $4.8^{m}$ | $0.72^{m}$ | $0.4^{m}$ |
| wasp: flight muscle | 71.2 | 16.7 | 154.0 | 148.0 | 3.2 | 1.2 | - |
| honey-bee: flight muscle | 49.7 | - | 58.0 | 40.0 | 4.8 | 1.0 | 0.8 |
| bumble-bee: flight muscle | 45.9 | 10.8 | 228.0 | 66.0 | 3.2 | 0.5 | 2.8 |
| poplar hawk moth: flight muscle | 42.7 | 3.7 | 6.4 | 18.0 | 3.2 | 1.0 | - |
| silver Y moth: flight muscle | 25.9 | 0.8 | 100.0 | 82.0 | 8.0 | 1.0 | 4.4 |
| yellow underwing moth: flight muscle | 39.6 | 9.0 | - | - | 9.6 | 1.2 | 4.4 |
| tsetse fly: flight muscle | 26.4 | 0.9 | 4.6 | - | - | - | - |

TABLE III (cont'd.)

| ANIMAL: MUSCLE | MAXIMUM ENZYME ACTIVITIES ($\mu$mol.min.$^{-1}$.g$^{-1}$) | | TCA CYCLE EQUIVALENT FLUX ($\mu$mol $C_3/C_2$.min$^{-1}$.g$^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|
| | OGDH | PDH | HK | PFK | DGL | TGL | CPT |
| fleshfly: flight muscle | 32.8 | 14.6 | 34.0 | - | 4.8 | 1.0 | 1.2 |
| blowfly: flight muscle | 46.7 | 14.3 | 70.0 | 86.0 | 3.2 | 0.7 | 0.4 |
| housefly: flight muscle | 38.1 | 17.3 | - | 62.2* | - | - | - |
| dogfish: red muscle | 2.2 | 3.2 | 3.8 | - | - | < 0.1 | < 0.04** |
| dogfish: white muscle | < 0.1 | 0.1 | 0.2 | - | - | < 0.1 | < 0.04** |
| trout: red muscle | 1.8 | 3.2 | 5.2 | - | - | 0.5 | 0.8 |
| trout: white muscle | <0.1 | 0.1 | 3.2 | - | - | - | - |
| frog: sartorius | 0.1 | 0.4 | 2.6 | - | - | - | - |
| pigeon: pectoral muscle | 3.2 | 4.9 | 6.0 | - | - | 1.7 | 12.8 |
| chicken: pectoral muscle | <0.1 | 0.2 | 2.2 | - | - | - | - |
| pheasant: pectoral muscle | 1.5 | 0.9 | 4.6 | - | - | 0.2 | 0.1 |
| rabbit: semitendinosus | 0.9 | 0.5 | 3.8 | - | - | 0.7 | 2.0 |
| rabbit: adductor longus | 0.7 | 0.4 | 0.6 | - | - | 0.2 | 0.2 |
| rat: heart | 6.8 | 6.9 | 12.2 | - | 7.2 | 1.4 | 8.8 |
| rat: skeletal muscle | 2.4[s] | 1.2[s] | 3.8[q] | - | - | 0.2[q] | 7.6[q] |

*This value was supplied by B. Surholt (personal communication).

** These values are from Zammit & Newsholme (1979).

the rates of oxidation of glycerides in terms of TCA cycle equivalents. In Table III, the assumption has been made that glycerol is unimportant. This is primarily because the contribution that glycerol oxidation would make to the energy production during the oxidation of glycerides is negligible, i.e. this contribution would be 8% and 5% of that of diglyceride and triglyceride oxidation respectively (see footnote).

There is some evidence that the glyceride lipases and carnitine palmitoyltransferase catalyse reactions which are far displaced from equilibrium, and the use of the enzymes as rate indicators for glyceride hydrolysis and fatty acid oxidation respectively is discussed in Newsholme & Start (1973), pp. 298-300 and Crabtree & Newsholme (1972b). The importance of glycerol as a fuel can be assessed by comparison of these activities with glycerol kinase activities. The $V_{max}$ values of glycerol kinase are lower than the $V_{max}$ values of triglyceride lipase (TGL) in terms of $C_3$ or $C_2$ fragment of substrate converted. In addition, in the isolated rat heart, glycerol is released into the medium under conditions when triglyceride is being oxidised (Crabtree & Newsholme, 1972b and Garland & Randle, 1964b). These two facts suggest that when triglyceride is being oxidised, glycerol is not oxidised as fast as the fatty acid residues. In insects, the major exogenous lipid fuel is diglyceride (see below). Evidence has been presented that glycerol is oxidised by insect

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Footnote: Each mol of palmitate gives rise to 130 mol ATP: 34 mol during $\beta$-oxidation, and 96 mol ATP from the oxidation of 8 mol of acetyl-CoA via the TCA cycle. Thus, assuming that the fatty acid residues of glycerides are mainly palmitate, triglyceride oxidation gives rise to 390 mol ATP and diglyceride oxidation gives rise to 260 mol ATP. Glycerol oxidation gives rise to 7 mol ATP during the conversion of pyruvate, less 1 mol in the glycerol kinase reaction, plus 15 mol ATP from the oxidation of pyruvate via the TCA cycle. Thus, the net ATP production from one mol of glycerol is 21 mol. (21/260 = 0.08, 21/390 = 0.05).

flight muscle (Candy et al., 1976). However, maximum activities of glycerol kinase from Crabtree & Newsholme (1972b) are lower than the maximum rate of operation of the TCA cycle. For example, in the desert locust flight muscle $V_{max}$ of glycerol kinase is 0.5 $\mu$mol.min$^{-1}$.g$^{-1}$ and the $V_{max}$ of OGDH is 20.8 $\mu$mol.min$^{-1}$.g$^{-1}$. Thus it appears that glycerol is oxidised by insect flight muscle at a very slow rate; this agrees with an earlier conclusion of Sacktor (1970), p. 322.

Having established that the oxidation of glycerol can be disregarded, the contribution of other fuels to the TCA cycle can be considered, i.e. of glucose, diglyceride and triglyceride and fatty acids. The estimates of the flux through the TCA cycle from these fuels are presented in Table III, together with the $V_{max}$ values of PDH and OGDH.

Evidence has been presented that phosphofructokinase can be used as an indicator of flux from carbohydrate oxidation in insects (see above). Comparison of estimated through the TCA cycle from carbohydrate (i.e. 2 x $V_{max}$ of phosphofructokinase) with $J_{max}$ through the TCA cycle (i.e. $V_{max}$ of OGDH) suggests that in many insect flight muscles, carbohydrate alone can support the maximum rate of the TCA cycle. This is not the case for the following insects: African locust, giant waterbug, summer chafer and poplar hawk moth. This suggests that the maximum rate of utilisation of carbohydrate cannot support the maximum rate of the TCA cycle in these insects, so that an alternative, supplementary fuel must be used for maximum energy requirements. There is evidence that lipids and proline can be used as fuels for flight in insects (see Sacktor, 1965, 1970, for review).

Sacktor suggested that insects with the asynchronous fibrillar type of muscle (e.g. Diptera, Hymenoptera) utilise mainly carbohydrate, and that the insects with synchronous muscle (e.g. Lepidoptera, Orthoptera, Odonata)

utilise mainly fat during flight. However, many exceptions to this have been found and it is now thought that no simple generalisation can be made about which insects utilise which fuel. It will aid discussion of the use of supplementary fuels by considering each insect in turn.

African locust (Locusta migratoria)

Comparison of the $V_{max}$ of PFK with the $V_{max}$ of OGDH, in terms of TCA cycle equivalents, shows that this insect can only support approximately 60% of its maximum energy requirements from carbohydrate. In this insect, the supplementary fuel is likely to be lipid. Lipid has advantages over carbohydrate as a storage fuel in flying animals, since the hydration of glycogen would make isocaloric quantities eight times heavier than lipid (see Chapter One, p. 24 and Weis-Fogh, 1952). In addition, lipid is likely to be used by migrating animals (Weis-Fogh, 1952; Odum, 1965). Evidence from R.Q. values during flight suggests that some insects utilise mainly fat as a fuel for flight (Sacktor, 1970 for review).

The major lipid fuel in insect haemolymph is diglyceride (Tietz, 1967; Chino & Gilbert, 1965), although it is stored in the fat body as triglyceride. Thus, although the $V_{max}$ values of triglyceride lipase (TGL) in insect flight muscle are approximately ten-fold lower than the $V_{max}$ values of diglyceride lipase (DGL) in the flight muscle, it is not thought that the former enzyme limits the utilisation of lipid in insect flight muscle. Although unesterified fatty acids only form 5% of the lipid fuels in insect haemolymph (diglycerides constitute 84%), the concentration of these fatty acids increases four-fold during flight in the African locust (Beenakkers, 1973). If the uptake and utilisation of fatty acids are not rate limiting in insect flight muscle, this suggests that fatty acids may also serve as a fuel during flight.

The $V_{max}$ values of DGL and CPT presented in Table III suggest that diglycerides and fatty acids could support 20% and 33% of maximum energy re-

quirements, respectively. (CPT activities represent the flux through fatty acid oxidation from exogenous fatty acids and those derived from both endogenous and exogenous diglycerides.) Thus it appears that, in Locusta, carbohydrate can only support 60% of maximum energy requirements, and total lipid fuels can only support 33% of amximum energy requirements. During prolonged flight, muscle glycogen stores will be rapidly depleted. In the blowfly, which utilises mainly carbohydrate during flight, the concentration of glycogen in the flight muscle decreased from approximately 8 mM to 0.75 mM after 10 minutes of flight and then stayed steady at this concentration during prolonged flight (Sacktor, 1970). Presumably, glycogen stores from the fat body may also be mobilised (see Sacktor & Wormser-Shavit, 1966), but the weight restriction prevents large deposits of glycogen. During migration in Locusta, then, a problem arises in the supply of fuels after the depletion of carbohydrate stores.

One possible process which could produce carbohydrate may be gluconeogenesis in the fat body. A possible precursor for gluconeogenesis is glycerol, and as mentioned above, the rate of oxidation of glycerol by flight muscle is much lower than that of diglyceride oxidation, and thus glycerol may be released into the haemolymph to serve as a gluconeogenic precursor, during prolonged flight. Other possible substrates for gluconeogenesis are amino acids: these may be derived from the breakdown of the flight muscles in extreme circumstances (Matthews & Matthews, 1978, p. 92), or could be derived from the breakdown of proteins in the fat body. However, there have been no quantitative measurements on rates of gluconeogenesis during prolonged flight in insects; such measurements should be carried out to assess the importance of this process. In man, during prolonged exercise, the rate of gluconeogenesis in the liver increases 3-fold (Wahren, 1977, and see later discussion).

A further possibility which might augment the rate of fuel supply is that the $V_{max}$ of DGL and CPT may increase shortly before the migratory phase of the locust. If this is so, then it may be important to collect the insects when they are in such a state. It has been noted that overcrowding can give rise to endocrine changes which induce the migratory phase in locusts (Barrass, 1974).

A useful comparison can be made at this stage with the Desert locust, Schistocerca gregaria. Weis-Fogh measured R.Q. values during flight in this insect, and found that, in the first half hour of prolonged flight, the energy was derived from carbohydrate oxidation, whereas later in flight, the energy was derived from lipid oxidation (Weis-Fogh, 1952). That carbohydrate can support flight is upheld by the data in the present work where the $V_{max}$ of OGDH is approximately equal to the maximum flux through the TCA cycle from glucose ($2 \times V_{max}$ of HK) and lower than the maximum flux from total carbohydrate ($2 \times V_{max}$ of PFK). However, comparison of the flux from diglyceride and fatty acids with the $J_{max}$ through the TCA cycle suggests that lipid fuels can only support approximately 50% of the maximum energy demands of flight. Presumably, during prolonged flight, the same problem of supplementary fuels occurs, as in the African locust, Locusta, and the same postulated solutions apply.

Finally, it may be mentioned that during the migration of these locusts, occasional stops occur, when the locust swarms ingest large quantities of food (Barrass, 1974). These stops may be occasioned by the depletion of all the available stores of fuel, and represent a 're-fuelling' time, for the repletion of fat and glycogen stores. This is discussed more fully in Weis-Fogh (1952).

Tsetse fly

The concentration of proline in the Haemolymph of tsetse flies is very high, and this decreases rapidly after a period of flight. It has been

demonstrated that proline serves as an important fuel source for flight in the tsetse fly (Bursell, 1963, 1965, 1966, 1967). The pathway, occurring in the flight muscles, is proposed to convert proline to alanine, $CO_2$, NADH and reduced flavoprotein, and is shown in Figure 5, Chapter One. The pathway involves only a segment of the TCA cycle from 2-oxoglutarate to oxaloacetate. A calculation reveals that the number of molecules of ATP that are produced by the conversion of 1 molecule of proline into alanine by this pathway is 14, a value comparing favourably with the 15 molecules of ATP produced by the complete oxidation of pyruvate via the TCA cycle. Hence, it seems likely that OGDH is not part of a classic TCA cycle, and cannot therefore be used as an indicator of $J_{max}$ through the TCA cycle, and may possess other control properties. If it can be assumed that the $V_{max}$ gives an indication of $J_{max}$ through the OGDH reaction, it may give an indication of $J_{max}$ through the proline oxidation pathway. Indeed, OGDH may be a better indicator of flux through proline oxidation than proline dehydrogenase, which has already been suggested for this role. The $V_{max}$ of proline dehydrogenase is 40 $\mu$mol. $min^{-1}.g^{-1}$ (Crabtree & Newsholme, 1970) and the $V_{max}$ of OGDH is 26.4 $\mu$mol.$min^{-1}$ $.g^{-1}$. Thus the $V_{max}$ of OGDH is more likely to represent the maximum flux through the pathway, as the residues must pass through this reaction. In addition, a calculation made by Crabtree & Newsholme (1975) p.450, from the oxygen uptake from another small dipteran fly gave rise to a value of 25 $\mu$mol. $min^{-1}.g^{-1}$ at 25$^{o}$C for flux through the proline oxidation pathway of the tsetse fly, and this is nearer to the $V_{max}$ of OGDH than that of proline dehydrogenase.

From this index, it can be seen that the flux through the 'Bursell' pathway can provide a rate of ATP generation similar to that in other small flying insects. In the fleshfly, a slightly larger Dipteran fly, the $J_{max}$ through the TCA cycle is 32.8 $\mu$mol.$min^{-1}.g^{-1}$.

The classic TCA cycle does not appear to be entirely lacking in the tsetse fly, however. Unfortunately, the $V_{max}$ of PDH may not provide a good indicator of the $J_{max}$ from carbohydrate into the TCA cycle in insects (see Chapter Five). However, maximum activities of HK, phosphorylase, CS and total ICDH have been measured, and found to be 2.3, 1.8, 74.0 and 29.0 $\mu$mol.min$^{-1}$.g$^{-1}$ respectively. If the flux from total carbohydrate (i.e. $V_{max}$ of HK plus $V_{max}$ of phosphorylase multiplied by 2: 8.2 $\mu$mol $C_2/C_3$.min$^{-1}$.g$^{-1}$) is assumed to be oxidised via the TCA cycle, then this may represent the maximum flux through the first span of the cycle. The $V_{max}$ of neither CS nor total ICDH can give a quantitative index of the first span of the cycle, but these values are much lower than in other insects, especially in other Dipterans; e.g. in the blowfly, Calliphora vomitoria, the $V_{max}$ values of CS and total ICDH are 418.0 and 177.0 mol.min$^{-1}$.g$^{-1}$ respectively.

Although the concentration of proline is high in the haemolymph of the tsetse fly (170 mM, as opposed to 7 mM in the house fly haemolymph; see footnote, Bursell, 1963; Price, 1961) it is depleted during the first two minutes of flight and the flies can fly for several minutes longer (Hargrove, 1976). This suggests that either the tsetse flies can utilise an alternative, stored fuel or that they can synthesise proline almost as fast as they can utilise it, at least for a period of minutes. Lipid is stored in the fat body of tsetse flies, but McCabe & Bursell (1975) show that the flight muscles are apparently unable to oxidise fatty acids. (Unfortunately, DGL, TGL and CPT $V_{max}$ values are not available for the tsetse fly flight muscle). There is evidence that the lipids are used instead to provide carbon for proline synthesis in the fat body (McCabe & Bursell, 1975). Other amino acids, presumably derived from the blood meal, contribute to supply the necessary ammonium ions and oxaloacetate

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Footnote: The concentration of proline in the tsetse fly haemolymph has been calculated from Bursell (1963), assuming that his data in $\mu$g. l$^{-1}$ refer to $\mu$g proline.

required for the proposed pathway of proline synthesis, but alanine probably plays a major role as this is the product of proline oxidation by the flight muscles. It is not known whether the rate of proline formation from fat and amino acids in the fat body is sufficiently rapid to contribute to fuel utilisation by the muscles during prolonged flight, but this possibility has been raised by Bursell (1977).

Further evidence that lipids are not important as a fuel for flight in the tsetse fly derives from the fact that glycerol kinase activity is very low (Crabtree & Newsholme, 1972b) and this may correlate with a low capacity for fatty acid oxidation. In addition, the diglyceride concentration in the haemolymph is very low (McCabe & Bursell, 1975) and diglyceride is believed the major lipid fuel present in the haemolymph of fat-utilising insects (see above).

Poplar hawk moth

Zebe (1954) measured the R.Q.s of various moths in flight and found them to be near to 0.7, thus indicating the use of fat. However, in many moths, $V_{max}$ values of HK and PFK are high, and suggest that carbohydrate may be an important fuel. This is not surprising since many moths feed in their adult life on nectar, as do other Lepidopterans (Johnson, 1976; Van Handel & Nayar, 1972). In fact, nectar, like honey, is a more concentrated form of carbohydrate than glycogen (i.e. values of $mg.cal^{-I}$ are 0.11, 0.33, 0.67 and 0.88 for fat, honey, nectar and glycogen respectively: Weis-Fogh, 1967b). If nectar can be retained in the gut, it could be considered as a form of storage fuel.

A survey of the maximum activities of HK and PFK in different moths shows that those moths which do not feed in the adult stage (primarily members of the families Arctiidae and Notodontidae, but also the silk moth, Bombix mori) have low activities of HK ($V_{max}$ in the region of 5 $\mu$mol $C_6.min^{-1}.g^{-1}$) while

those that do feed in the adult stage (i.e. members of the families Sphingidae, Noctuidae and Geometridae) have high activities of HK ($V_{max}$ in the region of 20 to 60 $\mu$mol $C_6.min^{-1}.g^{-1}$) (Crabtree & Newsholme, 1975, p. 447 and B. Surholt, personal communication).

The moths that have been investigated in the present survey all belong to families which feed in the adult stage i.e. the silver Y moth and yellow underwing in Noctuidae, and the poplar hawk moth in Sphingidae. However, the poplar hawk moth appears to be an exception. From four species of Sphingids that have been investigated, only the poplar hawk moth has low activities of HK and PFK (3.2 and 9.0 $\mu$mol $C_6.min^{-1}.g^{-1}$ respectively, see Table III), compared with a $V_{max}$ of OGDH of 42.7 $\mu$mol $C_3/C_2.min^{-1}.g^{-1}$. Apprently, this moth has a microscopic proboscis, and does not appear to be able to feed in the adult stage, unlike other Sphingids (B. Surholt, personall communication). Thus it seems likely that the poplar hawk moth may use fat as a fuel for flight, as this could be laid down in the fat body during the larval and pupal stages of the moth.

Comparison of the $V_{max}$ values of DGL and OGDH in this insect show that diglyceride may only support 8% of the $J_{max}$ of the TCA cycle, and hence the maximum energy demands of flight. Total carbohydrate can support only 42% (see above). This raises a problem about how this insect can support flight.

Proline levels have not been measured in the haemolymph of the poplar hawk moth but proline could be used to supplement carbohydrate and fat utilisation during flight (see later). However, $V_{max}$ of proline dehydrogenase is 2.4 $\mu$mol.$min^{-1}.g^{-1}$ and this is too low to suggest a major contribution from proline as a supplementary fuel.

Another possible process which might supply fuel for flight is gluconeogenesis. However, it is not known whether the process of gluconeogenesis is rapid enough during flight to contribute a significant amount of glucose for

flight (see above). One possible explanation for the discrepancy between the rates of utilisation of available fuels and the $J_{max}$ of the TCA cycle in the poplar hawk moth may be that the activities of CPT and DGL may vary during the life of the insect. This has been suggested before, in the case of the African locust, and could be related to changes in full flying competence (see above).

Johnson (1976) suggests that a variety of factors such as dietary history, age, migratory status and continual use of flight muscles may affect full flying competence. The poplar hawk moth is a poor flier and does not live long after egg laying (B. Surholt, personal communication). It is possible that the full capacity for flight in this moth is only present immediately after hatching, and is important in dispersal before egg laying; the activities of DGL and CPT may decrease after this period. The $V_{max}$ of OGDH was measured immediately after hatching, and before egg laying. If the DGL and CPT activities were measured later in the life of the moth, then it may have lost its full flying competence. (This occurs in the silver Y moth; see Johnson, 1976). This raises a general problem in insects, when comparing the estimates of rates of fuel utilising pathways, which may contribute to the maximum rate of the TCA cycle. If full flying competence does vary with factors in the wild, then it may be difficult to control these. Ideally, activities of enzymes to be compared should be measured from the same insects, caught in the wild. In laboratory raised insects, every effort should be made to standardise such conditions as diet and crowding, as these may influence full flying competence (Johnson, 1976), and hence the $V_{max}$ of some enzymes from fuel-utilising pathways.

Giant waterbug

This insect is unusual in that no trace of a fat body has been found,

but large fat droplets of neutral lipid have been demonstrated in the flight muscle by electron microscopy (Ashurst & Luke, 1968). From a comparison of the activities of DGL, CPT and OGDH, it appears that the giant waterbug can support all the energy demands of flight from lipid. However, the activity of TGL is low and although the nature of the neutral lipid was not established by Ashurst & Luke (1968) nor by Crabtree & Newsholme (1975), (who measured the concentration of glyceride in a variety of flight muscles), the stored lipid is more likely to be triglyceride. This is because triglyceride is more hydrophobic than diglyceride, and this is the property which confers that advantages that fat has over carbohydrate as a storage fuel.) However, until the nature of the storage lipid in the flight muscles of this insect is established, or until the control properties of TGL are more fully understood, it is difficult to say which is the major fat fuel for flight in this insect. It is known, however, that although the waterbug is mainly aquatic, it is a strong flier, and flight is important in dispersal (J. Ashe, personal communication). Under these circumstances, fat may be an important fuel as suggested in Chapter One.

Johnson (1976) suggests that some Heteropterans (the sub-order of Hemipterans which contain the giant waterbugs) lose the ability to fly, and the muscles histolyse. This could be due to being kept in water tanks for several months, without the use of their wings, or it could be due to the diet. However, Lethocerus cordofanus, unlike other species of African waterbug, does not lose the ability to fly when kept in activity (J. Ashe, personal communication).

Discussion on insect fuels

At the beginning of this discussion, it was suggested that many insects, other than those discussed separately above, could support the $J_{max}$ through

the TCA cycle, i.e. the maximum energy demands of flight from carbohydrate. During sustained flight HK gives a better indication of the contribution of carbohydrate to flight, as the involvement of glycogen oxidation is more likely to occur at the initiation of flight. This is because muscle glycogen is easily mobilised, and the reserves are low. Reference has already been made to the rapid decrease in muscle glycogen during flight in the blowfly (see above). Presumably, when the energy stores in muscle are exhausted, fuels must be mobilised from the fat body and/or the gut. However, the reserves of glycogen in the fat body are also limited, due to the problem of weight. Although trehalose concentrations in the haemolymph are high (approximately 60 mM) it must be realised that for sustained flight, this carbohydrate would also be depleted, after fat body glycogen had been exhausted. It is suggested that insects utilising carbohydrate could maximise their store by maintaining high concentrations of trehalose in the haemolymph, storing nectar in their guts, and even perhaps undergoing gluconeogenesis during flight. Many carbohydrate utilising insects, however, are not capable of sustained flight, and need to feed frequently.

The question arises as to why insects utilise such a variety of fuels for flight, i.e. glycogen, trehalose, diglyceride and proline. The answer may lie in different solutions to the problems of storage, mobilisation, transport, supply to the muscles and uptake by the muscles. The advantage of fat as a storage fuel in flying animals has already been stressed. However, the transport of such a fuel in the haemolymph is a problem. In insects, high concentrations of substrates must be maintained in the haemolymph (see below). High concentrations of free fatty acids are toxic, unless bound to protein, and high concentrations of protein may produce an adverse osmotic balance. In insects, transport of fat as diglyceride may represent the solution to this problem, as diglyceride is more hydrophilic than triglyceride.

The high concentrations of substrates in the haemolymph may reflect the difficulty of distributing fuels to the muscles; the 'open blood system' of invertebrates with the haemolymph bathing all the organs of the body is not likely to be as efficient as the 'closed blood system' of the vertebrates, and high concentrations of substrates may facilitate diffusion or uptake into the muscle fibres. The efficiency of the tracheolar system, however, in distributing oxygen to the flight muscles is high enough to provide adequate oxygen to meet the demands of flight (Weis-Fogh, 1967a). Thus, providing enough fuels can be supplied to the muscles, they will be able to oxidise them completely via the TCA cycle.

Why proline should be used as a fuel is difficult to explain. Other insects, such as the roaches and beetles have high activities of proline dehydrogenase (Crabtree & Newsholme, 1970) and so proline utilisation may be more widespread than originally thought. Sacktor (1976) suggests that one reason for the use of proline as a fuel might be that, since there is no net charge on the proline molecule, uptake of proline into muscle cells and mitochondria could be facilitated. There may also be a connection with the diet, e,g. the blood meal in the tsetse fly, although proline does not predominate amongst the amino acids of haemoglobin. It would be interesting to compare proline levels in the haemolymph and proline synthesis in the fat body in other flying carnivorous insects such as Dytiscus (the great water beetle) with other possible proline utilising insects, such as the cockroach.

Vertebrates: Fish

Fish are unlike higher vertebrates in having only two major types of muscle: the white muscle forms the main muscle mass while the lateral strip of red muscle, forming only 8% of the total weight, is the muscle which is used in continual swimming activity (Bone, 1966; Webb, 1971). This lateral

muscle has been described as containing high levels of mitochondria and lipid droplets (Lin et al., 1974) and high activities of cytochrome oxidase and lipolytic enzymes (Bone, 1966). It has always been deduced that such characteristics correlate with a high aerobic capacity, but even the enzymes whose $V_{max}$ values were measured are believed to catalyse reactions at equilibrium (see later discussion in Chapter Five) and, like the other characteristics, are only qualitative indications of aerobic capacity. However, on the basis of the work reported in this thesis it is now possible to estimate with reasonable accuracy the $J_{max}$ through the TCA cycle in the red and white muscles of fish (see Chapter Five). In addition, comparisons with activities of other enzymes of fuel utilising pathways can give an indication of the contribution of different fuels.

In the present study, two fish have been investigated: one a teleost, the trout, which is an active swimming fish, and the other an elasmobranch, the dogfish, which may spend much time in the substratum (Bone, 1966) and does not possess the streamlining (as an adaptation to fast swimming) that the trout does. The storage of lipid is also different in these two fish. Elasmobranches store most of their triglyceride in the liver, while teleosts store most of their triglycerides in an abdominal adipose tissue, and/or in adipocytes which are distributed throughout the muscle fibres (Love, 1970).

The $V_{max}$ values of PDH and OGDH in heart and red muscle from both these fish are very similar (2 to 3 $\mu$mol.min$^{-1}$.g$^{-1}$), which is surprising considering the different behaviour of the two fish. Presumably, the maximum capacity for energy production must always be present, whether or not it is used for long periods of time. Comparison of the activities of other fuel utilising enzymes with the $J_{max}$ through the TCA cycle shows that carbohydrate oxidation could support maximum energy requirements in both types of muscle, in both

fish. However, carbohydrate storage is limited compared with fat storage, and this would limit the use of carbohydrate as a fuel during sustained exercise.

In the dogfish, both CPT and TGL activities are very low: comparison of the $V_{max}$ values of CPT and OGDH suggest that fatty acids can support less than 4% of the $J_{max}$ of the TCA cycle. This suggests that fatty acids are not an important fuel for swimming in this fish. Indeed, in recent work on the fuel utilisation of elasmobranchs and teleosts, Zammit & Newsholme (1979) show that ketone bodies are an important fuel in the dogfish, in common with other elasmobranchs.

In the trout, comparison of the activities of CPT with OGDH suggest that fatty acid oxidation could support 44% of the $J_{max}$ of the TCA cycle in the red muscle. However, in the red muscle of the mackerel, another similar teleost, activities of CPT suggest that a $J_{max}$ through the TCA cycle of 2.4 $\mu$mol.min$^{-1}$.g$^{-1}$ could be supported entirely from fatty acids (Zammit & Newsholme, 1979). It is possible that trout reared in trout hatcheries lose the capacity for maximum rates of fatty acid oxidation (Webb, 1971). However, if fatty acid oxidation cannot supply the maximum energy demands in the trout, then gluconeogenesis could supplement the fuel utilisation as suggested before for insects. The substrates glycerol and lactate would be available for gluconeogenesis: glycerol is released from the abdominal adipose tissue during mobilisation of triglycerides (Zammit & Newsholme, 1979) and lactate may be produced from the white muscle during vigorous activity. Webb (1971) noticed that at very high speeds, white muscle was also involved in the swimming activity of the trout. The specialisation of white muscles for the use of anaerobic glycolysis is discussed in Chapter Five.

In addition, another possible source of gluconeogenic precursors is amino acids from body muscle. To quote from Bone (1966): "For the fish, it

is but a light penalty to carry about a large mass of normally inactive muscle; not so for the terrestrial animal". This may suggest that in fish, the body muscle could be spared for use in gluconeogenesis during exercise. However, it is not known whether gluconeogenesis would be rapid enough during maximum exercise to supplement fuel utilisation in this way (see later).

Amphibia

Unfortunately, no activities of fat utilising enzymes are available for the frog or African clawed toad. The $J_{max}$ of the TCA cycle in the sartorius muscle of the frog is very low (0.1 $\mu$mol.min$^{-1}$.g$^{-1}$) and this gives quantitative confirmation of earlier suppositions that the muscle had little capacity for aerobic metabolism. Indeed, Helmreich et al. (1965) refer to this muscle as anaerobic. The importance of anaerobic glycolysis in this muscle is discussed in Chapter Five. Nevertheless, it appears that the low $J_{max}$ of the TCA cycle in this muscle could be supported by glucose utilisation.

Birds

In migratory flight, R.Q. values for birds range from 0.7 to 0.8 suggesting that fat is a major fuel source. Fat amounting to 20 to 50% of the body weight may be used up during migration (Tucker, 1972). Pigeon pectoral muscle has been shown to contain large numbers of mitochondria and lipid droplets (Ashurst, 1969; George & Jyoti, 1955; and Chapter One, p. 21). Thus, the cumulative evidence would suggest that fat is an important fuel in pigeons. Considerations of weight are important in flying animals, especially in migration (Odum, 1965; Weis-Fogh, 1952) and therefore birds would be expected to store fat rather than carbohydrate.

Comparisons of enzyme activities from Table III show that in pigeon pectoral muscle, muscle triglycerides could support 53% of maximum energy requirements and fatty acid oxidation could entirely support the maximum

energy demands of flight. Thus, we have quantitative data on the importance of fat utilisation in the pigeon pectoral muscle. This is a particularly important finding, since the amount of carbohydrate stored must be very limited in these animals; weight versus lift is crucial in the power output of flying animals (Weis-Fogh, 1952). In addition, it is unlikely that gluconeogenesis from body protein would occur, since there is little excess protein: the pectoral muscle forms 20% of the body weight and this is essential for flight.

The two other birds investigated are the chicken (a domesticated Galliform) and the pleasant (also a member of the Galliformes). These game birds (Galliformes) are mainly ground-living birds and possess only a limited capacity for flight, which is restricted to flapping into the air and gliding down to cover (Welty, 1955, and see Chapter One, p. 23).

The pectoral muscle of the chicken is white and that of the pleasant appears pink. The reason for this difference in pigmentation is not clear, though the redness of muscle does not necessarily correlate with aerobic capacity (see Crabtree & Newsholme, 1975, pp. 426-427 and Chapter One, pp. 16-23). The $J_{max}$ of the TCA cycle in the pectoral muscle of the chicken is very low ($\lesssim 0.1$ $\mu$mol. $min^{-1}.g^{-1}$) and is the first quantitative indication of the lack of aerobic capacity of this muscle. (The importance of anaerobic energy production in this muscle is introduced at the end of this Chapter, and discussed in Chapter Five.) Somewhat surprisingly, the $J_{max}$ of the TCA cycle is higher in the pheasant pectoral muscle (1.5 $\mu$mol. $min^{-1}.g^{-1}$) The difference between the chicken and pheasant pectoral muscles may be due to the domestication of the chicken. Pennycuick (1975) suggests that the power of flight may be lost during domestication by selection for characteristics inconsistent with the maintenance of the capacity for flight in the chicken. However, the reason for the loss of flight in the chicken is not precisely known, and the

significance of the occurrence of a higher rate of the TCA cycle in pheasant pectoral muscle is not clear.

Mammalian skeletal muscle

Mammalian skeletal muscle usually contains a mixture of red and white fibre types. The muscles investigated in the present study include the rat soleus (predominantly red), the rabbit semitendinosus (predominantly red) and the rabbit adductor longus (predominantly white). The $J_{max}$ of the TCA cycle in the rabbit adductor longus muscle is very low, and this supports previous assumptions that mammalian white skeletal muscle is mainly anaerobic (see Needham, 1971, pp. 451-483).

TGL and CPT activities are available for the rabbit muscles, and for the rat quadriceps. The deeper fibres of the rat quadriceps are more red than the outer fibres, and may have a similar metabolism to the rat soleus (Rennie & Holloszy, 1977). If the assumption is made that the deeper fibres of the quadriceps were used for the assay of HK, TGL and CPT, then these activities can be compared with those of PDH and OGDH in the soleus muscle. From this comparison, it appears that muscle triglyceride could support 8% of the $J_{max}$ of the TCA cycle in the rat soleus. More importantly, fatty acids (that is, including exogenous fatty acids) could entirely support the maximum $J_{max}$ of the TCA cycle in the soleus muscle.

The contributions of triglyceride and fatty acids to the $J_{max}$ of the TCA cycle in the rabbit adductor longus are 28.5 and 100% respectively. However, this muscle is believed to rely mainly on anaerobic energy production during contraction (see Chapter Five). It is possible that fats may be utilised by white muscles at rest, since this would help to conserve carbohydrate fuels. This process would be important during starvation to conserve carbohydrate for glucose-dependent tissues such as the brain. (Fatty acid oxidation inhibits PDH and glycolysis, and this process of the sparing of carbohydrate, originally called the glucose-fatty acid cycle, has been referred to several times in Chapter One).

In the rabbit semitendinosus, triglyceride and fatty acids can contribute 70% and 100% respectively to the $J_{max}$ of the TCA cycle. Thus, in this red muscle also, fatty acids could entirely support maximum aerobic energy demands.

The relative contributions of these fat fuels to muscle during exercise depends on a variety of factors. In insects, it was suggested that muscle energy stores were easily mobilised, but rapidly depleted so that fuels would then have to be mobilised from the fat body. This may also apply to vertebrate muscles, with the liver serving a similar function to that of the fat body in insects. In Chapter One, Table IV gives the relative contributions of glucose, fatty acids and endogenous fuels to the oxygen uptake of the leg muscles of exercising man (taken from Felig & Wahren, 1975). It can be seen that the relative importance of free fatty acids as a fuel for exercise increases with the endurance of the exercise, while that of blood glucose stays relatively constant. The importance of endogneous triglyceride in muscle is not clear, but is believed to contribute to exercise only during the initial stages and may be more important in starvation (Essen, 1977; Masoro, 1977).

Another possible source of fuel during exercise may be provided by the process of gluconeogenesis. This has already been postulated in the insects as a possible way of providing supplementary fuels during flight. Indeed, there is some data on the importance of this process in exercise in man. Wahren (1977) shows that, although the relative contribution of gluconeogenesis to liver glucose production decreases in the resting state from 25% to 16% after 40 minutes of mild exercise, during prolonged mild exercise there is a 3-fold rise in the absolute rate of gluconeogenesis, and the relative contribution to liver glucose output rises to 45%. The importance of gluconeogenesis during prolonged exercise is stressed by the fact that in four hours of exercise, there is a 75% depletion of total liver glycogen stores, so that in the marathon run, for example, gluconeogenesis may have to supplement the glucose supply (Wahren, 1977).

Mammalian hearts

From the activity of TGL compared with $J_{max}$ through the TCA cycle, triglycerides could support only 20% of the maximum energy requirements. Fatty acid oxidation, on the other hand, could entirely support the maximum rate of the TCA cycle, as has been shown for other vertebrate red muscles.

Hearts belong to the class of muscle types referred to in Chapter One as group a: continuously contracting muscles (p. 19). Therefore it is important that fuel is continually supplied to the muscle. Hearts have a good blood supply and therefore should be adequately supplied with oxygen, blood glucose and fatty acids when these fuels are available. However, at the onset of a sudden increased work load, oxygen and exogenous fuels may be temporarily limiting. Under these conditions, endogenous glycogen may be utilised and converted to lactate (see Chapter One, p. 20). However, this process is likely to be used only in emergencies. The capacity of hearts for anaerobic glycolysis is discussed in Chapter Five. Normally, however, the heart will take up lactate, and a recent preparation of the working, perfused heart could operate for longer than an hour with only 10 mM-lactate and 1 mM-pyruvate in the perfusate (Taegtmeyer et al., 1979).

Endogenous triglyceride is not thought to be an important energy source in hearts. It appears to be oxidised by isolated rat hearts, perfused by the Langendorff technique, in the absence of other fuels in the perfusate (Denton & Randle,1967). Perfused rat hearts can also be induced to utilise endogenous triglyceride under other circumstances e.g. an increased work load (Crass et al. 1971) and addition of catecholamines to the perfusate (Crass, 1977) but the importance in vivo of this endogenous fuel in hearts is not known.

In summary, fats can support maximum aerobic energy requirements in hearts and red muscles of vertebrates, and in the insect, the giant waterbug. It is

a major fuel in migratory insects and birds. Carbohydrate can also support maximum rates of the TCA cycle, but the point is made that carbohydrate stores may be limited and therefore will restrict the duration of the exercise. In many other muscles, supplementary fuels may be used during sustained exercise, e.g. proline in insects, ketone bodies in elasmobranchs. The proposal has been made that gluconeogenesis may help support the maximum demand for fuels in some muscles during continual activity. Evidence for this has been given in man.

2) Comparison of lactate dehydrogenase (LDH) with 2-oxoglutarate dehydrogenase (OGDH) activities in vertebrate white muscles and insect flight muscles

Lactate dehydrogenase (LDH) is necessary for anaerobic glycolysis in muscles to regenerate the $NAD^+$ required from the NADH produced. In insect flight muscles, the activity of LDH is very low, and as the enzyme is believed to catalyse an equilibrium reaction (see Crabtree & Newsholme, 1972a), the flux through the reaction will be even less than the $V_{max}$ *in vitro*. This suggests that anaerobic glycolysis may be unimportant in insect flight muscle.

As the $V_{max}$ of OGDH can be used as an index of $J_{max}$ through the TCA cycle, and hence of the aerobic capacity of a muscle, the values of $V_{max}$ of OGDH and $V_{max}$ of LDH are presented in Table IV in insect flight muscle and vertebrate white muscle, for comparison.

It is interesting to note that insect flight muscles have a very high rate of aerobic metabolism, and low LDH activities, while vertebrate white muscles have a very low rate of aerobic metabolism and high LDH activities. The importance of anaerobic metabolism in muscles will be discussed in the next Chapter.

TABLE IV

MAXIMUM ACTIVITIES OF 2-OXOGLUTARATE DEHYDROGENASE AND LACTATE DEHYDROGENASE IN INSECT FLIGHT MUSCLES AND VERTEBRATE WHITE MUSCLES

The maximum activities ($V_{max}$) of OGDH are taken from Table II. The $V_{max}$ of lactate dehydrogenase are taken from Crabtree & Newsholme, (1972a).

| TYPE OF MUSCLE | MAXIMUM ENZYME ACTIVITIES ($\mu mol.min^{-1}.g^{-1}$) | |
|---|---|---|
| | OGDH | LACTATE DEHYDROGENASE |
| Insect flight muscles: | | |
| African locust | 44.2 | 1.6 |
| desert locust | 20.8 | 2.9 |
| cockroach | 48.6 | 1.5 |
| giant waterbug | 15.2 | 1.3 |
| chafer | 57.5* | 4.4+ |
| common wasp | 71.2 | 1.5 |
| bumble-bee | 45.9 | 1.8 |
| poplar hawk moth | 42.7 | 3.2 |
| tsetse fly | 26.4 | 1.5 |
| fleshfly | 32.8 | 1.7 |
| Vertebrate white muscles: | | |
| dogfish white | $<0.1$ | 330.0 |
| frog sartorius | 0.1 | 398.0 |
| chicken pectoral | $<0.1$ | 870.0 |
| rabbit adductor longus | 0.7 | 311.0 |

*This value was taken from the summer chafer (Amphimallon solstitialis).

+This value was taken from the cockchafer (Melolontha melolontha).

# CHAPTER FIVE

# GENERAL DISCUSSION

## INTRODUCTION

In an attempt to find a more quantitative index of aerobic metabolism in muscle (i.e. TCA cycle flux) which not only was more precise than the subjective criterion of 'redness', but could also be applied across the animal kingdom, a search for a rate-indicating enzyme of the TCA cycle was instigated. Studies on the enzymes of the TCA cycle have revealed several enzymes catalysing non-equilibrium reactions: PDH, CS, NAD-ICDH and OGDH. Since these enzymes may also catalyse flux generating steps, they are candidates for the role of indicators of flux through the TCA cycle. Comparison of $V_{max}$ values with estimated $J_{max}$ derived from oxygen uptake data eliminated CS and NAD-ICDH (see Alp et al., 1976, Chapter One, pp. 58-64, and Chapter Four). The remaining enzymes, PDH and OGDH were shown to be satisfactory indicators of flux through the TCA cycle and pyruvate oxidation respectively (see Chapter Four). A comparative survey of the $V_{max}$ values of these enzymes was therefore carried out and the results provide a framework of knowledge about the rates of aerobic metabolism in these muscles. This knowledge can be used in conjunction with evidence of maximum rates of other fuel utilising pathways to discuss the relationship between the metabolism and the physiology in different muscles. Discussion in Chapter Four dealt mainly with the contribution of fuels to the TCA cycle in muscles. In this Chapter, the importance of pyruvate oxidation and the TCA cycle in different muscles, the relative importance of aerobic versus anaerobic metabolism in muscles and the use to which an index of $J_{max}$ through the TCA cycle can be put will be discussed.

The importance of the TCA cycle and pyruvate oxidation in muscle

As the $V_{max}$ values of OGDH agree well with estimates of maximum fluxes through the TCA cycle in both vertebrate and insect muscle, it can be used as an index across the whole animal kingdom. On this basis, the muscles investigated can be divided into four groups: group 1 includes muscles whose $V_{max}$ of OGDH is greater than 20 $\mu$mol.min$^{-1}$.g$^{-1}$ at 25$^{o}$C; group 2, muscles whose $V_{max}$ of OGDH lies between 5 and 20 $\mu$mol.min$^{-1}$.g$^{-1}$; group 3, muscles whose $V_{max}$ of OGDH lies between 1 and 5 $\mu$mol. min$^{-1}$.g$^{-1}$ and group 4, whose $V_{max}$ of OGDH lies below 1 $\mu$mol.min$^{-1}$.g$^{-1}$. The arrangement of these groups is shown in Table I. It can be seen that group 1 comprises insect flight muscles only. This confirms the reports that this muscle is highly aerobic, and has a high power output (see Sacktor, 1970, for review). Weis-Fogh (1961, 1967b) shows that the maximum power output of insect flight muscle is 10-fold greater than that of vertebrate muscle. This is borne out by the present study, where the highest $V_{max}$ of OGDH in insect flight muscle (71.5 in wasp flight muscle) is approximately 8-fold higher than the highest $V_{max}$ of OGDH in vertebrate muscle (9.0 in rabbit heart), and approximately 20-fold higher than the highest $V_{max}$ of OGDH in vertebrate skeletal muscle (3.2 in pigeon pectoral muscle).

In Chapter One, pp. 16-23, a classification of muscles was given, based on their physiological function; the flight muscles of insects correspond to the second group of muscles, group (b). That is, they are used from time to time, for long periods of contraction. Other examples of this type of muscle, given in Chapter One, include pigeon pectoral muscle. However, the $J_{max}$ of the TCA cycle in insect flight muscle is much higher than that of the pigeon pectoral muscle. Thus, although both animals fly for long periods of time, and are known to lay down extensive stores of fat for these flights (see Chapters One and Four) the power output of insect flight muscle is exceptionally high. This may be related to the size of the insect. Many metabolic processes

TABLE I

GROUPS OF MUSCLES CLASSIFIED ACCORDING TO THEIR $V_{max}$ OF 2-OXOGLUTARATE DEHYDROGENASE

$V_{max}$ values of OGDH are expressed in $\mu mol.min^{-1}.g^{-1}$.

| GROUP 1<br>>20 | GROUP 2<br>5 to 20 | GROUP 3<br>1 to 5 | GROUP 4<br><1 |
|---|---|---|---|
| flight muscles of:<br>silver Y moth<br>yellow underwing moth<br>poplar hawk moth<br>wasps (both spp.)<br>honey-bee<br>bumble-bee<br>dung beetle<br>rosechafer<br>summer chafer<br>cockroach<br>locusts (both spp.)<br>blowfly<br>fleshfly<br>housefly<br>tsetse fly | flight muscles of:<br>giant diving beetle<br>giant water bug<br>whelk radular retractor<br>hearts of:<br>rat<br>rabbit<br>mouse<br>sheep<br>chicken | hearts of:<br>pigeon<br>cow<br>pig<br>dogfish<br>trout<br>frog<br>toad<br>radular retractor of:<br>limpet<br>winkle<br>red muscles of:<br>dogfish<br>trout<br>pectoral muscles of:<br>pheasant<br>pigeon<br>rat soleus muscle<br>rat diaphragm<br>human thigh muscle<br>rabbit semitendinosus<br>toad sartorius | frog sartorius<br>frog gastrocnemius<br>toad gastrocnemius<br>white muscles of:<br>dogfish<br>trout<br>rabbit adductor longus<br>chicken pectoral muscle |

and other physiological variables, when plotted on a logarithmic scale, are linearly proportional to body weight (Schmidt-Nielsen, 1975, pp. 253-256). These different scale effects related to the size of an animal are discussed by Hill (1950).

Group 2 includes some vertebrate hearts, the whelk radula retractor, and the flight muscles of the giant diving beetle (Dytiscus) and the giant water-bug. The latter two insects are mainly aquatic, but they are also good fliers, and flight is important for dispersal in these insects. The problem was raised in Chapter Four that the rate of fuel utilisation for full flying competence may depend on such factors as starvation or overcrowding. It is also known that some waterbugs, when kept confined in water tanks for several months, may lost the capacity for flight (Johnson, 1976). It is possible that, since in the present work both these insects had been kept in captivity in water tanks for more than a month and continually fed, that the $J_{max}$ through the TCA cycle was lower than when these insects possess full flying competence. Both insects could be made to fly in the laboratory but they may not have possessed the full potential power output. If this is so, then this could be the reason for the lower $J_{max}$ in these insects, than those in group 1.

Vertebrate hearts and the whelk radular muscle are both muscles which are used continually (or more or less continuously, in the case of the whelk). These correspond to the first group of muscles, group (a), arranged according to physiological function, described in Chapter One, pp. 16-23. These muscles are those which contract continually and serve a vital function such as maintaining posture, or pumping blood around the body, or feeding. During continual contraction, muscles require a continual supply of fuel, and in vertebrates, this occurs via the blood system which also supplies oxygen. Thus, provided a blood supply is available, aerobic metabolism of the fuels is possible, and from the higher ATP production per mol of substrate shown in

Table I, Chapter One, is energetically desirable. The whelk is large for a Gastropod (20 g weight as opposed to the limpet which weighs about 7 g) and needs a large intake of food to supply its energy requirements. As a carrion feeder, it possesses a sharp, rasping radula (feeding organ) and a large retractor muscle to operate it (Owen, 1966). The muscle is very red in colour, reflecting the high concentration of myoglobin in the muscle (Alyakrinskaya, 1974). Although myoglobin concentration does not necessarily correlate with aerobic capacity of a muscle (see Crabtree & Newsholme, 1975, for discussion) molluscs have an open blood system (haemocoele) which may not be as efficient as the closed blood system of vertebrates, and thus some facilitation of diffusion of oxygen may be required for this aerobic muscle. This example shows that highly aerobic muscle may occur in invertebrates other than insect flight muscles.

Within the vertebrate hearts, only the birds and mammals have a complete double circulation of blood, i.e. a four chambered heart. Fish possess a two-chambered heart, and amphibia a three-chambered heart. The evolution of the double circulation allows a higher peripheral blood pressure to be developed by the heart. Thus, the power output of the hearts of birds and mammals may have to be greater to maintain a high blood pressure to overcome the resistance of the many blood vessels and capillaries. This difference is reflected in the present work, where the hearts of birds and mammals have a higher $V_{max}$ of OGDH (3.8 to 9.0) than the hearts of fish and amphibia (3.3 to 3.5). The larger mammals such as the sheep, pig and cow have lower $V_{max}$ of OGDH from their hearts than the smaller mammals. This may reflect the scale effects of smaller mammals, referred to above (see Hill, 1950). The 'basal metabolic rate', which is related to the power output of an animal, has been shown to be inversely proportional to the body weight in mammals (Brody *et al.*, 1934).

Group 3, apart from containing the hearts of other vertebrates, also contains vertebrate muscles which are considered predominaly 'red'. These may be ranked according to their $J_{max}$ of the TCA cycle in the following way: pigeon pectoral > rat soleus and rat diaphragm > dogfish red muscle > trout red muscle > pheasant pectoral muscle > toad sartorius. However, it must be pointed out that this represents the $J_{max}$ through the TCA cycle at 25°C, for the purposes of comparison, and that the $J_{max}$ *in vivo* will be different: the temperature *in vivo* will vary to 40°C in birds (Welty, 1955), 37°C in mammals to 15°C in fish and amphibia (see Appendix VII and VI). Some the muscles in this group fall into group (a) in Chapter One, on the basis of their physiological function, and some into group (b). That is, the trout and dogfish red muscle and the rat diaphragm can be regarded as continually contracting muscles, like the hearts described above, while the other muscles may be used in periods of exercise from time to time. It can be seen then that the classification of muscles according to physiological function, carried out in Chapter One, pp. 16-23, does not correlate exactly with the classification of muscles on the basis of the $J_{max}$ through the TCA cycle. This is partly because, although the physiological function of two muscles may be similar, their power output may vary depending on the size of the animal, and the amount and efficiency of supply of the fuels and oxygen.

It was suggested in Chapter One, p. 19, that the power output of hearts would be expected to be greater than that of diaphragms, in the same animal. In the rat, this is borne out by the evidence of the $J_{max}$ of the TCA cycle in these two muscles: $V_{max}$ values of OGDH are 6.8 and 2.4 $\mu$mol.min$^{-1}$.g$^{-1}$ in rat heart and diaphragm respectively.

It is interesting that the sartorius muscle of the African clawed toad (*Xenopus laevis*) comes into group 3 (see Table I). The name of this animal

is a misnomer, as it is classified as a frog (Frazer, 1973) but the animal is entirely aquatic, and is a powerful swimmer, as exemplified by the commotion these animals create when disturbed in their aquaria. Thus, the $V_{max}$ of OGDH of both muscles investigated, sartorius and gastrocnemius, in this animal are greater than those of the same muscles in the common British frog, Rana temporaria. It is to be assumed that Xenopus encounters higher temperatures than does Rana in the wild, and as poikilotherms cannot maintain a body temperature much different from that of their surroundings, the flux through the TCA cycle will be even greater in Xenopus muscles than those of Rana. It was noted by the author during dissection of these animals that the skeletal muscles of Xenopus appeared more 'red' than those of Rana. However, as mentioned before, 'redness' does not necessarily correlate with aerobic capacity. It is suggested that Xenopus muscles require a greater aerobic capacity to support continual swimming activity than the skeletal muscles of Rana, which may rely more on anaerobic energy production, to support the occasional bursts of contraction required in jumping (see later).

In group 4, some predominantly 'white' vertebrate muscles occur, together with other muscles that contain mixed fibre types, e.g. human thigh muscle (see Saltin et al., 1977). The specialisation of these vertebrate white muscles for a greater dependence on anaerobic energy production for exercise has already been suggested by a comparison of the their $V_{max}$ values of LDH (which are high) with those of OGDH (which are very low) in Chapter Four.

The evidence of the anaerobic nature of 'white' muscles has been discussed by Needham (1971). Historically the demonstration of low myoglobin content, few mitochondria, and low activities of cytochrome oxidase, was followed by the measurement of high activities of flux-generating glycolytic enzymes (i.e. phosphorylase and phosphofructokinase, Crabtree & Newsholme, 1972a). Low

activities of CS and total ICDH had also been found in white muscle (Alp et al., 1976) but these cannot be used as a quantitative index of the rate of the TCA cycle in these muscles. The use of the $V_{max}$ of OGDH as an index of $J_{max}$ of the TCA cycle provides the first quantitative confirmation of the low aerobic capacity of vertebrate white muscles. The rates of ATP turnover from aerobic and anaerobic pathways is compared in various muscles in Table III, and the relative importance of these processes is discussed later. It is interesting to note that although the pheasant and chicken pectoral muscles are mainly 'white', the $J_{max}$ of the TCA cycle is higher in pheasant pectoral muscle. This point is discussed in Chapter Four, p.152.

There is a continual spectrum between dogfish white muscle and pigeon pectoral muscle on the basis of the $J_{max}$ of the TCA cycle. The arbitrary grouping into groups 3 and 4 divides this spectrum from the 'more red' to the 'more white', but as pointed out in Chapter One, most muscles of higher vertebrates contain a mixture of fibre types, and the $V_{max}$ of OGDH of a whole muscle extract can only give an index of the overall aerobic capacity of the muscle.

Relationship between PDH and OGDH maximum activities

As pointed out in Chapter Four, the $V_{max}$ of PDH is primarily an index of the $J_{max}$ through pyruvate oxidation. On the other hand, the $V_{max}$ of OGDH gives an estimate of the $J_{max}$ through the TCA cycle regardless of the fuel type: the TCA is the final oxidation pathway for fatty acids, ketone bodies, carbohydrates and most amino acids. The $V_{max}$ also appears to give an index of the $J_{max}$ through proline oxidation in some insects. The relationship between the $V_{max}$ of PDH and the $V_{max}$ of OGDH could give an indication of the relative importance of carbohydrate as a source of fuel in muscle. The ratio of $V_{max}$(PDH)/$V_{max}$(OGDH) is presented in Table II.

In mammalian and other vertebrate hearts, and in vertebrate red muscles

TABLE II

THE RATIO OF THE MAXIMUM ACTIVITIES OF PYRUVATE DEHYDROGENASE TO THOSE OF 2-OXOGLUTARATE DEHYDROGENASE IN VERTEBRATE AND INVERTEBRATE MUSCLES

The $V_{max}$ values of PDH and OGDH are taken from Chapter Four, Table II.

| MUSCLE | RATIO OF $V_{max}$(PDH)/$V_{max}$(OGDH) |
|---|---|
| Mammalian hearts: | |
| rat | 1.01 |
| mouse | 1.35 |
| rabbit | 0.71 |
| guinea pig | 0.40 |
| sheep | 0.97 |
| pig | 1.08 |
| Other vertebrate hearts: | |
| dogfish | 0.77 |
| trout | 1.09 |
| frog | 1.38 |
| toad | 0.97 |
| chicken | 0.83 |
| pigeon | 1.59 |
| pheasant | 1.89 |
| Vertebrate red muscles: | |
| dogfish | 0.70 |
| trout | 1.80 |
| pigeon pectoral | 1.53 |
| rat soleus | 0.50 |
| rat diaphragm | 0.79 |
| rabbit semitendinosus | 0.56 |
| Vertebrate white muscles: | |
| trout | > 3.0 |
| frog sartorius | 4.0 |
| frog gastrocnemius | 7.0 |
| toad sartorius | 0.5 |
| toad gastrocnemius | 1.0 |
| chicken pectoral | > 2.0 |
| rabbit adductor longus | 0.57 |

TABLE II (cont'd.)

| MUSCLE | RATIO OF $V_{max}$(PDH)/$V_{max}$ (OGDH) |
|---|---|
| Molluscan radula retractors: | |
| whelk | 0.04 |
| limpet | 0.24 |
| periwinkle | 0.19 |
| Insect flight muscles: | |
| yellow underwing moth | 0.23 |
| silver Y moth | 0.03 |
| poplar hawk moth | 0.09 |
| wasp | 0.23 |
| queen bumblebee | 0.24 |
| rosechafer | 0.07 |
| African dung beetle | 0.02 |
| giant waterbug | 0.01 |
| cockroach | 0.30 |
| desert locust | 0.50 |
| African locust | 0.12 |
| blowfly | 0.31 |
| fleshfly | 0.45 |
| housefly | 0.45 |
| tsetse fly | 0.03 |

the ratio is approximately unity. Read et al. (1977) also measured this ratio in mammalian hearts, and found the same result. Thus, the maximum rate of operation of the TCA cycle in these muscles could theoretically be supported by carbohydrate utilisation alone. During rest and especially after a carbohydrate meal, the R.Q. of a whole animal approaches unity, indicating that the muscles are utilising carbohydrate (Newsholme, 1976b). However, as carbohydrate stores are limited, during continual exercise or after an overnight fast, the R.Q. approaches 0.7, indicating the use of lipid (Newsholme, 1976b). This overall picture of the metabolism of a whole animal is likely to be true for muscles also, as muscles constitute a high proportion of body weight, at least in vertebrates. Indeed, R.Q. values measured across the human arm, at rest and during exercise support the conclusion that fat may be used during exercise (Essen, 1977). However, the relative dependence of muscle on carbohydrate and lipid fuels is influenced by many factors, as well as starvation and exercise (for reviews on the subject, see Opie (1968) on the heart and various papers in Milvy (1977)).

In vertebrate white muscles, the ratio of $V_{max}$ (PDH)/$V_{max}$(OGDH) shows great variation. There may be several reasons for this. One is that the technical difficulties that arise in measuring low activities of OGDH and PDH in vertebrate white muscle may have introduced errors which are exaggerated when a ratio of activities is taken. However, in many vertebrate muscles, unlike the insect muscles, PDH activity is greater than that of OGDH. This raises the interesting speculation that the fate of acetyl CoA may not be exclusively oxidation via the TCA cycle. Although acetyl CoA is a precursor for fatty acid synthesis in adipose tissue and ketone body formation in the liver, this is unlikely to be the case for skeletal muscles. However, acetyl CoA plays an important general role as an acetyl group donor. In the kidney, N-acetyl glutamate, produced by the acetylation of glutamate, is an activator of the ornithine to citrulline reaction in the urea cycle. In nervous tissue,

acetyl groups are required for the synthesis of the neurotransmitter, acetylcholine, and high concentrations of N-acetyl glutamate and N-acetyl aspartate are found in these tissues. N-acetyl glutamate is a precursor of $\gamma$-amino butyric acid, an important inhibitory neurotransmitter in brain. It would be interesting to discover whether these acetylated amino acids are present at high concentrations in muscle. A more universal role of acetylation appears to be implicated in the binding of histones to chromosomes and the control of DNA transcription during the cell cycle (see Ord & Stocken, 1975, for review). This suggests that the acetylation of proteins may change their conformation, and in enzymes, may change their activity. Acetylation has been suggested as an alternative to phosphorylation for covalent modification in an interconversion cycle of enzymes (see p.77). An interconversion cycle confers sensitivity on the response of the activity of the enzyme to substrate concentration (see Chapter One, p. 32). However, the suggestion that acetyl CoA could be used as an acetyl donor for interconversion cycles in muscle is as yet speculative; alternative roles for acetyl CoA in muscle are not known. It is noteworthy in this context that the role of the coupling enzyme used in the assay of PDH i.e. arylamine acetyltransferase, occurring in high activities in pigeon liver, is not completely understood.

In molluscs and insects, the ratio of $V_{max}$(PDH)/$V_{max}$(OGDH) is very low; in most cases less than 0.5. This could suggest that carbohydrate was not as important a fuel in these muscles as in vertebrate muscles. However, there is evidence to the contrary in many insects, especially Hymenopterans and Dipterans (Weis-Fogh, 1952; Crabtree & Newsholme, 1972a; and see Chapter Four). Hexokinase activities are high enough to account for total dependence on trehalose for a period of flight. The low ratios in insects may therefore indicate the fact that the maximum activity of PDH is not being measured. It was suggested

in Chapter One that the control properties of insect PDH were less well understood than those of vertebrate PDH, and that less confidence could be placed in the use of the $V_{max}$ of PDH as an indicator of $J_{max}$ through pyruvate oxidation. If it is assumed that the $V_{max}$ values of hexokinase (multiplied by 2) can indicate $J_{max}$ through the TCA cycle (and comparison of these values with oxygen uptake data suggest that they may do so in some insects, see footnote) then the $V_{max}$ values of PDH are lower than the predicted maximum flux through the reaction. This suggests that the correct conditions for the activation of PDH in insect flight muscle were not used in this work and that further studies of the properties of the enzyme must be carried out before PDH can be used as an index of pyruvate oxidation.

Although all the ratios of $V_{max}$(PDH)/$V_{max}$(OGDH) are low for insect flight muscles in Table II , in five insects the ratio appears exceptionally low, and may represent a qualitative difference between these insects and the others. The very low ratios are observed in the tsetse fly, the giant waterbug, the silver Y moth, the African dung beetle and the rose chafer. In Chapter Four, proline oxidation was shown to be an important energy yielding process in the tsetse fly and the chafers (presumably also in the rose chafer, although the activity of proline dehydrogenase has not been measured in this chafer). As the $V_{max}$ of proline dehydrogenase is also high in beetles, this may also be true for the African dung beetle (see Chapter Four). Thus, in these insects, the very low ratio of PDH to OGDH may be explained by a low glycolytic capacity

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Footnote: If $J_{max}(O_2)$ represents through the TCA cycle estimated from oxygen uptake, and $J_{max}$ (HK) represents $J_{max}$ through the TCA cycle estimated from 2 x HK activities, the following comparison can be made:

| INSECT FLIGHT MUSCLE | $J_{max}(O_2)$ | $J_{max}$(HK) |
|---|---|---|
| honey-bee | 53.0 | 58.0 |
| bumble-bee | 46.1 | 228.0 |
| blowfly | 66.3 | 70.0 |
| cockroach | 22.3 | 36.0 |
| desert locust | 20.7 | 23.0 |

(The data are taken from Crabtree & Newsholme, 1972a, and rates are expressed as $\mu$mol $C_3/C_2$. $min^{-1}.g^{-1}$ at 25°C.)

and a dependence on non-carbohydrate fuels. (The low ratio in the silver Y moth is surprising, however, as this moth is believed to feed in the adult stage, and it has high activities of HK and PFK; see Chapter Four).

Comparison of $V_{max}$ of OGDH and PDH with the $V_{max}$ of phosphorylase and PFK

Crabtree & Newsholme (1972a) showed that the $V_{max}$ values of phosphorylase and PFK were good indicators of the maximum rate of glycolysis in muscle (phosphorylase for the flux from glycogen and PFK for the total flux from carbohydrate fuels). Some muscles have a low rate of the TCA cycle (see above) and it would be interesting to compare the rate of ATP formation from the anaerobic breakdown of glycogen to lactate with the rate of ATP formation from the TCA cycle in these and other muscles, in order to see how the energy demands of these muscles may be met.

Rates of ATP production have been calculated from the $V_{max}$ values of PDH, OGDH, phosphorylase and PFK and the results are presented in Table III. In the calculation of the rates of ATP production from the activities of phosphorylase and PFK, it has been assumed that the muscles can operate anaerobically. From the high activities of LDH in these muscles (see Crabtree & Newsholme, 1972a, and Chapter Four, Table IV), flux through to lactate is possible and this has been shown to be the case in a number of vertebrate muscles.

In general, the rates of ATP production are higher from the anaerobic process (i.e. via phosphorylase and PFK) than from the aerobic process (i.e. via PDH and the TCA cycle) (see Table III). This is presumably to compensate for the fact that the number of molecules of ATP produced from one molecule of substrate is much lower via anaerobic glycolysis than via the TCA cycle (see Chapter One, Table II). Thus, in order to supply the same rate of ATP production, anaerobic glycolysis must operate much faster than the TCA cycle (almost 10-fold).

It can be seen that the highest rates of ATP production via anaerobic glycolysis are found in muscles where the rate of ATP production via the TCA cycle is low, i.e. pheasant pectoral, chicken pectoral, and trout white muscle.

TABLE III

MAXIMUM RATES OF ATP TURNOVER DERIVED FROM MAXIMUM ACTIVITIES OF 2-OXOGLUTARATE DEHYDROGENASE, PYRUVATE DEHYDROGENASE, PHOSPHORYLASE AND PHOSPHOFRUCTOKINASE IN VERTEBRATE MUSCLES

The activities of the enzymes are converted to rates of ATP turnover in the following way: the $V_{max}$ OGDH was multiplied by 15; the $V_{max}$ of PDH was multiplied by 15 assuming that all the acetyl CoA produced from the reaction was oxidised via the TCA cycle; the $V_{max}$ values of phosphorylase and phosphofructokinase (PFK) were multiplied by 3, assuming that the muscles are anaerobic. Unless otherwise indicated, the $V_{max}$ values of phosphorylase and PFK were taken from Crabtree & Newsholme (1972a). The following superscripts indicate the actual muscles used: $^{q}$, quadriceps; $^{s}$, soleus; $^{g}$, gastrocnemius.

| ANIMAL: MUSCLE | MAXIMUM RATES OF ATP TURNOVER CALCULATED FROM THE $V_{max}$ VALUES OF: | | | |
|---|---|---|---|---|
| | OGDH | PDH | phosphorylase | PFK |
| Dogfish: heart | 49.5 | 37.5 | - | 103.8* |
| : red muscle | 33.0 | 48.0 | 72.0 | 42.6* |
| : white muscle | <1.5 | <1.5 | 372.0 | 180.6* |
| Trout : heart | 49.5 | 54.0 | - | 258.6* |
| : red muscle | 27.0 | 48.0 | 84.0 | 83.4* |
| : white muscle | <1.5 | 4.5 | 288.0 | 606.0* |
| Frog : heart | 52.5 | 72.0 | - | 120.0* |
| : sartorius | $<1.5^{g}$ | $10.5^{g}$ | 174.0 | $222.0^{g*}$ |
| Pigeon : heart | 64.5 | 102.0 | - | 103.8* |
| : pectoral | 48.0 | 73.5 | 108.0 | 144.0 |
| Chicken: heart | 90.0 | 75.0 | - | 105.6* |
| : pectoral | <1.5 | 1.8 | 498.0 | 864.0* |
| Pheasant: heart | 70.5 | 133.5 | - | 102.6* |
| : pectoral | 22.5 | 13.5 | 720.0 | 1,116.0* |
| Rabbit : semitendinosus | 13.5 | 7.5 | 48.0 | 48.0 |
| : adductor longus | 10.5 | 6.0 | 180.0 | 156.0 |
| Rat : heart | 102.0 | 103.5 | 72.0 | 86.4* |
| : skeletal muscle | $36.6^{s}$ | $18.0^{s}$ | $300.0^{q}$ | $398.4^{g*}$ |
| Mouse : heart | 133.5 | 180.0 | - | 137.4* |

*These values were taken from Zammit et al. (1978).

However, in the hearts, rates of ATP production via aerobic and anaerobic processes are similar. This suggests that although hearts are continually active, and have been shown to have a relatively high rate of the TCA cycle, these muscles can support the same energy requirements from anaerobic glycolysis e.g. when an increased work load temporarily increases energy demand over oxygen supply. Although such a situation may be regarded as occurring only infrequently, it stresses the importance of this organ to the well-being of the animal that the capacity for ATP production is equally high whether from endogenous glycogen or exogenous substrates. The importance of glycolysis from glycogen to lactate in tissues normally capable of aerobic metabolism is discussed by Krebs (1972). He suggests that the role in muscle would be to supply energy when the oxygen becomes limiting, as suggested above.

In other muscles, the degree to which a muscle is capable of producing high rates of ATP both aerobically and anaerobically may reflect the mixture of aerobically and anaerobically specialised fibres in the muscle. It has been noted above that many muscles in higher vertebrates are composed of a mixture of fibre types. In fish, the two discrete muscle types might exemplify the two extremes. Indeed, the lowest rates of ATP production from anaerobic glycolysis are found in the dogfish red muscle, and the lowest rates of ATP production from aerobic metabolism are found in dogfish white muscle. Presumably, in higher animals, it was advantageous to evolve muscles which were multi-functional, and could produce energy under different conditions.

The importance of the TCA cycle for energy production in continual contraction has been stressed above. The importance of anaerobic energy production i.e. glycogen breakdown to lactate in the 'white' muscles is that endogenous fuel stores can be mobilised rapidly, and that for short-term and violent contractions, such as those required in escape reactions, a complex system of blood capillaries supplying fuel and oxygen to the muscle is not required.

That some muscles can be specialised in this way represents an economical saving in the animal as a whole (see Chapter One, pp.23-24).

Comparison of the $V_{max}$ of OGDH with the $V_{max}$ of other enzymes of the TCA cycle

As OGDH has been shown to be a 'rate-indicator' for the TCA cycle, comparison of its $V_{max}$ with the $V_{max}$ of other enzymes of the TCA cycle may demonstrate the equilibrium nature of the reactions catalysed by these reactions. In Chapter One, pp.33-36, it was stated that one way of identifying the equilibrium nature of a reaction was to compare the $V_{max}$ of the enzyme *in vitro* with the $J_{max}$ through the reaction *in vivo*. If the $V_{max}$ is much greater than the $J_{max}$, then the reaction may be near-equilibrium *in vivo*. Since the $V_{max}$ of OGDH has been shown to represent the $J_{max}$ through the TCA cycle, it can be used for comparison in this way, assuming that the flux is the same through all the reactions of the TCA cycle during maximum activity.

$V_{max}$ values of succinate dehydrogenase (SDH) and OGDH are presented in Table IV. It can be seen that the $V_{max}$ values of SDH do not greatly exceed the $V_{max}$ values of OGDH in many muscles, although in some, notably rat and mouse heart, rat diaphragm, chicken pectoral trout white and dogfish white muscle, the $V_{max}$ of SDH is more than 3-fold higher than the $V_{max}$ of OGDH. The main problem with the assay of SDH is that, since the enzyme involves part of the electron transport chain, the supply of electrons (or protons) *in vitro* is unlikely to be similar to that *in vivo* (see below), and this may affect the measurement of the $V_{max}$ of SDH.

Malate dehydrogenase $V_{max}$ values have been reported by Dawson & Romanul (1964) for four muscles from the rat. Activities in $\mu mol.min^{-1}.g^{-1}$ (wet weight) were: 190.0, 434.0, 437.0 and 997.0 for white gastrocnemius, red gastrocnemius, soleus muscle and heart respectively. These activities exceed greatly the $V_{max}$ of OGDH from the same or similar muscles, and this suggests that malate dehydrogenase catalyses a reaction, which is near-equilibrium

TABLE IV

MAXIMUM ACTIVITIES OF 2-OXOGLUTARATE DEHYDROGENASE AND SUCCINATE DEHYDROGENASE IN MUSCLES FROM VERTEBRATES AND INVERTEBRATES

The $V_{max}$ values of succinate dehydrogenase (SDH) are taken from Crabtree & Newsholme (1975). The $V_{max}$ values of OGDH are taken from Chapter Four, Table II. The superscripts represent the actual animal used: $^{c}$, cockchafer, $^{s}$, summer chafer; $^{p}$, Phormia; $^{c}$, Calliphora

| ANIMAL: MUSCLE | MAXIMUM ENZYME ACTIVITIES | | RATIO OF $V_{max}$(SDH)/ $V_{max}$(OGDH) |
|---|---|---|---|
| | SDH | OGDH | |
| African locust:flight muscle | 20.0 | 44.2 | 0.5 |
| cockroach: flight muscle | 57.0 | 48.6 | 1.2 |
| giant waterbug: flight muscle | 27.0 | 12.0 | 2.3 |
| chafer: flight muscle | $30.0^{c}$ | $57.5^{s}$ | 0.5 |
| bumble-bee: flight muscle | 128.0 | 45.9 | 2.8 |
| fleshfly: flight muscle | 80.0 | 32.8 | 2.4 |
| blowfly: flight muscle | $45.0^{p}$ | $46.7^{c}$ | 1.0 |
| dogfish: red muscle | 4.5 | 2.2 | 2.0 |
| : white muscle | 0.6 | $<0.1$ | $>6.0$ |
| trout: red muscle | 2.7 | 1.8 | 1.5 |
| : white muscle | 0.6 | $<0.1$ | $>6.0$ |
| pigeon: pectoral muscle | 9.0 | 3.2 | 2.8 |
| chicken: pectoral muscle | 0.3 | $<0.1$ | $>3.0$ |
| pheasant: pectoral muscle | 0.5 | 1.5 | 0.3 |
| rabbit: heart | 20.0 | 9.0 | 2.2 |
| : semitendinosus | 1.8 | 0.9 | 2.0 |
| : adductor longus | 0.6 | 0.7 | 0.9 |
| rat: heart | 36.9 | 6.8 | 5.4 |
| : diaphragm | 9.7 | 2.4 | 4.0 |
| mouse: heart | 33.0 | 8.9 | 3.7 |

*in vivo*. This supports the earlier conclusion of Pette & Luh (1962). In addition, malate dehydrogenase activities have been reported by Hoek *et al*. (1976) for a variety of insect flight muscles. If the assumption is made that 1 g of insect flight muscle contains 200 mg protein, these activities can be expressed as 542.0, 128.0, 121.0, 458.0, 450.0 and 176.0 $\mu$mol.min$^{-1}$.g$^{-1}$ for the honey-bee, African dung beetle, cockroach, locust, fleshly and tsetse fly flight muscle respectively. These activities also exceed by approximately 10-fold the $V_{max}$ values of OGDH, and show that malate dehydrogenase probably catalyses a near-equilibrium reaction *in vivo* in insect flight muscle. Cytochrome oxidase activities have sometimes been used as an indication of the aerobic capacity of a tissue. For example, George & Talesara (1961) give data of cytochrome oxidase activities expressed as oxygen uptake of extracts of pigeon muscle fibres, supplied with excess reduced cytochrome c, which can be converted (assuming the conversion factors given in Appendices IV and VI) into cytochrome oxidase activities, e.g. 26 $\mu$mol.min$^{-1}$.g$^{-1}$ for pigeon pectoral muscle. This value is in excess of the $V_{max}$ of OGDH and would suggest that the enzyme is near-equilibrium in the cell.

Similarly, Baldwin *et al*. (1972) measured cytochrome oxidase activities in skeletal muscles of the rat, and expressed the activities as $\mu$l $O_2$.min$^{-1}$.g$^{-1}$. By dividing by 22.4 and by 3, it is possible to convert these activities to $\mu$mol $C_3/C_2$.min$^{-1}$.g$^{-1}$ (see Appendix VI). Cytochrome oxidase activities expressed in this way are 2.5, 12.4 and 9.2 $\mu$mol.min$^{-1}$.g$^{-1}$ for white quadriceps, red quadriceps and soleus muscle respectively. These values are greater than the $V_{max}$ of OGDH in the same or similar tissues. However, cytochrome oxidase is part of the cytochrome chain, and as mentioned above, there are problems inherent in the assay of such enzymes *in vitro*. Wilson *et al*. (1974) investigated the redox state of cytochrome c isolated in mitochondria separated from ascites tumour cells and isolated rat liver cells, and discovered that over a range of values of the concentration ratio of $NAD^+$/NADH (in the presence and

absence of respiratory substrate) the ratio of oxidised cytochrome c to reduced cytochrome c was between 2 and 5. Thus, from their data it appears that cytochrome c, and hence cytochrome $a_3$, is probably mainly in the oxidised form *in vivo* (Wilson *et al.*, 1974). Thus, electrons or protons (if these are considered to represent the flow through the pathway, i.e. the pathway substrate) may be rate-limiting *in vivo*. Thus, as in the case of CS, this enzyme may not be a good indicator of TCA cycle flux (or flux through the electron transport chain) as the assay *in vitro* may supply the enzyme with a higher substrate concentration than exists *in vivo* and give an overestimate of the $V_{max}$ of the enzyme.

TCA cycle flux in tissue other than muscle

The $V_{max}$ of OGDH may give a useful index of $J_{max}$ through the TCA cycle not only in muscle, where the power output in terms of contractile activity can be easily appreciated, but also in other tissues. $V_{max}$ values for OGDH and PDH for rat and mouse brain and kidney are presented in Table V , together with estimates of maximum flux ($J_{max}$) through the TCA cycle in rat brain and rat kidney. These estimates are derived from oxygen uptake data from A-V differences across the brain (Carlsson *et al.*, 1977) and from oxygen uptake of the isolated perfused kidney (Ross & Bullock, 1976) (see Appendix VI). For comparison, $V_{max}$ values of CS and total ICDH in brains are also presented in Table VI, and are taken from Sugden & Newsholme (1975).

It can be seen that in the rat kidney the $V_{max}$ of OGDH and the $J_{max}$ through the TCA cycle estimated from oxygen uptake data are in good agreement. This supports the assumption that the $V_{max}$ of OGDH can give an indication of the $J_{max}$ through the TCA cycle in tissues other than muscle. It also shows how important aerobic metabolism is in the kidney as a whole: the $V_{max}$ of OGDH in rat and mouse kidney is similar to that in the hearts of these animals. This demonstrates that, in the rat and mouse at least, the work done by the

TABLE V

MAXIMUM ACTIVITIES OF PDH AND OGDH IN BRAIN AND KIDNEY FROM THE MOUSE AND THE RAT, AND ESTIMATES OF THE MAXIMUM RATE OF FLUX THROUGH THE TCA CYCLE IN THESE TISSUES

The assay systems of PDH and OGDH are described in Chapter Three. Three determinations were carried out for each value of $V_{max}$ and the figure in brackets is $S_w$, an estimate of the standard deviation (Dean & Dixon, 1951). Maximum activities of citrate synthase (CS) and total isocitrate dehydrogenase (ICDH) are presented for comparison, and are taken from Sugden & Newsholme (1975). The estimates of maximum flux through the TCA cycle are derived from oxygen uptake data (see Appendix VI ).

| ANIMAL | TISSUE | ESTIMATE OF $J_{max}$ THROUGH TCA CYCLE AT 25°C ($\mu$mol $C_3/C_2$.min$^{-1}$.g$^{-1}$) | MAXIMUM ENZYME ACTIVITIES ($\mu$mol $C_3/C_2$.min$^{-1}$.g$^{-1}$) | | | |
|---|---|---|---|---|---|---|
| | | | OGDH | PDH | CS | TOTAL ICDH |
| Mouse | brain | - | - | 5.1 (0.1) | 24.3 | 5.0 |
| | kidney | - | 8.3 (0.7) | 3.8 (0.4 | - | - |
| Rat | brain | 1.0[1] | - | 2.4 (0.7) | 13.6 | 1.9 |
| | kidney | 7.9[2] | 6.9 (0.9) | 4.2 (0.5) | - | - |

1. Carlsson et al. (1977)
2. Ross & Bullock (1976)

kidney in active transport consumes as much energy as the work done by the heart in pumping blood around the body. This high $J_{max}$ of the TCA cycle may be related to the fact that since the kidney must receive a very good blood supply in order to serve its function, it is therefore supplied with ample oxygen, glucose and other blood-borne fuels. Since the $V_{max}$ of OGDH is greater than the $V_{max}$ of PDH, this could suggest that carbohydrate alone cannot support maximum energy requirements. However, an alternative explanation may be that, since OGDH in kidney plays a role in the glutamine pathway, which is important in producing $NH_3$ for acid balance, the $V_{max}$ of OGDH is relatively higher, i.e. that it represents the flux not only through the TCA cycle, but also through the glutamine pathway. This is supported by the fact that the ratio $V_{max}$(OGDH)/$V_{max}$(total ICDH) is much higher in kidney than in vertebrate muscles (E.A. Newsholme, personal communication).

In brain, there were technical difficulties in measuring the activity of OGDH. Sonication destroyed the activity, and the use of Triton X-100 in the cuvette produced interference, as clarification of the turbid extract continually occurred, presumably due to the solubilising of lipid components of brain tissue by the detergent. However, the $V_{max}$ of PDH is in reasonable agreement with the $J_{max}$ through the TCA cycle estimated from the data of Carlsson et al. (1977). McIlwain gives a value for oxygen uptake of brain slices during electrical stimulation which is equivalent to 0.6 $\mu$mol.min$^{-1}$.g$^{-1}$ (McIlwain, 1966). PDH has also been measured in various portions of the brain of the cat, but the values are lower than those for the rat or the mouse reported in the present work (see Table V; Blass et al., 1975; McIlwain & Bachelard, 1971). In rat brain mitochondria, the maximum activity of PDH was found to be 53.5 nmol.min$^{-1}$.(mg protein)$^{-1}$ (Jope & Blass, 1975). If it is assumed that brain tissue contains 60 mg mitochondrial protein, then this value can be expressed as 3.2 $\mu$mol.

$min^{-1}.g^{-1}$ (wet weight). This value is similar to the value for rat brain presented in Table V. It was shown in Chapter One, p. 44 that the flux through PDH in brain was in close agreement with $V_{max}$ of PDH *in vitro* (Cremer & Teal, 1974). It therefore seems likely that PDH may be a good 'flux-indicator' in brain. It can be seen from Table V that the activity of total ICDH does not greatly exceed the activity of PDH in brain. This suggests that the $J_{max}$ through the TCA cycle must be similar to the $J_{max}$ through ICDH and that ICDH may be a 'rate-indicator' for the TCA cycle in brain, unlike its role in muscle (see Chapters One and Four). The activity of CS, however, does exceed the $J_{max}$ through pyruvate oxidation and the TCA cycle, as it does in muscle.

Brain usually depends on carbohydrate utilisation for its energy requirements (McIlwain & Bachelard, 1971; Hawkins *et al.*, 1971). Jope & Blass have shown that, in isolated brain mitochondria, PDH is totally in the active form (Jope & Blass, 1975). In addition, nervous tissue as a whole depends mainly upon aerobic metabolism, and therefore the $V_{max}$ of PDH may give an indication of the flux through carbohydrate utilising pathways. However, the $V_{max}$ of hexokinase expressed as $\mu mol.C_3/C_2.min^{-1}.g^{-1}$ in rat brain is 27.8 at 25°C, compared with a $V_{max}$ of PDH of 2.4 (Sugden & Newsholme, 1973). This suggests that the glycolytic capacity in brain is greater than the maximum flux through PDH. Reasons for this high glycolytic capacity have been suggested by Sugden & Newsholme (1973). These authors suggest that the large capacity of the glycolytic pathway in nervous tissue may provide a short term mechanism for the production of energy by anaerobic glycolysis if the brain should become hypoxic. It is interesting in this context that the $V_{max}$ of PFK does not greatly exceed the $V_{max}$ of hexokinase; this fact, together with the fact that the brain produces very little lactate (Rowe *et al.*, 1959; Hawkins *et al.*, 1971) suggests that glucose rather than glycogen may be used in anaerobic

energy production. An alternative explanation for the high activities of hexokinase may be that, if the glial cells and the neuronal cells have a different metabolism and are not always maximally active at the same time, then the activities of PDH and hexokinase *in vitro* represent only the sum of these different tissue types (see below).

Potential use of OGDH and PDH as 'rate-indicator' enzymes

It has been suggested earlier that the role of OGDH as indicator of flux through the TCA cycle may extend to tissues other than muscle. The evidence from the comparison of $V_{max}$ of OGDH with $J_{max}$ through the TCA cycle in kidney supports this suggestion. It would therefore be of interest to look at the $J_{max}$ of the TCA cycle in different tissues within the same organ, e.g. the kidney cortex and medulla.

In other tissues where fat biosynthesis was important (e.g. mammary gland, adipose tissue) a comparison of the $V_{max}$ of OGDH may provide evidence on two of the pathways which supply and remove acetyl CoA, thus indirectly providing evidence on the rate of acetyl CoA provision for fat biosynthesis. This has been done for lactating mammary glands from various mammals (Read *et al.*, 1977). These workers found that in non-ruminant mammary glands (.e.g rat, mouse, rabbit, guinea pig) the $V_{max}$ of PDH was much higher than the $V_{max}$ of OGDH, suggesting a greater rate of production of acetyl CoA than the rate of its removal by the TCA cycle for aerobic energy production. Indeed, the difference between the two activities was similar to the estimated rate of conversion of pyruvate to acetyl CoA for fat synthesis in a cell preparation from lactating mammary gland of the rat (Read *et al.*, 1977).

The $V_{max}$ of PDH may be used as an indication of the $J_{max}$ through pyruvate oxidation (and hence through the TCA cycle in brain, see above). A comparison of the $V_{max}$ of PDH in glial cells with the $V_{max}$ of PDH in neuronal cells may provide an answer to the problem of high rates of glycolytic flux in whole

brain homogenates, and to whether these two types of cell have different types of metabolism. In addition, subcellular fractions of brain can be prepared which contain the vesicles for neurotransmitter release i.e. synaptosomes. It would be interesting to compare the $J_{max}$ of the TCA cycle at the site of neurotransmitter release with the $J_{max}$ of the TCA cycle along the axon of the nerve. It may be possible that OGDH can be measured in synaptosomes without the problems inherent in measuring the activity in extracts of whole brain (see above).

Similarly, measurement of the $V_{max}$ of OGDH and PDH may provide a means of investigating the metabolism of subcellular fractions from other tissues not only as a quantitative index of the mitochondrial activities, but to compare mitochondria from different sources. In addition, the fibre types IIA and IIB in muscle, referred to in Chapter One, p.18, could be distinguished on the basis of the $J_{max}$ through the TCA cycle, instead of using different criteria in different muscles.

The $V_{max}$ of OGDH may also be measured in tumour cells and tissue cultures to monitor the change in aerobic metabolism in these transformed tissues.

## CONCLUSION

This thesis proposes that, compared with other attempts to estimate the aerobic metabolism of a tissue, the measurement of the $V_{max}$ of OGDH *in vitro* provides a more precise and reasonably quick quantitative index of the maximum flux through the TCA cycle in muscles across the animal kingdom and across a range of different tissues. Secondly, that PDH may supplement this role in vertebrate tissues to give a quantitative index of the maximum flux through pyruvate oxidation. Further work on PDH in insects may reveal aspects of control which render the measurement of the $V_{max}$ of PDH a similarly useful tool across the animal kingdom, and across a wide range of tissues.

Professor Sir Hans Krebs, who discovered and named the TCA cycle in 1937, elucidated the enzymic reactions and demonstrated its existence in many living tissues. In the late 1940s, he wrote in an article entitled "Le Cycle des Acides Tricarboxylliques"

> "Une autre lacune dans la connaissance des réactions génératrices d'énergie concerne les méchanismes qui contrôllent les vitesses de ces réactions et les ajustent aux besions de l'organisme considéré comme un tout"
>
> (Biochemie Medicale, 10$^{e}$)

> ("Another gap in the understanding of the energy producing reactions" that is, of the TCA cycle, "concerns the mechanisms which control the rate of these reactions and adjust them to the needs of the animal as a whole")

In the subsequent 35 years, further knowledge was built upon his foundations; knowledge of the control of the pathway, of the mechanisms of action of the constituent enzymes, of the localisation within the mitochondria. But no simple index of the rate of the cycle in different tissues "adjusted to the needs of the animal as a whole" was known. Studies of the importance of the TCA cycle in muscle have failed to find a relatively simple measurement of its maximum rate in order to compare this in different tissues. Read et al. (1977) were the first workers to propose OGDH for this role and this thesis demonstrates that it can fulfill it.

# APPENDIX I

## Derivation of the relationship between the standard free energy change of a reaction, the mass action ratio and the equilibrium constant

Classical thermodynamics relates the free energy change of reaction ( $\Delta G$) to its displacement from equilibrium.

Consider the reaction

$$A \rightleftharpoons B$$

The Gibb's free energy equation states that

$$\Delta G = \Delta G^{o} + RT \ln \frac{[B]}{[A]} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (i)$$

where $\Delta G^{o}$ is the standard free energy change, and [A] and [B] are the concentrations of substrate and product. When the reaction is at equilibrium, $\Delta G$ is zero and hence

$$\Delta G^{o} = -RT \ln \frac{[B_e]}{[A_e]}$$

where $[B_e]$ and $[A_e]$ are the equilibrium concentrations of product and substrate

$$= -RT \ln k \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (ii)$$

where k is the equilibrium constant of the reaction. Combining (i) and (ii), we have

$$\Delta G = -RT \ln k + RT \ln \frac{[B]}{[A]} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (iii)$$

$$\therefore \Delta G = -RT \ln \left( \frac{[A]}{[B]} \cdot k \right) \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (iv)$$

The ratio of the concentrations of products (in this case [B] ) to those of substrates (in this case [A] ) as measured in a tissue is known as the mass action ratio and is designated by the symbol $\Gamma$ (Bücher & Rüssman, 1964) so that substituting into equation (iv)

$$\Delta G = -RT \ln \frac{K}{\Gamma} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (v)$$

# APPENDIX II

## DERIVATION OF THE RELATIONSHIP BETWEEN THE RATIO OF THE RATES OF THE FORWARD AND BACKWARD REACTIONS IN VIVO AND THE MASS ACTION RATIO

If $V_f$ is the rate in the forward direction and $V_r$ is the rate in the reverse direction, $k_{+1}$ and $k_{-1}$ are the rate constants for the forward and reverse directions respectively, we can develop the following equations.

Consider the reaction

$$\longrightarrow A \underset{V_r}{\overset{V_f}{\rightleftharpoons}} B \longrightarrow$$

Then

$$V_f = k_{+1}[A] \quad \text{.....(i)}$$

$$V_r = k_{-1}[B] \quad \text{.....(ii)}$$

Combining (i) and (ii)

$$\frac{V_f}{V_r} = \frac{k_{+1}[A]}{k_{-1}[B]} \quad \text{.....(iii)}$$

At equilibrium, $V_f = V_r$ so that

$$\frac{k_{+1}}{k_{-1}} = \frac{[B_e]}{[A_e]} = K \quad \text{(see Appendix 1)} \quad \text{.....(iv)}$$

Substituting back into equation (iii)

$$\frac{V_f}{V_r} = K\frac{[A]}{[B]}$$

But $[A]/[B] = 1/\Gamma$ (see Appendix 1), therefore

$$\frac{V_f}{V_r} = \frac{K}{\Gamma}$$

# APPENDIX III

## SOURCES OF MATERIALS, EQUIPMENT AND ANIMALS

### Materials

All chemicals and enzymes were obtained from Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG, except for the following:

MSH, creatine phosphate: Sigma (London) Chemical Co. Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH

EDTA, Triton X-100, glycerol, inorganic reagents: BDH Chemicals Ltd., Broom Road, Poole, Dorset, BH12 4NN

creatine phosphate: Calbiochem Ltd., 79-81, South St., Bishop's Stortford Hertfordshire CM 23 3AL

p (p-aminophenylazo)-benzene sulphonic acid was a gift from the Zoology Department, University of Oxford.

arylamine acetyltransferase was prepared from pigeon livers as described in Chapter Three.

1 l bottles of AR acetone: Hopkin & Williams, P.O. Box 1, Romford, Essex. RM1 1HA.

Winchesters of organic solvents : Fisons Scientific Apparatus Ltd., Bishop Meadow Rd., Loughborough, Leicestershire.

The cyanide reagent was made up as follows:

2.72 g $KH_2PO_4$

1 g of bovine serum albumin

60 mg EDTA

were dissolved in distilled water and adjusted to pH 7.0 with 1M potassium hydroxide. 0.78 g potassium cyanide were added to this solution, and the solution made up to 1 l.

APPENDIX III (cont'd.)

Equipment

The Silverson homogeniser was obtained from Silverson Machines Ltd., Waterside, Chesham, Bucks. HP5 1PQ.

The Polytron (model PT 10-35) was obtained from Northern Media Supply Ltd., Crosslands Lane, Newport Road, North Cave, Brough, East Yorkshire, HU 15 2PG.

Animals

Pigeons were obtained from the Oxford City Council Department of Health, and from Park Farm, Northmoor, Oxon.

Insects were caught locally and were used on the day of capture.

Locusts (Schistocerca and Locusta) were obtained from the Department of Zoology, University of Oxford and were maintained in locust cages in the laboratory.

Fleshfly and housefly pupae, and larval stages of Locusta were received as a gift from Shell Biosciences, Tunstall, nr. Sittingbourne, Kent, and were hatched in cages in the laboratory.

Blowfly larvae (Calliphora vomitoria)were obtained from Bioserve Ltd., 38-42, Station Rd., Worthing, Sussex, BN11 1JP, and were hatched in cages in the laboratory.

Cockroaches, rose chafers, African dung beetles and giant waterbugs were obtained from stocks maintained in the Department of Zoology, University of Oxford.

Giant diving beetles were caught in a local pond and kept in tanks in the Department of Zoology, University of Oxford.

Summer chafers were caught locally whilst foraging at dusk in June.

Trout were obtained from Bibury Trout Farm, Bibury, Cirencester, Gloucestershire.

Dogfish, whelks, limpets and winkles were obtained from the Marine Laboratory, Marine Biological Association of the U.K., Plymouth, Devon.

APPENDIX III (cont'd.)

Pheasants and chickens were obtained from Park Farm, Northmoor, Oxon.

Frogs (Rana temporaria) were obtained from T. Gerrard, Worthing Rd., East Preston, W. Sussex, BN16 1AS.

African clawed toads (Xenopus laevis)were obtained from stocks maintained by Dr. J. Knowland, Department of Biochemistry, University of Oxford.

Tsetse flies were a gift from Dr. A.M. Jordan, Tsetse Research Laboratories, Department of Veterinary Medicine, Langford House, Langford, Bristol. BS18 7DU.

Poplar hawk moth larvae were a gift from Mr. Parsons, of Berks, Bucks and Oxfordshire Naturalist Trust, and were hatched in the laboratory.

# APPENDIX IV

## CALCULATION OF INTRAMITOCHONDRIAL METABOLITES

### CONVERSION FACTORS FOR RAT HEART

1) Fresh weight = 5 x dry weight of heart (Opie & Owen, 1975)

$\therefore$ dry weight = 20% fresh weight

$\therefore$ total water in preparation = 80% fresh weight

2) Extracellular water = 50% fresh weight (v/w) (Opie & Owen, 1975)

$\therefore$ intracellular water = 30% fresh weight

= 0.3 $ml.g^{-1}$ (fresh weight)

3) 30 mg mitochondrial protein per g fresh weight (Haslam & Krebs, 1968)

4) 1 $\mu$l matrix water and 2 to 3 $\mu$l extramatrix water associated with mitochondria per mg mitochondrial protein (Williamson et al., 1973)

$\therefore$ per 1 g fresh weight of heart, there are 30 $\mu$l matrix and 90 to 120 $\mu$l extramitochondrial water, i.e. total water associated with mitochondria = 120 to 150 $\mu$l

But total intracellular water = 300 $\mu l.g^{-1}$

$\therefore$ cytosolic water = 300 - mitochondrial water

= 180 to 150 $\mu l.g^{-1}$

and matrix water = 30 $\mu l.g^{-1}$

Boundary Condition A assumes that all the metabolite measured is contained in the matrix water

$\therefore$ to convert tissue concentration in $\mu mol.g^{-1}$ to mitochondrial concentration in M, divide by 30 (for $\mu mol.g^{-1}$ fresh weight)

or by 150 (for $\mu mol.g^{-1}$ dry weight)

Boundary Condition B assumes that all the metabolite measured is equally distributed between mitochondria and cytoplasm.

$\therefore$ to convert tissue concentration in $\mu mol.g^{-1}$ to mitochondrial concentration in M, divide by 300 (for $\mu mol.g^{-1}$ fresh weight)

or by 1500 (for $\mu mol.g^{-1}$ dry weight)

APPENDIX IV (continued)

Rat heart:

For cytosolic concentrations, divide by 165 (to convert $\mu$mol.g$^{-1}$fresh weight to M

or by 825 (to convert $\mu$mol.g$^{-1}$dry weight to M

assuming that the volume of mitochondria is negligible when compared with that of the cytosol.

CONVERSION FACTORS FOR INSECT FLIGHT MUSCLE

1) Dry wieght = 28% fresh weight (Rowan,1975)

total water = 72% fresh weight

2) Inulin space = 30% total volume (Rowan,1975)

intracellular water= 72 - 30 = 42% total volume

= 0.42 ml.g$^{-1}$(fresh weight)

3) Mitochondrial protein

= 60 mg.(g fresh weight)$^{-1}$ (Klingenberg, 1964)

4) Matrix volume =2.5 $\mu$l.(mg mitochondrial protein)$^{-1}$(Hansford & Lehninger, 1972)

In 1g fresh weight flight muscle, there are 150 $\mu$l matrix water.

But total intracellular water = 420 $\mu$l.g$^{-1}$

cytosolic water = 420 - 150 = 270 $\mu$l.g$^{-1}$

To convert tissue concentration in $\mu$mol.g$^{-1}$ to mitochondrial concentration in M,

under Boundary condition A : divide by 150

under Boundary condition B : divide by 420

For cytosolic concentrations, divide by 270 (to convert $\mu$mol.g$^{-1}$ fresh weight to M).

# APPENDIX V

## CALCULATION OF EQUILIBRIUM CONSTANT

$$\Delta G^{o'} = - RT \ln K_{eq}$$

where R = universal gas constant = 1.987 $cal.mol^{-1}K^{-1}$

T = absolute temperature in K

$\Delta G^{o'}$ = standard free energy change

$K_{eq}$ = thermodynamic equilibrium constant

EXAMPLE 1

$$citrate^{3-} \xrightarrow{aconitase} isocitrate^{3-} \qquad \Delta G^{o'} = 1.59 \text{ Kcal}$$

T = 298 K (assumed)

R = 1.987 $cal.mol^{-1}K^{-1}$

$$\Delta G^{o'} = - RT \ln K_{eq}$$

$$\ln K_{eq} = - \frac{\Delta G^{o'}}{RT}$$

$$= - \frac{1590}{1.987 \times 298}$$

$$= - 2.685$$

$$K_{eq} = 0.068$$

EXAMPLE 2

$$2\text{-oxoglutarate}^{2-} + NAD^{+} + CoA \xrightarrow{OGDH} \text{succinyl CoA} + NADH + CO_2 + H^{+}$$

$\Delta G^{o'} = - 8000 \ cal.mol^{-1}$

T = 298 K (assumed)

R = 1.987 $cal.mol^{-1}K^{-1}$

$$\Delta G^{o'} = -RT \ln K_{eq}$$

$$\ln K_{eq} = - \frac{\Delta G^{o'}}{RT}$$

$$= \frac{8000}{1.987 \times 298}$$

$$= 13.5$$

$$K_{eq} = 7.4 \times 10^{5}$$

# APPENDIX VI

## CALCULATION OF THE MAXIMUM FLUX THROUGH THE TCA CYCLE FROM OXYGEN UPTAKE DATA

### OXYGEN UPTAKE DATA

| ANIMAL OR PREPARATION | MAX. $O_2$ UPTAKE | MUSCLE AS % BODY WEIGHT % | TEMPERATURE OF MUSCLES $^{o}C$ |
|---|---|---|---|
| honey-bee | 100 $ml.hr^{-1}.g^{-1}$ (1) | 17 (2) | 40 (3, 21) |
| bumble-bee | 186 $ml.hr^{-1}.g^{-1}$ (4) | * | 40 (3, 21) |
| silver Y moth | 40 $ml.hr^{-1}.g^{-1}$ (5) | 20 (6) | 35 (7, 21) |
| cockroach | 36 $ml.hr^{-1}.g^{-1}$ (8) | 20 (assume) | 30 (9) |
| locust | 30 $ml.hr^{-1}.g^{-1}$ (10) | 18 (2) | 35 (11) |
| blowfly | 187 $ml.hr^{-1}.g^{-1}$ (12) | 35 (2) | 35 (2) |
| trout | 586 $mg.hr^{-1}.g^{-1}$ (14) | 8.6 (15) | 15 (assume) |
| pigeon | 200 $ml.min^{-1}.kg^{-1}$ (16) | 20 (13) | 43.3 (16) |
| rat heart | 43.5 $\mu mol.min^{-1}.g^{-1}$ (17)** | - | 35 (17) |
| rat hindlimb | 32.7 $\mu mol.min^{-1}.(15g)^{-1}$ (18)** | | 35 (18) |
| man thigh muscle | 11.2 $ml.min^{-1}.(100\ g)^{-1}$ (19)** | | 37 (assume) |

*Data are given in $ml.hr^{-1}.g^{-1}$ (wet weight of muscle)

**The data from these preparations are given in terms of g wet weight muscle.


(1) Jongbloed & Wiersma (1934)

(2) Weis-Fogh (1952)

(3) Krogh & Zeuthen (1941)

(4) Kammer & Heinrich (1974)

(5) Zebe (1954)

(6) Crabtree & Newsholme (1972b)

(7) Heinrich & Bartholomew (1971)

(8) Polacek & Kubista (1960)

(9) Hofmanova et al. (1966)

(10) Krogh & Weis-Fogh (1951)

(11) Weis-Fogh (1967a)

(12) Davis & Fraenkel (1940)

(13) Greenewalt (1962)

(14) Webb (1971)

(15) Webb (1970)

(16) Butler et al. (1977)

(17) Taegtmeyer et al. (1979)

(18) Rennie & Holloszy (1977)

(19) Asmussen et al. (1939)

(20) Field et al. (1939)

(21) Dorsett (1962)

APPENDIX VI (cont'd.)

TCA cycle equivalents of oxygen uptake during utilisation of various fuels

(i.e. $\mu$mol $C_3/C_2$ per $\mu$mol $O_2$)

palmitate - 8 turns of the TCA cycle per 23 mol $O_2$ = 2.875

glucose - 2 " " " " " " 6 mol $O_2$ = 3.0

pyruvate - 1 " " " " " " $2\frac{1}{2}$ mol $O_2$ = 2.5

acetate - 1 " " " " " " 2 mol $O_2$ = 2.0

lactate - 1 " " " " " " 3 mol $O_2$ = 3.0

For major fuels, TCA cycle equivalents = 2.875 - 3.0 $\mu$mol $C_3/C_2.(\mu\text{mol } O_2)^{-1}$

The conversion factor is therefore assumed to be 3.

Calculation of TCA cycle flux from oxygen uptake data

e.g. honey bee

$O_2$ uptake = 100 $ml.hr^{-1}.g^{-1}$ (whole body)

Assuming that the resting rate of $O_2$ uptake is negligible in comparison with the rate during flight, and assuming that the increase in oxygen uptake is due to the flight muscles alone, this corresponds to an oxygen uptake of

$$= 100 \times \frac{100}{17} \text{ ml } O_2.hr^{-1}.g^{-1} \text{ (flight muscles)}$$

$$= 100 \times \frac{100}{17} \times \frac{1}{60} \text{ ml } O_2.min^{-1}.g^{-1}$$

1 $\mu$mol $O_2$ occupies 22.4 $\mu$l of $O_2$

$$\therefore O_2 \text{ uptake} = \frac{100 \times 100 \times 1000}{17 \times 60 \times 22.4}$$

$$= 437.675 \ \mu\text{mol } O_2.min^{-1}.g^{-1} \text{ at } 40^{\circ}C$$

Assuming a $Q_{10}$ of 2 over the range 25°C to 40°C, the rate at 25°C = 0.36 x the rate at 40°C (see Appendix VII)

$$= 157.563 \ \mu\text{mol } O_2.min^{-1}.g^{-1} \text{ at } 25^{\circ}C.$$

TCA cycle flux = 53 $\mu$mol $C_3/C_2.min^{-1}.g^{-1}$ at 25°C

# APPENDIX VII

## ESTIMATION OF FLUX RATES AT DIFFERENT TEMPERATURES

A $Q_{10}$ of 2.0 is assumed for metabolic rates and enzyme reactions over the whole range of 15°C to 40°C.

If $T_1$ = the higher temperature in K

$T_2$ = the lower temperature in K

$k_1$ = the rate of the reaction at $T_1$

$k_2$ = the rate of the reaction at $T_2$

Then $k_1 \propto e^{-E_{A/RT_1}}$ and $k_2 \propto e^{-E_{A/RT_2}}$

where $E_A$ = energy of activation of the reaction

$R$ = universal gas constant = 1.987 $cal.mol^{-1}.K^{-1}$

$$\ln \frac{k_1}{k_2} = \frac{-E_A}{RT_1} + \frac{E_A}{RT_2}$$

$$\ln \frac{k_1}{k_2} = \frac{-E_A}{R} \cdot \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \quad \text{.....(i)}$$

Assuming that $Q_{10} = 2$ over the range 25°C to 35°C

$T_1$ = 308 K

$T_2$ = 298 K

and $$\ln 2 = \frac{-E_A}{R} \cdot \left( \frac{298 - 308}{308 \times 298} \right)$$

$$\ln 2 = \frac{-E_A}{R} \cdot \left( \frac{-10}{308 \times 298} \right)$$

$$\therefore \frac{E_A}{R} = \frac{\ln 2 \times 308 \times 298}{10} \quad \text{.....(ii)}$$

Substituting into (i)

$$\ln \frac{k_1}{k_2} = \left( \frac{\ln 2 \times 308 \times 298}{10} \right) \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \text{.....(iii)}$$

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