Pyruvate Metabolism by Isolated Rat Liver Mitochondria as a Function of Adenosine Diphosphate Controlled Respiratory State*

S. C. Stuart and G. R. Williams

ABSTRACT: The addition of adenosine diphosphate (ADP) to isolated rat liver mitochondria utilizing pyruvate causes marked changes in the pattern of metabolism of this substrate. Of these effects, some, such as the inhibition of malate synthesis and the incorporation of carboxyl-labeled pyruvate into citrate, occur equally as well in the presence of oligomycin and thus represent direct actions of ADP upon enzymes involved in pyruvate metabolism. On the other hand other effects, such as the disappearance of endogenous malate or the change in the β-hydroxybutyrate/acetoacetate ratio, do not occur when oligomycin is present and thus are the consequence of the effect of ADP on the electron transport system. The results indicate that an important relationship between respiratory control and the rate of tricarboxylic acid cycle activity exists on the pathway between malate and citrate. This rate-limiting interaction is discussed.

It was first demonstrated by Lardy and Wellman (1952) that, under appropriate conditions, the rate of respiration of isolated mitochondria may be severely limited in the absence of a system which can utilize the high-energy intermediates (or discharge the high-energy state) formed concomitantly to the uptake of oxygen by these preparations. When a phosphate acceptor such as ADP[1] is added to these mitochondria the respiration rate increases many fold and certain well-defined transitions occur in the redox state of the mitochondrial respiratory carriers (Chance and Williams, 1955). Such an increase in the uptake of oxygen clearly implies a greater flux of substrate through the metabolic sequences which supply reducing equivalents to the dehydrogenases of the respiratory chain and, in the case of compounds for which alternative enzymic pathways exist, it is possible that major qualitative changes in their pattern of metabolism may occur. The general field has been the subject of a detailed review by Greville (1966) but comparatively little experimental information is available about the relationship between respiratory control and substrate metabolism, although Von Korff (1965) has commenced a detailed study of this relationship in heart mitochondria and the work of Berry (1965) on rat liver cells is clearly relevant. In recent experiments from this laboratory a clear-cut effect of phosphate acceptor systems on the operation of the tricarboxylic acid cycle was observed (Figure 12 of Williams, 1965) and these experiments prompted the investigations reported here. While this work was in progress the experiments of Lardy *et al.* (1965) and Walter *et al.* (1966) were reported and although the work of this group was directed toward a somewhat different problem and used a different experimental approach much of their data is complementary to that reported here.

One of the principal problems is to distinguish between three types of effect of adenosine diphosphate upon mitochondrial metabolism: (a) direct effects as an allosteric ligand on enzymes such as pyruvic carboxylase (Keech and Utter, 1963) and the NAD^+-linked isocitric dehydrogenase (Chen and Plaut, 1965; Klingenberg *et al.*, 1965); (b) indirect effects brought about as a consequence of the marked changes in the redox state of nicotinamide nucleotide which may be expected to alter the kinetics and equilibria of dehydrogenase reactions; (c) indirect effects due to the synthesis of ATP, *e.g.*, the inhibition of citrate synthase (Hathaway and Atkinson, 1965; Shepherd and Garland, 1966) or the relief of oxaloacetate effects (Williams, 1961). A similar classification of ADP effects has been made by Klingenberg (1966). The distinction between the two former types of actions is made relatively simple by the use of the inhibitor oligomycin (Lardy *et al.*, 1958) which will inhibit effects of ADP mediated *via* the redox carriers without interfering with its action as an allosteric ligand. This present report is primarily concerned with the use of oligomycin to permit such a distinction and to indicate some of the sites at which ADP can influence pyruvate metabolism by isolated rat liver mitochondria.

Materials and Methods

The mitochondria were isolated from the livers of male rats weighing about 250 g using the homogeniza-

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[1] Abbreviations used: ADP, adenosine diphosphate; NAD^+, oxidized nicotinamide-adenine dinucleotide; ATP, adenosine triphosphate; NADH, reduced nicotinamide-adenine dinucleotide.

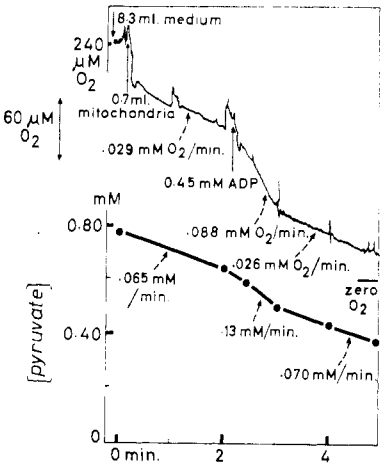

FIGURE 1: Correlation of oxygen consumption (polarograph tracing, upper curve) and pyruvate disappearance (lower curve) in a mitochondrial suspension. Additions were made and samples withdrawn as indicated.

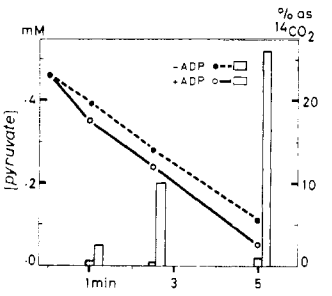

FIGURE 2: The effect of ADP addition upon the proportion of ^{14}C appearing as $^{14}CO_2$ from C-2-labeled pyruvate. Techniques of pyruvate analyses (circles) and $^{14}CO_2$ collection (bars) are described under Methods. The right-hand ordinate indicates the counts recovered as a percentage of the counts corresponding to the decrease in pyruvate concentration at a given time.

tion and differential centrifugation procedure described by Lardy and Wellman (1952) but in the lightly buffered mannitol–sucrose medium introduced by Chance and Hagihara (1960). The final pellet was suspended in the same medium to give a volume approximately one-tenth of the volume of liver used for the preparation. Such suspensions contain approximately 60 mg of mitochondrial protein/ml. The medium used for the experiments contained: 0.05 M Tris–HCl, 0.05 M KCl, 0.005 M $MgSO_4$, 0.010 M sodium phosphate, and 0.15 M sucrose, pH 7.4.

Respiration was measured polarographically in an open rotating cup using a bare platinum cathode and a silver anode (Williams, 1960). The stock mitochondrial suspension was diluted into ten times the volume of the medium described above. Samples for metabolite assay were withdrawn by pipet and added to an equal volume of cold 0.6 M perchloric acid, the time of mixing being noted. When oxygen uptake was not being monitored the incubation was carried out in a rotating centrifuge tube (Ferguson and Williams, 1966). Metabolite assays were carried out on the deproteinized suspensions after neutralization with KOH and removal of precipitated potassium perchlorate. Pyruvate was assayed by the lactic dehydrogenase technique, the oxidation of NADH being followed to completion spectrophotometrically. Other enzymatic assays were performed fluorimetrically using an Eppendorf fluorimeter modified according to Estabrook and Maitra (1962). The system used for the assay of malate was that recommended by Hohorst (1963) and for isocitrate and citrate that recommended by Siebert (1963). For this latter assay aconitase was prepared by the method of Morrison (1954) except that acetone was used as precipitant instead of alcohol (Siebert, 1963; Ferguson and Williams, 1966). At the level of citrate assays reported below it is not possible to use citrate itself as a stabilizer for aconitase. We are grateful to Dr. J. R. Williamson for informing us of the introduction by Dr. Nelson Goldberg of 1,2,3-propanetricarboxylic acid as a stabilizer and this compound was used in place of citric acid in the aconitase preparations used in these experiments. Acetoacetate was determined by the method of Walker (1954) and β-hydroxybutyrate by the method of Berry (1963). The β-hydroxybutyrate assay system was used to standardize the acetoacetate determination. Doubly distilled water was used throughout and the chemicals used were of the highest purity commercially available. The oligomycin used was obtained from Sigma and was stated to be a mixture of 15% oligomycin A and 85% oligomycin B. Intramitochondrial nicotinamide nucleotide reduction and oxidation were measured by a dual wavelength spectrophotometer (American Instrument) at 340 − 360 mμ.

When it was necessary to collect $^{14}CO_2$ the incubation was carried out in a rocking reaction vessel (Williams, 1965) and $^{14}CO_2$ was determined by trapping in phenylethylamine (Woeller, 1961) supported on a solid substrate (Stuart and Williams, 1966c), followed by dissolution of the [^{14}C]carbamate in toluene containing 0.4% (w/v) 2,5-diphenyloxazole and 0.01% (w/v), 1,4-bis-2-(5-phenyloxazolyl)benzene, and counting in a liquid scintillation counter. The reaction was terminated by the addition of an equal volume of 0.6 M perchloric acid and shaking was continued for 2 min to ensure complete elimination of $^{14}CO_2$. Radioactive malate and citrate were isolated by paper chromatography in the butanol–formic acid–water (40:10:50) system (Lugg and Overell, 1948). Carrier acids were added to facilitate location by cresol green and the spots were cut out and counted directly on the paper in the scintillation fluid described above. [^{14}C]Pyruvate was obtained from New England Nuclear Corp.; the precautions suggested by Von Korff (1964) were observed in making stock solutions of this material, but the solutions were not used later than 4–6 weeks after purchase.

Throughout this paper all metabolite concentrations are reported as the apparent molarity present in the

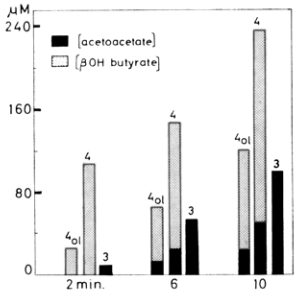

FIGURE 3: The effect of ADP (state 3) and ADP plus oligomycin (state 4_{ol}) upon the production of ketone bodies and the proportion of acetoacetate to β-hydroxybutyrate. The initial concentration of pyruvate was 1.0 mM and that of ADP was 3.8 mM. Oligomycin was present at a concentration of 8.0 μg/ml.

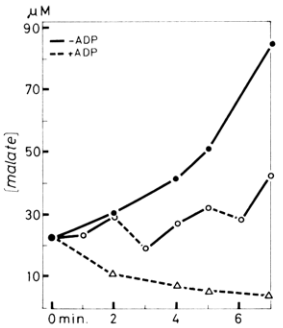

FIGURE 4: Effect of ADP addition upon the production of malate from pyruvate. The initial concentration of pyruvate was 0.75 mM and that of ADP was 2.9 mM (bottom curve). In the middle tracing state 3 was established briefly by two additions of 0.25 and 0.20 mM ADP as indicated.

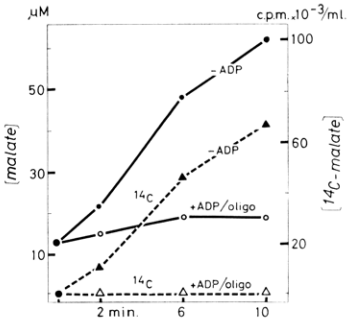

FIGURE 5: Effect of ADP and of ADP plus oligomycin upon the production of malate from pyruvate. The initial concentration of carboxyl-labeled pyruvate was 0.56 mM and 5.35×10^5 cpm/ml. ADP was added at a concentration of 3.3 mM and oligomycin at 5.5 μg/ml.

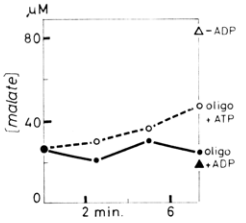

FIGURE 6: Partial restoration of oligomycin-blocked malate synthesis by ATP. Initial concentrations were, pyruvate, 1.0 mM; ADP, 7.4 mM; ATP, 5.0 mM; oligomycin 12.0 μg/ml.

suspension after tenfold dilution as described above. No attempt has been made to determine whether the metabolites are intra- or extramitochondrial. The terminology of mitochondrial states used is that introduced by Chance and Williams (1955), *i.e.*, mitochondria to which no ADP has been added or which have exhausted added ADP by phosphorylation are referred to as being in state 4. Mitochondria in the presence of ADP are normally in state 3 but for the purposes of this paper it is useful to refer also to state 4_{ol} which indicates mitochondria to which ADP has been added in the presence of oligomycin.

Results

Pyruvate Utilization. It will be seen in Figure 1 that the rate of oxygen uptake by a suspension of rat liver mitochondria is stimulated only threefold by the addition of ADP when 0.80 mM pyruvate is used as the substrate. Such a low degree of stimulation compares unfavorably with the respiratory control ratios observed using the same mitochondria with glutamate or β-hydroxybutyrate as added substrate. The lower trace indicates that the stimulation of pyruvate disappearance is even less marked but an effect of added ADP is clearly observable. It should be noted that in state 3 and in state 4 the $\Delta O_2/\Delta$pyruvate ratios are, respectively, 0.68 and 0.45 indicating that there is very little complete oxidation of pyruvate under either circumstance. This suggestion is borne out by studies using C-2-labeled pyruvate (Figure 2). Here it will be seen that there is little appearance of $^{14}CO_2$ from this source in state 4 and that there is still only a relatively small percentage of the utilized pyruvate appearing as $^{14}CO_2$ in state 3. However, both the over-all $\Delta O_2/\Delta$pyruvate ratio and the appearance of $^{14}CO_2$ from C-2-labeled pyruvate do indicate a marked shift toward complete oxidation upon the addition of ADP.

Ketone Body Production. Figure 3 shows that the addition of ADP causes marked changes both in the quantity and nature of the "ketone bodies" produced. There is a very much greater production of acetoacetate plus β-hydroxybutyrate in state 4 and a much smaller proportion is in the form of acetoacetate. In state 3 the ratio acetoacetate/acetoacetate + β-hydroxybutyrate is 1.00 at all times whereas in state 4 it is 0.0 at 2 min

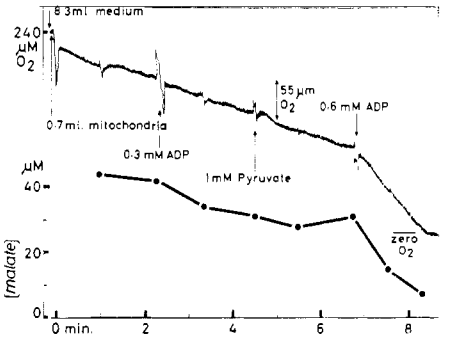

FIGURE 7: Necessity of pyruvate for the effect of ADP upon the level of endogenous malate. Additions and sampling times as indicated. Upper curve, polarograph tracing of oxygen tension; lower curve, malate concentration.

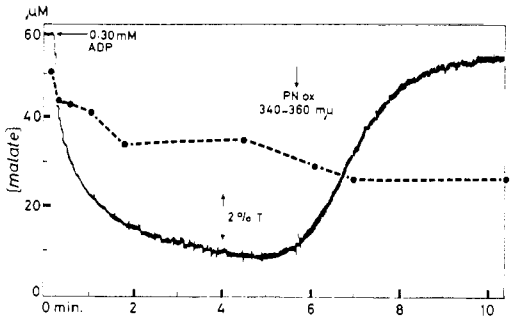

FIGURE 8: Simultaneous recording of nicotinamide nucleotide oxidation (solid line) and malate disappearance (dashed line) upon addition of ADP. The vessel from which samples were withdrawn for malate analyses was held in a bath at 6° from which coolant was passed through the cell block of the Aminco-Chance spectrophotometer. The cuvet was therefore about 1° higher in temperature than this vessel. A first cycle of nicotinamide nucleotide oxidation and reduction was performed and the samples withdrawn for malate analysis during a second cycle shown in this figure.

and rises to only 0.2 at 10 min. In the presence of oligomycin ADP is able to reduce the ketone body production to rates characteristic of state 3 but the proportion of acetoacetate and β-hydroxybutyrate in the mixture resembles that found in the absence of added ADP.

Malate Production and Disappearance. In contrast to the case of acetoacetate and β-hydroxybutyrate, the malate concentration of the system does not start from zero. The mitochondria as prepared contain endogenous malate at a level of about 5 nmoles/mg of protein. Figure 4 shows that if the mitochondria metabolize pyruvate in state 4, the level of malate in the incubation mixture may increase four to five times whereas upon addition of ADP the level of malate falls. If repeated small additions of ADP are made, the malate concentration will fall during state 3 and will then rise during

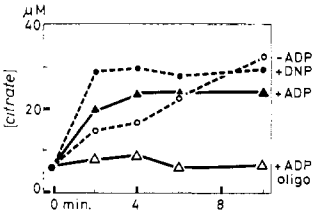

FIGURE 9: Mitochondrial citrate concentration during the metabolism of pyruvate by mitochondria. Initial concentrations were: pyruvate, 0.56 mM; ADP, 3.3 mM; oligomycin, 5.5 µg/ml; 2,4-dinitrophenol, 55 µM.

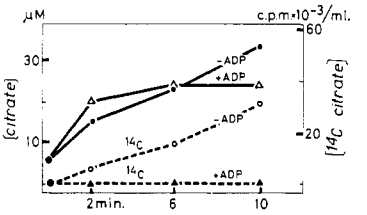

FIGURE 10: Appearance of ^{14}C in citrate during pyruvate metabolism. Pyruvate and ADP concentrations as in the previous figure. Carboxyl-labeled pyruvate was present initially at 5.3×10^5 cpm/ml.

the period following exhaustion of the added ADP. In the presence of oligomycin a state of affairs results which clearly shows the effects of ADP upon malate to be dual in nature (Figure 5). ADP can still completely inhibit malate synthesis as shown by the failure of malate to rise and the nonappearance of [^{14}C]malate from carboxyl-labeled pyruvate. Control experiments show that oligomycin alone will inhibit malate synthesis but this inhibition is partially reversible by ATP (Figure 6). On the other hand in the presence of oligomycin and ADP although the malate no longer rises as in state 4, neither does it fall as it would if ADP were added alone. The action of ADP in causing the fall in malate is therefore brought about by an oligomycin sensitive system. This decrease in malate brought about by ADP requires also in some preparations the presence of pyruvate (or butyrate) (Figure 7). This dependency is not observed in all mitochondrial preparations. In many preparations the addition of ADP alone will reduce the malate content of the system to such low values that it is difficult to observe a further effect of pyruvate. It may be that these preparations have more extensive endogenous sources of acetyl-CoA. The difficulty may therefore be circumvented by an incubation with low concentrations of succinate (0.25 mM) which will raise the malate concentration of the system. When this is done ADP will then bring about a fall in the malate concentration but the situation now resembles that of Figure 7 in that the decrease does not proceed indefinitely and a further fall will occur if pyruvate is added. Since the addition of exogenous succinate is

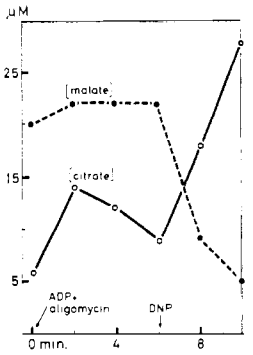

FIGURE 11: Cross-over of malate and citrate concentration upon addition of 2,4-dinitrophenol. The mitochondria were in state 4_{oi}, ADP being present at a concentration of 0.87 mM, oligomycin at 4.3 μg/ml. 2,4-Dinitrophenol was added as indicated at a concentration of 61 mM. Initial pyruvate concentration was 0.5 mM.

an additional complication such experiments have only been performed to support the above explanation for our inability to obtain consistently data such as that of Figure 7. It is important also to observe that although the drop in malate concentration in state 3 can be reversed during a subsequent period in state 4 (Figure 3, middle tracing), the changes are not closely synchronized with fluctuations in the redox state of the intramitochondrial nucleotides (Figure 8).

Citrate Production and Disappearance. The case of citrate resembles that of malate in that the mitochondria as isolated contain endogenous metabolite and that one therefore observes net changes from a finite value at zero time. The changes in citrate (Figure 9) differ far less from states 3 to 4 than do the corresponding changes in malate although the more rapid initial increase in the presence of ADP and a higher final value in its absence are consistently observed. On the other hand Figure 10 demonstrates that the origin of this citrate differs markedly between the two states. In state 4 the incorporation of ^{14}C from carboxyl-labeled pyruvates closely parallels the accumulation of citrate, whereas the citrate produced in state 3 arises from nonradioactive sources. In the presence of oligomycin and ADP, citrate accumulation does not occur (Figure 9) and citrate remains at approximately the value found at zero time, as does malate (see above). When 2,4-dinitrophenol is added to such a system there are marked reciprocal changes in malate, which falls, and citrate, which rises. Such changes are shown in Figure 11. In similar experiments the addition of 5.0 mM ATP to the system did not influence the extent or rate of the "crossover."

Discussion

The above data show that the metabolism of pyruvate by rat liver mitochondria in state 4 possesses the following characteristics: (a) a high production of β-hydroxybutyrate plus acetoacetate; (b) a high proportion of β-hydroxybutyrate in that mixture; (c) an accumulation of malate; (d) an efficient incorporation of C-1-labeled pyruvate into malate; (e) a slow, prolonged accumulation of citrate; (f) an efficient incorporation of C-1-labeled pyruvate into citrate. These characteristics should be contrasted with those of state 3: (a′) a lowered production of β-hydroxybutyrate plus acetoacetate; (b′) a negligible proportion of β-hydroxybutyrate in that mixture; (c′) a disappearance of endogenous malate; (d′) negligible incorporation of C-1-labeled pyruvate into malate; (e′) a rapid but limited accumulation of citrate; (f′) a failure of ^{14}C from the carboxyl group of pyruvate to appear in citrate. The six-paired characteristics listed above distinguish the metabolic states of mitochondria and the transition between any named characteristic on the first list and the corresponding characteristic on the second may be brought about by the addition of ADP.

It is useful also to list the corresponding characteristics of the state achieved when ADP is added together with oligomycin (state 4_{oi}): (a″) a lowered production of "ketone bodies;" (b″) a high proportion of β-hydroxybutyrate in that mixture; (c″) malate neither accumulates nor disappears; (d″) negligible incorporation of C-1-labeled pyruvate into malate; (e″) citrate remains approximately at the endogenous level; (f″) negligible incorporation of C-1-labeled pyruvate into citrate.

Before turning to the significance of these findings it should be emphasized that one of the chief hazards in interpretation lies in the fact that our experiments were not designed to determine the location of the metabolites within the diluted mitochondrial suspension. Even in the cases of malate and citrate which must surely be intramitochondrial in the well-washed stock suspension it may be that these intermediates will diffuse into the medium containing electrolytes into which the stock suspension is diluted.

The most easily identified action of ADP is its inhibition of the incorporation of carbon from C-1 of pyruvate into malate and citrate (d and f above). The effect is not sensitive to oligomycin and must therefore be brought about by a direct effect of ADP upon some mitochondrial enzyme system. There are two enzymes which might be implicated in the carboxylation of pyruvate; malic enzyme (EC 1.1.1.40) and pyruvate carboxylase (EC 6.4.1.1.). The former is extramitochondrial in origin and is not known to be influenced by adenine nucleotides. It therefore seems most reasonable to relate this action of ADP to the inhibition of pyruvate carboxylase (Keech and Utter, 1963). This conclusion is reinforced by data such as those of Figure 6 where it is shown that oligomycin itself inhibits the carboxylation process but that the inhibition may be partially overcome by added ATP. In the presence of oligomycin there will be no possibility of generating mitochondrial ATP which is itself one of the substrates for pyruvic carboxylase. The incomplete relief of this inhibition by exogenous ATP may be related to per-

meability barriers for this nucleotide (Chappell and Crofts, 1965; Pfaff *et al.*, 1965). This interpretation is in agreement with that of Walter *et al.* (1966) on the effect of ADP on CO_2 fixation. A similar conclusion concerning the effect of ADP on pyruvate carboxylation has been reached by Berry (1965) though he did not demonstrate the effect to be oligomycin insensitive and was thus unable to exclude the possibility of the effect being a consequence of the changed redox state of the nicotinamide nucleotides. On the other hand our demonstration that ADP has an "antiketogenic" effect (a above) is in direct contrast with Berry's results. In his experiments the "ketogenic" effect of ADP was clearly related to a relative deficiency of C_4-dicarboxylic acids, the shortage arising as a result of the inhibition of pyruvate carboxylation discussed above. In long-term (60 min) experiments of the type performed by Berry such a consequence might well occur. It is noteworthy that expt 2 of Table IX in Berry (1965) shows that a reduction of the incubation time to 30 min comes close to abolishing the ketogenic effect of ADP. Figure 3 of this paper demonstrates that a primary effect of ADP and one which is oligomycin insensitive is to reduce the production of acetoacetate plus β-hydroxybutyrate. It is less easy to identify this action of ADP with any known effect of this nucleotide on isolated enzymes. The insensitivity to oligomycin not only rules out the involvement of nicotinamide nucleotides but also makes it unlikely that a fall in intramitochondrial ATP is involved.

Of the oligomycin-sensitive effects the one most clearly related to the NAD^+/NADH ratio is the change in the ratio of β-hydroxybutyrate to acetoacetate. The change is in the expected direction, *i.e.*, when the NAD^+/NADH ratio rises in state 3 little β-hydroxybutyrate appears and the converse holds in state 4. These results, however, do not serve to indicate whether the β-hydroxybutyrate arises by reduction of acetoacetate or whether as has been suggested (Wakil and Bressler, 1962) β-hydroxybutyrate is the primary product and is then oxidized to acetoacetate. The time course of events in state 4 might lend some support to the latter interpretation. Another problem concerns the quantitative relationship found. The difference in redox potential at pH 7 between the β-hydroxybutyrate/acetoacetate couple and the NAD^+/NADH couple is about 40 mv (Clark, 1960), and the equilibrium constant should therefore be about 20. The values of NAD^+/NADH observed in this laboratory (using the state 3–state 5 transition as 100%) are about 20 (state 3) and 0.5 (state 4) in fair agreement with the values originally reported (Chance and Williams, 1955). The corresponding values of β-hydroxybutyrate/acetoacetate are 0 (state 3) and 4 (state 4) giving an experimentally observed equilibrium constant of about 2. Such a discrepancy may mean that the nicotinamide nucleotide associated with mitochondrial β-hydroxybutyrate dehydrogenase has a much higher NAD^+/NADH ratio than the average mitochondrial pool or that the effective pH at the reaction site is about 6.0. It is not possible to choose between these alternatives on the basis of the present data but the possibility of using this technique as a probe of mitochondrial pH is of considerable interest.

The case of β-hydroxybutyric dehydrogenase where the equilibrium constant at pH 7 $\simeq$ 20 may be contrasted with that of malic dehydrogenase where the corresponding constant is about 10^5 (Naval and Wolfe, 1962). In this case the equilibrium mixture will contain almost exclusively malate even when the NAD^+/NADH ratio is considerably higher than the value of 20 reported above for state 3. The oligomycin-sensitive drop in malate brought about by ADP must therefore require not only a change in the NAD^+/NADH ratio but also a removal of oxaloacetate. This suggestion is borne out both by the failure to observe a simple relationship between malate and the observed changes in nicotinamide nucleotide redox state (Figure 8) and the observation that the fall in malate may not occur in the absence of pyruvate (or butyrate), presumably as a source of acetyl-CoA (Figure 7). The problems of malate oxidation have been discussed by Greville (1966), Klingenberg (1966), and Tager and Slater (1963) but our evidence does not seem to favor the involvment of an energy-requiring step in oxaloacetate removal. In the presence of glutamate, ^{14}C is transferred quantitatively from malate to aspartate when ADP is added (S. C. Stuart and G. R. Williams, unpublished observations). On the other hand, the concept proposed by Shepherd *et al.* (1965) of a reductive role for malic dehydrogenase in state 4 is strongly supported by our evidence.

The final case to be considered is that of citrate (e and f above). Although an accumulation of citrate occurs in both states 3 and 4, the origins of this citrate differ markedly. In the absence of ADP, the citrate is clearly derived from C_4-dicarboxylic acids into which pyruvate has been incorporated as a C_3 unit. On the other hand, when ADP is added, the synthesis of citrate presumably is related to the concomitant drop in malate and the plateau in citrate accumulation is a reflection of the exhaustion of endogenous malate. This interpretation is supported by experiments such as that of Figure 11 in which a "crossover" occurs between malate and citrate. Indeed this state 4_{ol}–state 3 transition identifies an important control site between malate and citrate. The rate of malate to citrate conversion will be influenced by the intrinsic activities of malic dehydrogenase and citrate synthase, by any specific activators and inhibitors of these enzymes and by the concentrations of NAD^+ and acetyl-CoA available. The oligomycin sensitivity of the crossover points to the NAD^+/NADH ratio as being of importance. The shifts in the redox steady state of the nicotinamide nucleotides are thus not merely automatic effects of the change in flow of reducing equivalents but are shown, in this instance as well as that of the acetoacetate/β-hydroxybutyrate ratio, to be significant causal determinants of mitochondrial metabolism.

On the other hand, the data presented also clearly show that the effects of ADP upon mitochondrial metabolism are not confined to those brought about

by changes in the redox state of the nicotinamide nucleotides but that the metabolic changes resulting from the state 3–state 4 transition (or vice versa) must be the resultant of the oligomycin-sensitive effects plus allosteric effects on specific enzymes such as pyruvic carboxylase. The over-all picture is therefore complex and must be even more complicated when the effects of ATP produced in the system have to be taken into account. Such effects may not however be as important in tricarboxylic acid cycle regulation as has been claimed (Hathaway and Atkinson, 1965; Shepherd and Garland, 1966) since we have been unable to demonstrate significant changes in the pattern of metabolism reported here when ATP is added, and, in successive cycles with ADP, no obvious differences are seen in later cycles when ATP has accumulated. As noted in the Experimental Section, attempts to demonstrate specifically an effect of ATP on the malate–citrate crossover were unsuccessful.

The emphasis which our experimental data force us to place upon the malate–citrate region of the tricarboxylic acid cycle as a control site does not mean that other effects such as the ADP control of the NAD^+-linked isocitric dehydrogenase are of no importance in the regulation of this pathway although certainly the accumulation of citrate in state 3 is not easy to reconcile with the role suggested for this activation by Klingenberg (1966). Nor do our data necessarily contradict the suggestion made by one of us (Williams, 1965) and by others (R. W. Von Korff, personal communication) that in isolated heart mitochondria the rate-controlling step of the tricarboxylic acid cycle is at the succinic dehydrogenase step, at least in the absence of an active system for removal of oxaloacetate. The active carboxylating systems of liver would be expected to bring about a very different metabolic pattern from that of heart and the role of this system in gluconeogenesis and lipogenesis (Lardy, 1966) would make control systems which may be appropriate for muscle less appropriate for liver.

Acknowledgments

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Stimulation of Oxidative Phosphorylation in Mitochondria by Potassium in the Presence of Valinomycin*

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ABSTRACT: Conditions have been described under which the valinomycin induced transport of K^+ into mitochondria stimulates the phosphorylation of endogenous nucleotides. These same conditions stimulate the rate of phosphorylation of added adenosine 5′-diphosphate but respiration is also elevated, even beyond the rates obtainable with uncoupling agents. Thus the P/O ratios do not rise above the values conventionally obtained. Similar effects can also be obtained with the macrolide actin ion transport inducers, but not the gramicidins; induced Na^+ transport will not substitute for induced K^+ transport. It is suggested that a component reaction of oxidative phosphorylation is K^+ activated and regulated by the level of K^+ available at a specific mitochondrial locus. This locus is deficient in K^+ in mitochondria as normally isolated, but can become saturated during the energy-dependent transport of K^+ induced by antibiotics.

It has been reported by Pressman and Lardy (1952) that K^+ helps sustain the prolonged respiration of phosphate acceptor free mitochondria and stimulates the short-term phosphorylation of appropriately treated mitochondria (1955). It was concluded that the K^+ sensitivity of mitochondria is more closely associated with phosphorylation than with oxidative processes, and governed by the levels of K^+ associated with the mitochondria and only indirectly by the extramitochondrial $[K^+]$. Gamble (1957) also observed close relationships between K^+ binding and oxidative phosphorylation in both intact mitochondria and mitochondrial fragments. Recently, however, Opit and Charnock (1962, 1965) have challenged the capability of K^+ to stimulate either mitochondrial respiration or phosphorylation.

The property of valinomycin and other antibiotics to induce an extensive transport of K^+ and other alkali ions into mitochondria (Moore and Pressman, 1964; Pressman, 1965b) has provided a means of reexamining this question within a somewhat different context. The present paper provides evidence that valinomycin-induced K^+ accumulation in mitochondria stimulates both mitochondrial respiration and phosphorylation of the bound adenine nucleotides of mitochondria as well as added ADP.[1] The maximal, state 3 (*cf.* Chance and Williams, 1955) respiration rate with a given substrate system, under the influence of appropriate levels of valinomycin and K^+, can attain values well above the maximal respiration rate produced by adding an uncoupler such as 2,4-dinitrophenol (DNP). Under a wide range of conditions the ADP/O ratio is maintained at the usual values as the phosphorylation of ADP is also stimulated beyond rates commonly obtained in state 3. A preliminary report of this work has been published (Höfer *et al.*, 1966).

Materials and Methods

Rat liver mitochondria were isolated essentially as described by Schneider (1948). The original homogenization was carried out in 0.25 M sucrose containing 1 mM EDTA (pH 7.4), followed by three washings with 0.25 M sucrose alone, and the mitochondria were stored in a suspension equivalent to *ca.* 50 mg of protein/ml, until added to the experimental system.

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[1] Abbreviations used: ADP and ATP, adenosine 5′-di- and triphosphates, respectively; DPNH and TPNH, reduced di- and triphosphopyridine nucleotides, respectively; ~P, high-energy, *i.e.*, anhydride bond, phosphate.