The Effects of Adenine Nucleotides on Pyruvate Metabolism in Rat Liver

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(Received 21 August 1964)

1. The effects of adenine nucleotides on pyruvate metabolism by isolated liver cells and isolated mitochondria have been investigated. The amount of pyruvate carboxylated has been estimated by determining the tricarboxylic acid-cycle intermediates, glutamate and aspartate accumulating in the incubation medium. The extent of pyruvate oxidation has been assessed by measuring oxygen uptake and the yield of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{pyruvate}$ and $[2\text{-}^{14}\text{C}]\text{pyruvate}$. 2. When catalytic amounts of adenine nucleotides (1–2 mM) were added to suspensions of isolated liver cells incubated with pyruvate an ATP:ADP ratio greater than 6:1 was maintained. Both pyruvate oxidation to acetyl-CoA and the oxidation of acetyl-CoA through the tricarboxylic acid cycle were stimulated but pyruvate carboxylation was not affected. The production of acetyl-CoA exceeded the capacity of the cells for the oxidation of acetyl-CoA and the excess was converted into ketone bodies. 3. If a low ATP:ADP ratio was maintained in isolated cells or mitochondria by incubating them with dinitrophenol or hexokinase, pyruvate carboxylation was grossly inhibited, oxygen uptake depressed and ketone-body formation stimulated. Measurement of oxaloacetate concentrations confirmed that under these conditions oxaloacetate was rate-limiting for the oxidation of acetyl-CoA via the tricarboxylic acid cycle. The inclusion in the incubation medium of fumarate (1.25 mM) completely prevented the ketogenic action of dinitrophenol or hexokinase. 4. When ADP (5 mM) was added to a suspension of isolated liver cells incubated with pyruvate an actual ADP concentration of about 1 mM was attained. This brought about effects on pyruvate metabolism similar to those obtained with dinitrophenol or hexokinase. 5. These results support the concept that the relative concentrations of adenine nucleotides within the liver cell may play a role in governing the rates of pyruvate oxidation and carboxylation. In addition, they provide further evidence that the availability of oxaloacetate in the liver cell can play a key role in determining whether acetyl-CoA arising from pyruvate is oxidized through the tricarboxylic acid cycle or converted into ketone bodies.

The investigation of the role of adenine nucleotides in the regulation of pyruvate oxidation and carboxylation in liver is complicated by the fact that many factors control the fate of pyruvate. These include the relative activities of various enzymes such as pyruvate dehydrogenase, pyruvate carboxylase, malic enzyme and lactate dehydrogenase, the concentration of cofactors such as NAD^+ , NADP^+ and acetyl-CoA, and the concentrations of other metabolites that interact with or compete with pyruvate. However, since the key enzymes concerned with pyruvate oxidation and carboxylation are located within mitochondria (Bartley, 1954; Keech & Utter, 1963; Utter, 1963)

it is possible to study the effects of adenine nucleotides on these processes in isolated liver cells and isolated mitochondria in which some of the complicating factors have been eliminated.

The metabolic behaviour of the mitochondria of isolated liver cells is similar to that of isolated mitochondria (Berry, 1962). Isolated cell suspensions have been preferred for the present work because they are easier and quicker to prepare, and cells can be obtained in much greater yield than isolated mitochondria. But, where possible, results obtained with cell suspensions have been confirmed with mitochondrial preparations.

The requirement of pyruvate carboxylase for ATP and the inhibition of this enzyme by ADP led Keech & Utter (1963) to suggest that the relative concentrations of ATP and ADP in the liver cell

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may regulate the fate of pyruvate. Evidence in favour of such a role for adenine nucleotides in the regulation of pathways of pyruvate metabolism in liver is provided by experiments reported in the present paper. It is shown that under suitable conditions ADP, at concentrations found *in vivo*, can stimulate the rate of pyruvate oxidation and suppress pyruvate carboxylation in isolated liver cells and isolated mitochondria.

In addition, these experiments support the concept that the availability of oxaloacetate in the liver cell can play a key role in determining whether acetyl-CoA, arising from pyruvate, is oxidized through the tricarboxylic acid cycle or, alternatively, is converted into ketone bodies (Lehninger, 1946; Krebs, 1960).

EXPERIMENTAL

[1-¹⁴C]Pyruvate and [2-¹⁴C]pyruvate were obtained from The Radiochemical Centre, Amersham, Bucks. On arrival the labelled pyruvate was dissolved in sufficient 0.4M-pyruvic acid (provided by Dr R. J. Haslam and prepared by the method of Haslam & Krebs, 1963) to give final specific activities of about 30 μ C/m-mole and stored at -15°. Immediately before use samples were neutralized and diluted with further unlabelled sodium pyruvate to give an appropriate specific activity.

Enzymes and cofactors. D-3-Hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides* (Williamson, Mellanby & Krebs, 1962) was prepared by Mr D. H. Williamson. The following enzymes and nucleotides were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany: lactate dehydrogenase, glucose 6-phosphate dehydrogenase, hexokinase, pyruvate kinase, adenylate kinase, malate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase, NAD⁺, NADH and NADP⁺. AMP, ADP and ATP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of isolated liver cells. Male Wistar albino rats weighing 250–350 g. were used in all experiments. Suspensions of isolated cells were prepared by a modification of the method of Branster & Morton (1957). A rat was anaesthetized with ether and the liver, portal canal and inferior vena cava were exposed, the latter being clamped just proximal to the left renal vein. A polythene cannula was inserted into the portal vein, leakage of blood round the cannula being prevented by a firmly fitting clamp. A 50 ml. syringe was attached to the cannula and the liver was perfused with about 45 ml. of 0.3M-sucrose solution in 0.01M-tris-HCl buffer, pH 7.4 (sucrose medium), at 37°. The perfusion pressure was gradually increased until the liver could not be distended further. The inferior vena cava was cut just distal to the left renal vein and the liver again perfused with 45 ml. of sucrose medium, with gentle massage of the surface of the organ to assist the flushing out of blood.

The blanched distended liver was removed, cut into small pieces, transferred to a glass homogenizer and gently dispersed with a loosely fitting Teflon pestle in 50 ml. of sucrose medium at 37°; 12 up-and-down strokes were always sufficient to disperse the cells. All subsequent procedures

were performed at 0–4°. The dispersion was filtered through two layers of nylon mesh to remove connective tissue and clumps of cells and the cells were separated from cell debris and erythrocytes by centrifuging the dispersion for 2 min. at 300g.

The cells were resuspended in 50 ml. of fresh medium and again centrifuged at 300g. They were now resuspended in 25 ml. of sucrose medium, centrifuged at 500g and taken up in the desired volume of fresh medium.

Preparation of isolated mitochondria. This was by the method of Schneider (1948), as modified by Werkheiser & Bartley (1957).

Incubations. The cells (1.33 ml.), suspended in sucrose medium, were added to Warburg vessels at 0° containing 2.67 ml. of solution. The final composition of the incubation medium was sucrose (0.1M), tris-HCl buffer, pH 7.4 (3.3 mm), KCl (0.05M), potassium phosphate buffer, pH 7.4 (10 mm), MgCl₂ (2.5 mm) and EDTA (1.25 mm). EDTA was included to prevent the mitochondrial swelling that occurs when isolated mitochondria or isolated liver cells are incubated with orthophosphate in the absence of added adenine nucleotide (Hunter & Ford, 1955; Berry, 1962). Mitochondria were incubated in a medium identical with that used for the incubation of isolated liver cells.

Oxygen uptake was measured in the conventional Warburg apparatus. The flasks, containing the complete reaction mixture in the main compartment and 0.2 ml. of 2N-KOH in the centre well, were incubated at 30° with oxygen as the gas phase. Flasks were equilibrated for 10 min. before the first reading.

Preparation of samples for analyses. At the end of the incubation the Warburg vessels were quickly removed from the bath and cooled in ice. To each vessel 1 ml. of 2N-perchloric acid was added followed by 3 ml. of water. If ¹⁴CO₂ was being collected 1 ml. of 2N-perchloric acid was tipped from the side arm and the vessel was allowed to shake after the addition of acid until no further change in gas pressure occurred (approx. 20 min.). The precipitated protein was removed by centrifuging and the supernatant transferred to a graduated tube. The volume was noted and 0.01 ml. of universal indicator was added. The tube was placed in ice on a magnetic stirrer and cautiously neutralized with ice-cooled 5N-KOH, the amount of KOH required being noted. The sample was allowed to stand at 2° for 30 min. and the precipitated potassium perchlorate removed by centrifuging. Analyses were carried out on the resultant supernatant.

Analytical techniques. The following metabolites were determined enzymically by standard spectrophotometric techniques: pyruvate (Bücher, Czok, Lamprecht & Latzko, 1962), lactate (Hohorst, 1962a), 2-oxoglutarate (Bergmeyer & Bernt, 1962), L-malate (Hohorst, 1962b), oxaloacetate (Hohorst & Reim, 1962), aspartate (Pfleiderer, 1962), glutamate (Bernt & Bergmeyer, 1962), ATP (Lamprecht & Trautschold, 1962), ADP and AMP (Adam, 1962). Succinate was determined with succinate oxidase from pigeon breast muscle (Krebs, 1937), but in every experiment the amount present was too low to be detected.

Fumarate was not determined, but the amount present was estimated on the basis of a malate:fumarate ratio 3.5 at 30° (Krebs, Smyth & Evans, 1940). Citrate was determined by the method of Taylor (1953). *cis*-Aconitate and isocitrate were not determined but were assumed to amount together to 10% of the citrate present (Krebs, 1953).

Ammonia was determined by diffusion in the Conway unit (Conway, 1947), followed by nesslerization.

Determination of ketone bodies. Acetoacetate was determined by the method of Walker (1954). In two experiments acetone was measured by the method of Thin & Robertson (1952), but in neither experiment did the amount of acetone present exceed 5% of the total ketone bodies accumulating. D-3-Hydroxybutyrate was determined either by quantitative conversion into acetoacetate (Berry, 1963) or by a modification of the method of Williamson *et al.* (1962) in which any malate likely to interfere with the determination was first removed by treatment of the sample with malate dehydrogenase and NAD⁺ at pH 9.4. Into a spectrophotometer cell was pipetted 1 ml. of tris-hydrazine buffer, pH 9.4 (10 ml. of 2M-tris, 1.0 ml. of hydrazine hydrate, 0.8 ml. of 0.1M-EDTA, sufficient 2N-NaOH to bring the pH to 9.4 and water to 40 ml.), 0.1 ml. of NAD⁺ (20 mg./ml.), the sample and water to give a final volume of 3.0 ml. Malate dehydrogenase (50 µg. of protein) was added and the reaction followed until there was no further increase in the extinction at 340 mµ. Sufficient 5N-HCl was then added to the cuvette to lower the pH to 8.6. The amount necessary was determined by titrating a measured portion of the buffer. The extinction at 340 mµ was measured again and D-3-hydroxybutyrate was determined as described by Williamson *et al.* (1962).

Radioactive-tracer techniques. The ¹⁴CO₂ trapped in 2N-KOH in the centre wells of the Warburg vessels was converted into Ba¹⁴CO₃ and plated on filter-paper disks, as described by Sakami (1955, p. 5). The plates were assayed for radioactivity in a gas-flow proportional counter (model D47; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). Counting was continued until 10000 counts had been recorded, and each plate was counted three times. The count was corrected for background, variations in the count of a standard source and for self-absorption (by correction to infinite thinness).

The specific activities of labelled pyruvate were estimated by converting it into ¹⁴CO₂, which was trapped in 2N-NaOH, plated as BaCO₃ and counted as above. This was achieved by combustion by the Van Slyke-Folch method as described by Sakami (1955, p. 1). The purity of the labelled pyruvate was checked as follows. [1-¹⁴C]Pyruvate was decarboxylated with yeast carboxylase and the ¹⁴CO₂ trapped in 2N-KOH and counted as above. [2-¹⁴C]Pyruvate was converted into lactate in the presence of lactate dehydrogenase and an excess of NADH. The lactate was then chromatographed in a descending direction with butan-1-ol-propionic acid-water (47:22:31, by vol.) (Benson *et al.* 1950). The lactic acid spot was located with a mica end-window Geiger-Müller tube, cut out and eluted with water. A measured portion of the eluate was taken for determination of lactate (Hohorst, 1962a) and another portion was burnt as for pyruvate. These results showed that over 98% of the radioactivity was present in C-1 of the original [1-¹⁴C]pyruvate, but about 5% of the counts in the [2-¹⁴C]pyruvate sample was present in impurities.

Dilution of labelled pyruvate. For the interpretation of results it was necessary to know to what extent labelled pyruvate was diluted by pyruvate produced endogenously by isolated liver cells. In agreement with the findings of Haslam & Krebs (1963) for liver homogenate there was no significant dilution of [1-¹⁴C]pyruvate by unlabelled material during an incubation of 60 min.

RESULTS

Assessment of results

Pyruvate carboxylation. When isolated liver cells are incubated with pyruvate, tricarboxylic acid-cycle intermediates accumulate in the medium. If ammonia is liberated by breakdown of added adenine nucleotide some glutamate and aspartate are also formed. The amount of carbon dioxide fixed by pyruvate during an incubation thus can be assessed by measuring the total amount of tricarboxylic acid-cycle intermediates, glutamate and aspartate accumulating.

Pyruvate oxidation. The two stages of pyruvate oxidation can be represented as follows:

Haslam & Krebs (1963) have shown that the yield of ¹⁴CO₂ from [1-¹⁴C]pyruvate gives an approximate indication of the extent of reaction (1). On the other hand, the yield of ¹⁴CO₂ from [2-¹⁴C]pyruvate should provide an indication of the amount of acetyl-CoA oxidized to completion through the tricarboxylic acid cycle in reaction (2). The values for the production of ¹⁴CO₂ have been taken in conjunction with values for oxygen uptake to assess the degree of pyruvate oxidation.

Effects of adenine nucleotides on pyruvate oxidation

Isolated liver cells lose many of their soluble constituents during the preparative procedures (Berry, 1962) and their adenine nucleotide content becomes greatly depleted (Table 1). They are therefore a suitable system for testing the effects of added nucleotides. When catalytic amounts of ADP (1–2 mM) were added to a cell suspension respiring in the presence of pyruvate most of the added ADP was rapidly converted into ATP. In the experiment recorded in Table 1 the resultant ATP:ADP ratio was 6.1:1 after 10 min. Some adenine nucleotide was broken down during the incubation but the ATP:ADP ratio remained greater than 6:1. Similar findings were obtained when the added nucleotide was ATP, a proportion being recovered as ADP.

This ready interconversion of ADP and ATP in tissue preparations probably accounts for the similar effects of 1–2 mM-ADP or -ATP on pyruvate metabolism by isolated liver cells (Table 2). Oxygen uptake was stimulated by between 58 and 87% and ketone-body formation by between 19 and 85%. An assessment of the amount of dicarboxylic acid synthesized from pyruvate in each experiment showed that pyruvate carboxylation was not

Table 1. *Action of isolated liver cells on catalytic quantities of added ADP*

The results refer to 4 ml. of isolated liver cells (67 mg. dry wt., equivalent to approx. 500 mg. fresh wt. of liver) incubated in the standard medium (see the text) in the presence of 20 mM-pyruvate at 30°. Values for nucleotides are expressed as μ moles.

Time (min.)		AMP	ADP	ATP	Total
	Cell content	0.11	0.22	0	0.33
	Nucleotide added...	0.16	3.57	0.03	3.76
0	Total found	0.27	3.79	0.03	4.09
10	Total found	0.07	0.46	2.81	3.34
30	Total found	0.03	0.27	2.36	2.66
60	Total found	0.03	0.26	1.98	2.27

Table 2. *Effects of catalytic amounts of adenine nucleotides on pyruvate metabolism in isolated liver cells*

The results refer to 4 ml. of isolated liver cells incubated in the standard medium (see the text) in the presence of 20 mM-pyruvate for 60 min. at 30°. In Expt. 4 the specific activity of [14 C]pyruvate was 1.5 μ C/m-mole and that of [14 C]pyruvate 3.0 μ C/m-mole.

Expt. no.	Dry wt. (mg.)	Added nucleotide		Metabolic changes (μ moles)	
				Control	Nucleotide added
1	41	ATP (1 mM)	O ₂	-12.3	-22.7
			Acetoacetate	+ 2.5	+ 3.3
			3-Hydroxybutyrate	+ 2.3	+ 2.4
			Cycle intermediates	+ 3.6	+ 6.5
2	35	ADP (1.5 mM)	O ₂	-18.3	-33.2
			Acetoacetate	+ 3.2	+ 3.9
			3-Hydroxybutyrate	+ 2.5	+ 3.3
3	35	ADP (2 mM)	O ₂	-21.6	-24.8
			Acetoacetate	+ 2.5	+ 6.1
			3-Hydroxybutyrate	+ 4.4	+ 2.3
			Cycle intermediates + glutamate + aspartate	+ 6.6	+ 4.9
4	51	ATP (1 mM)	O ₂	-19.2	-30.4
			Acetoacetate	+ 2.6	+ 3.6
			3-Hydroxybutyrate	+ 2.1	+ 5.1
			Cycle intermediates + glutamate + aspartate	+ 5.6	+ 8.2
			[14 C]Pyruvate $\rightarrow$ acetyl-CoA + 14 CO ₂	+ 15.4	+ 25.4
			[14 C]Pyruvate $\rightarrow$ 14 CO ₂ + H ₂ O	+ 2.2	+ 5.7

impaired in the presence of catalytic concentrations of adenine nucleotides (Table 2).

The values for the formation of 14 CO₂ obtained in Expt. 4 in Table 2 indicate that both the oxidation of pyruvate to acetyl-CoA and the oxidation of acetyl-CoA through the tricarboxylic acid cycle were increased by 1 mM-ATP. However, whereas an extra 10.0 μ moles of 14 CO₂ were formed from [14 C]pyruvate, only an extra 3.5 μ moles were produced from [14 C]pyruvate. This suggests that the ketogenic action of catalytic concentrations of ADP and ATP was due to a stimulation of acetyl-CoA formation in excess of the capacity of the cells for the oxidation of acetyl-CoA, the extra acetyl-CoA being converted into ketone bodies.

Effects of adenine nucleotides on pyruvate carboxylation

Effects of 2,4-dinitrophenol. To study the effects of a low ATP:ADP ratio on pyruvate metabolism by isolated liver cells, dinitrophenol was included in the incubation medium. Pilot experiments revealed that oxidative phosphorylation was completely prevented by 0.05–0.10 mM-dinitrophenol. Under these conditions the ATP content of the medium was immeasurably low after incubation of the cells even when 1 mM- or 2 mM-ATP was added at the start of the experiment.

Dinitrophenol (0.1 mM) caused a gross inhibition of dicarboxylic acid synthesis, depressed oxygen

Table 3. *Influence of dinitrophenol on pathways of pyruvate metabolism in liver cells*

The results refer to 4 ml. of isolated liver cells (51 mg. dry wt.) incubated in the standard medium (see the text) with the additions indicated below. The substrate was 20 mM-pyruvate. The incubation time was 60 min. and the temperature 30°. The specific activity of [1-¹⁴C]pyruvate was 1.5 μ C/m-mole, and that of [2-¹⁴C]pyruvate was 3.0 μ C/m-mole.

Vessel no....	Metabolic changes (μ moles)			
	1	2	3	4
Addition(s)...	ATP (1 mM)	Dinitrophenol (0.1 mM)	Fumarate (1.25 mM) + ATP (1 mM)	Fumarate (1.25 mM) + dinitrophenol (0.1 mM)
O ₂	-30.4	-16.1	-34.1	-57.7
Acetoacetate	+ 3.6	+ 11.25	+ 2.7	+ 0.59
3-Hydroxybutyrate	+ 5.1	+ 0.23	+ 3.1	+ 0.11
Total ketone bodies	+ 8.7	+ 11.5	+ 5.8	+ 0.70
[1- ¹⁴ C]Pyruvate $\rightarrow$ acetyl-CoA + ¹⁴ CO ₂	+ 25.4	+ 24.6	+ 20.0	+ 20.0
[2- ¹⁴ C]Pyruvate $\rightarrow$ ¹⁴ CO ₂ + H ₂ O	+ 5.7	+ 2.0	—	—
Cycle intermediates + glutamate + aspartate	+ 8.2	+ 1.06	+ 7.1	- 1.4

Table 4. *Accumulation of oxaloacetate and malate during incubation of liver cells with pyruvate*

The results refer to 4 ml. of isolated liver cells (55 mg. dry wt.) incubated in the standard medium (see the text) with the additions indicated below. The substrate was 20 mM-pyruvate. The incubation time was 25 min., and the temperature 30°. Values are expressed as μ moles/vessel.

Addition(s)...	None	Dinitrophenol (0.1 mM)	Fumarate (4 mM)	Fumarate (4 mM) + dinitrophenol (0.1 mM)
Malate accumulated	1255	0	6220	2840
Oxaloacetate accumulated	34.5	3.4	43.6	80.5

uptake by 47% and stimulated ketone-body formation by 32% when isolated liver cells were incubated with pyruvate (Table 3). The stimulation of ketone-body formation was accompanied by a large increase in the acetoacetate:3-hydroxybutyrate ratio. The yield of ¹⁴CO₂ from [1-¹⁴C]pyruvate was unaffected by dinitrophenol, but the yield from [2-¹⁴C]pyruvate was depressed by 65%.

These results indicate that the oxidation of pyruvate to acetyl-CoA was unaffected by dinitrophenol, whereas the oxidation of acetyl-CoA through the tricarboxylic acid cycle was grossly impaired. These findings can be explained by the assumption that the uncoupling of oxidative phosphorylation by dinitrophenol caused an inhibition of pyruvate carboxylation and decreased the supply of oxaloacetate available for the turnover of the tricarboxylic acid cycle. Oxidation of acetyl-CoA through the cycle was therefore depressed, with a resultant increase in the amount of acetyl-CoA diverted to ketone-body formation.

In another experiment the amounts of malate and oxaloacetate accumulating were measured (Table 4). In the presence of pyruvate alone 1.25 μ moles of malate and 34.5 μ moles of oxaloacetate accumulated. When 0.1 mM-dinitrophenol was included no malate and only 3.4 μ moles of

oxaloacetate were detected. In a volume of 4 ml. this represents an oxaloacetate concentration less than 1 μ M. This value is well below the *K_m* for oxaloacetate (about 10 μ M) in the citrate-synthase reaction (Kosicki & Srere, 1961).

When fumarate was included in the incubation medium to provide an alternative source of oxaloacetate, dinitrophenol no longer inhibited respiration but instead stimulated oxygen uptake by nearly 90% and decreased ketone-body formation over tenfold (Table 3, vessel 4). In the absence of dinitrophenol fumarate had no effect on oxygen uptake but caused a small decrease in ketogenesis from pyruvate by depressing the oxidation of pyruvate to acetyl-CoA (Table 3, vessel 3). The much greater antiketogenic effect of the combination of dinitrophenol with fumarate compared with fumarate alone can be accounted for by the observation that a much higher steady-state concentration of oxaloacetate was attained in the former instance (Table 4).

Effects of hexokinase plus glucose on pyruvate carboxylation. It is improbable that circumstances could arise *in vivo* whereby oxidative phosphorylation would be completely abolished without causing cell death. On the other hand, a situation can be envisaged in which an excessive demand for ATP

Table 5. *Effects of hexokinase plus glucose on pyruvate metabolism by isolated liver cells*

The results refer to 4 ml. of isolated liver cells (51 mg. dry wt. in Expt. 1; 81 mg. in Expt. 2; 52 mg. in Expt. 3) incubated in the standard medium (see the text) with the additions indicated below. The substrate was 20 mM-pyruvate. The incubation time was 60 min., and the temperature 30°.

Expt. no.	Addition(s)...	Metabolic changes (μ moles)				Other details
		None	Hexokinase	Fumarate	Fumarate + hexokinase	
1	O ₂	-30.4	-19.7	-34.1	-41.7	Medium contained ADP (1 mM); fumarate concn., 1.25 mM
	Acetoacetate	+ 3.6	+ 4.7	+ 2.7	+ 1.3	
	3-Hydroxybutyrate	+ 5.1	+ 5.5	+ 3.1	+ 4.9	
	[1- ¹⁴ C]Pyruvate $\rightarrow$ acetyl-CoA + ¹⁴ CO ₂	+25.4	+25.0	+20.0	+21.4	
	[2- ¹⁴ C]Pyruvate $\rightarrow$ ¹⁴ CO ₂ + H ₂ O	+ 5.7	+ 3.8	—	—	
2	Cycle intermediates + glutamate + aspartate	+ 8.2	+ 1.3	+ 7.1	+ 2.6	Medium contained ADP (1 mM); fumarate concn., 3.0 mM
	O ₂	-32.5	-20.3	-33.6	-55.4	
	Acetoacetate	+ 4.2	+17.3	+ 2.3	+ 4.6	
	3-Hydroxybutyrate	+ 8.4	+ 4.2	+ 4.0	+ 6.4	
	[1- ¹⁴ C]Pyruvate $\rightarrow$ acetyl-CoA + ¹⁴ CO ₂	+36.3	+38.1	+22.8	+37.0	
3	[2- ¹⁴ C]Pyruvate $\rightarrow$ ¹⁴ CO ₂ + H ₂ O	+ 4.6	+ 0.97	+ 1.8	+ 4.9	
	Cycle intermediates + glutamate + aspartate	+14.1	+ 1.8	+ 8.9	+ 8.9	
	O ₂	-20.3	-21.6	—	—	
	Acetoacetate	+ 3.4	+ 3.3	—	—	
	3-Hydroxybutyrate	+ 1.7	+ 1.7	—	—	
	[1- ¹⁴ C]Pyruvate $\rightarrow$ acetyl-CoA + ¹⁴ CO ₂	+17.8	+18.1	—	—	
	[2- ¹⁴ C]Pyruvate $\rightarrow$ ¹⁴ CO ₂ + H ₂ O	+ 4.2	+ 4.5	—	—	
	Malate	+ 2.2	+ 2.1	—	—	

Table 6. *Effects of AMP and ADP on the metabolism of pyruvate by isolated liver cells*

The results refer to 4 ml. of isolated liver cells (38 mg. dry wt. in Expt. 1; 35 mg. in Expts. 2 and 3; 92 mg. in Expt. 4; 55 mg. in Expt. 5) incubated in the standard medium (see the text). The substrate was 20 mM-pyruvate. The incubation time was 60 min., and the temperature 30°. All values are expressed as μ moles.

Expt. no.	Nucleotide added	Amount	O ₂ uptake	Ketone bodies formed			Nucleotide content after incubation		
				Acetoacetate	3-Hydroxybutyrate	Total	AMP	ADP	ATP
1	AMP	0	16.0	3.3	2.5	5.8	—	—	—
		5	19.1	3.7	1.3	5.0	—	—	—
		20	19.5	6.7	0.4	7.1	0.87	2.1	1.7
2	ADP	0	21.0	2.0	5.8	7.8	—	—	—
		40	19.5	10.9	0.3	11.2	3.9	9.9	10.2
3	ADP	6	30.9	4.1	2.8	6.9	0.3	0	1.6
		25	20.3	10.6	0.4	11.0	3.7	5.3	5.8
		50	18.4	10.3	0.3	10.6	10.8	14.5	9.9
4	ADP	4	47.5	10.7	7.5	18.2	—	—	—
		20	30.8	23.9	1.1	25.0	—	—	—
5	ADP	5	35.8	4.3	3.4	7.7	—	—	—
		20	18.0	11.1	0.2	11.3	—	—	—

might lower the concentration of ATP and raise that of ADP within the liver cell. This situation was induced in isolated liver-cell suspensions by including hexokinase plus glucose in the incubation medium.

In the presence of added adenine nucleotide (1 mM- or 2 mM-ADP or -ATP) hexokinase caused a gross inhibition of dicarboxylic acid synthesis when isolated liver cells were incubated with pyruvate (Expts. 1 and 2 in Table 5). This was accompanied by a depression of oxygen uptake and a stimulation of ketone-body formation. The

production of ¹⁴CO₂ from [2-¹⁴C]pyruvate was decreased but the yield from [1-¹⁴C]pyruvate was unaffected.

These effects of hexokinase were prevented by the inclusion of fumarate in the incubation medium. For example, when 1.25 mM-fumarate was present (Expt. 1 in Table 5) hexokinase stimulated oxygen uptake by 37% and its ketogenic action was abolished.

Hexokinase plus glucose had no significant effect on pyruvate metabolism by isolated liver cells if adenine nucleotide was omitted from the

incubation medium (Expt. 3 in Table 5). In contrast, dinitrophenol was effective whether or not adenine nucleotide was added. This suggests that the mitochondria were permeable to dinitrophenol but not to hexokinase.

Effects of high concentrations of ADP on pyruvate carboxylation. The effects of ADP on pyruvate carboxylation in isolated liver cells was also examined by adding high concentrations (5–12mm) of ADP to the incubation medium. The results of four experiments (Table 6) show that such concentrations of ADP consistently caused an inhibition of respiration and a stimulation of ketone-body formation when the cells were incubated with pyruvate. The increased ketone-body production was associated with a marked increase in the acetoacetate:3-hydroxybutyrate ratio. Measurement of adenine nucleotide concentrations in the medium at the end of some of these experiments revealed that significant quantities of AMP and ATP were formed from the added ADP (Table 6).

To follow the fate of added ADP throughout the course of an incubation, liver cells were incubated

with 5mm-ADP and the concentrations of adenine nucleotides (AMP, ADP and ATP) in the medium were measured at 10min. intervals. The results (Table 7) indicate that within 10min. much of the added ADP had been converted into AMP and ATP. As a result of these changes the ADP concentration was diminished to 1.4mm, and it declined gradually to 0.9mm during the incubation. The ATP concentration, which was 0.8mm after 10min., rose to 1.9mm by the end of the experiment, and the AMP concentration, 1.9mm after 10min., fell to 0.25mm by the end point. During the incubation a net loss of adenine nucleotide occurred, and by the end of the experiment 7.15 μ moles of nucleotide had been broken down.

In this particular experiment ADP decreased dicarboxylic acid synthesis from pyruvate by 81%, depressed oxygen uptake by 30% and stimulated ketone-body formation by 28% (Table 8). Both the inhibition of oxygen uptake and the stimulation of ketone-body formation were maintained throughout the course of the incubation. The yield of $^{14}\text{CO}_2$ from [2- ^{14}C]pyruvate was decreased by 52%, but the yield from [1- ^{14}C]pyruvate was not affected. The action of 5mm-ADP thus closely resembled that of dinitrophenol or hexokinase and, as with these agents, the inhibition of oxygen uptake and stimulation of ketone-body formation were prevented by the inclusion of fumarate in the incubation medium (Table 9).

In experiments with added ADP it was not feasible to exclude the possibility that AMP might also be a ketogenic agent. As a result of the equilibrium of the reaction catalysed by adenylate kinase:

$$[\text{AMP}] = 0.44 [\text{ADP}]^2/[\text{ATP}]$$

(Eggleston & Hems, 1952) liver cells rapidly convert some of the added ADP into AMP. Conversely, added AMP is converted into ADP, and in this case the situation is complicated by the breakdown

Table 7. *Action of isolated liver cells on added ADP*

The results refer to 4 ml. of isolated liver cells (67 mg. dry wt.) incubated in the standard medium (see the text) in the presence of 20mm-pyruvate and 18 μ moles of added ADP. The incubation temperature was 30°.

Time (min.)	Nucleotide content (μ moles)			Nucleotide disappearance (μ moles)
	AMP	ADP	ATP	
0	0.90	18.07	0.16	—
10	7.66	5.42	3.21	2.84
20	6.32	5.52	3.79	4.18
30	5.04	5.14	4.67	4.96
40	2.96	4.81	6.02	6.02
60	1.01	3.43	7.54	7.15

Table 8. *Effects of ADP (5mm) on pyruvate metabolism by isolated liver cells*

The results refer to 4 ml. of isolated liver cells (67 mg. dry wt.) incubated in the standard medium (see the text) in the presence of 20mm-pyruvate. The incubation time was 60min., and the temperature 30°. The specific activity of [1- ^{14}C]pyruvate was 1.5 $\mu\text{C}/\text{m-mole}$, and that of [2- ^{14}C]pyruvate was 3.0 $\mu\text{C}/\text{m-mole}$.

Addition...	Metabolic changes (μ moles)	
	ADP (1 mm)	ADP (5 mm)
O ₂	−31.3	−21.9
Acetoacetate	+ 7.2	+15.6
3-Hydroxybutyrate	+ 5.3	+ 0.4
Total ketone bodies	+12.5	+16.0
[1- ^{14}C]Pyruvate $\rightarrow$ acetyl-CoA + $^{14}\text{CO}_2$	+31.3	+30.8
[2- ^{14}C]Pyruvate $\rightarrow$ $^{14}\text{CO}_2$ + H ₂ O	+ 5.4	+ 2.6
Cycle intermediates + glutamate + aspartate	+ 7.6	+ 1.48

Table 9. *Effects of fumarate on the metabolism of pyruvate by isolated liver cells in the presence of high concentrations of ADP*

The results refer to 4 ml. of isolated liver cells (35 mg. dry wt. in Expt. 1; 40 mg. in Expt. 2) incubated in the standard medium (see the text) with the additions indicated below. The substrate was 20 mM-pyruvate. The incubation time in Expt. 1 was 60 min., and in Expt. 2 was 30 min.; the incubation temperature was 30°.

Expt. no.	Vessel no.... Addition(s)...	Metabolic changes (μ moles)				Other details
		1 None	2 ADP	3 Fumarate (1 mM)	4 Fumarate (1 mM) + ADP	
1	O ₂	-21.0	-19.5	-21.5	-25.3	ADP concn.
	Acetoacetate	+ 2.0	+10.9	+ 1.2	+ 1.2	in vessels 2
	3-Hydroxybutyrate	+ 5.8	+ 0.3	+ 3.4	+ 1.0	and 4,
	Total ketone bodies	+ 7.8	+11.2	+ 4.6	+ 2.2	12.5 mM
2	O ₂	-12.4	- 7.1	—	-15.7	ADP concn.
	Acetoacetate	+ 2.9	+ 4.3	—	+ 1.0	in vessel 1,
	3-Hydroxybutyrate	+ 1.2	+ 0.4	—	+ 0.7	1.25 mM; in
	Total ketone bodies	+ 4.1	+ 4.7	—	+ 1.7	vessels 2 and 4, 5 mM

Table 10. *Effects of dinitrophenol and hexokinase on the metabolism of pyruvate by mitochondria*

The results refer to 4 ml. of isolated liver mitochondria (17 mg. dry wt.) incubated in the standard medium for isolated cells (see the text) plus ATP (1 mM) and the additions indicated below. The substrate was 20 mM-pyruvate. The incubation time was 40 min., and the temperature 30°.

Expt. no.	Addition(s)...	Metabolic changes (μ moles)						Other details
		None	Hexokinase	Dinitrophenol	Fumarate (1 mM)	Fumarate (1 mM) + hexokinase	Fumarate (1 mM) + dinitrophenol	
1	O ₂	-13.7	-11.3	-17.2	-13.3	-22.3	-27.0	Concn. of dinitrophenol, 0.02 mM
	Pyruvate	-22.9	-17.9	-22.6	-19.8	-20.3	-22.6	
	Acetoacetate	+ 1.8	+ 6.4	+ 4.7	+ 1.1	+ 1.1	+ 1.1	
	3-Hydroxybutyrate	+ 1.6	+ 0.4	+ 0.5	+ 1.1	+ 1.0	+ 0.6	
	Total ketone bodies	+ 3.4	+ 6.8	+ 5.2	+ 2.2	+ 2.1	+ 1.7	Concn. of dinitrophenol, 0.05 mM
	Citrate	+ 3.9	+ 0.53	+ 1.8	+ 5.7	+ 0.64	+ 5.0	
	2-Oxoglutarate	+ 0.4	0	+ 0.3	+ 0.6	0	+ 0.6	
	Malate	+ 3.7	0	+ 0.98	+ 3.6	+ 0.7	+ 3.5	
	O ₂	-12.7	- 6.2	-10.3	-15.1	-22.0	-25.4	
	Pyruvate	-22.4	-19.9	-21.4	-18.1	-26.3	-26.9	
	Acetoacetate	+ 2.3	+ 7.2	+ 6.1	+ 1.0	+ 2.2	+ 0.8	
	3-Hydroxybutyrate	+ 0.6	+ 0.6	+ 0.2	+ 0.5	+ 0.3	+ 0.1	
	Total ketone bodies	+ 2.9	+ 7.8	+ 6.3	+ 1.5	+ 2.5	+ 0.9	

of much of the added nucleotide (Table 6). Purified pyruvate carboxylase is therefore likely to be a more suitable system for investigating a possible action of AMP on pyruvate carboxylation.

Effects of adenine nucleotides on pyruvate carboxylation by isolated mitochondria and homogenate. In the presence of hexokinase or dinitrophenol the behaviour of isolated mitochondria was very similar to that of isolated cells. In the experiments set out in Table 10 linear respiration was maintained throughout, much of the added pyruvate was removed and acetoacetate, 3-hydroxybutyrate and tricarboxylic acid-cycle intermediates accumulated in the medium. Hexokinase plus glucose inhibited respiration, decreased the accumulation of tricarboxylic acid-cycle intermediates and increased ketone-body formation by 100%. Dinitrophenol (0.02–0.05 mM) had similar effects. When

fumarate (1 mM) was included in the incubation medium both hexokinase and dinitrophenol stimulated oxygen uptake by between 50 and 100% but decreased ketone-body formation below the level observed with pyruvate alone. In the absence of hexokinase or dinitrophenol the presence of 1 mM-fumarate had no significant effect on oxygen uptake but decreased ketone-body formation by about one-third (Table 10).

It was not possible to demonstrate the effects of high concentrations of added ADP on the metabolism of pyruvate by mitochondria. In each experiment the ADP was rapidly and completely phosphorylated to ATP and no ketogenic activity was detected.

Liver homogenate proved unsuitable for demonstrating effects of adenine nucleotides on pyruvate metabolism. Added AMP or ADP was quickly

phosphorylated and maintained as ATP and had little effect on oxygen uptake or ketone-body formation. If dinitrophenol or hexokinase was added to maintain low concentrations of ATP, the added nucleotide was rapidly broken down with liberation of ammonia (cf. Krebs & Eggleston, 1962).

DISCUSSION

The stimulation of pyruvate oxidation brought about by the addition of catalytic quantities of adenine nucleotides to isolated liver-cell suspensions is in accordance with the long-established concept that the rate of cell respiration is determined by the availability of phosphate acceptor. The increase in ketone-body formation caused by the stimulation of pyruvate oxidation to acetyl-CoA (Table 2) is an example of a situation in which overproduction of acetyl-CoA leads to increased ketogenesis. On the other hand, when isolated liver cells or mitochondria are incubated with pyruvate and dinitrophenol or hexokinase the increased ketogenesis is due to a lack of oxaloacetate, which leads to a diversion of acetyl-CoA from oxidation to ketone-body formation. The extent to which these two mechanisms function *in vivo* is still uncertain (Krebs, 1960; van Itallie & Bergen, 1961).

The marked increase in the acetoacetate:3-hydroxybutyrate ratio brought about by dinitrophenol or 5 mM-ADP (Tables 3 and 6) may reflect an increase in the $\text{NAD}^+:\text{NADH}$ ratio within the mitochondria. Both dinitrophenol (Birt & Bartley, 1960) and ADP (Chance & Williams, 1956) bring about such an increase. Since 3-hydroxybutyrate dehydrogenase is located within the mitochondria (Lehninger, Sudduth & Wise, 1960) it is feasible that an increase in the mitochondrial $\text{NAD}^+:\text{NADH}$ ratio would cause a rise in the acetoacetate:3-hydroxybutyrate ratio.

The inhibition of pyruvate oxidation in mitochondria by dinitrophenol was first reported by Lipmann & Kaplan (1949) and confirmed by Judah (1951). These effects remained unexplained, although it was noted that the inhibition could be overcome if catalytic amounts of malate or larger quantities of ATP were added to the incubation medium (Judah, 1951). Birt & Bartley (1960) studied the effects of pyruvate and dinitrophenol on mitochondrial NAD and suggested that the inhibition of respiration that followed the addition of dinitrophenol may have been due to an impaired ability of the mitochondria to synthesize C_4 intermediates from pyruvate.

The present results support this explanation. Isolated liver cells and isolated liver mitochondria readily carboxylate pyruvate and thus provide sufficient dicarboxylic acid to promote the oxidation of acetyl-CoA formed from pyruvate through the

tricarboxylic acid cycle. If, however, dinitrophenol or hexokinase is present dicarboxylic acid synthesis is impaired and insufficient oxaloacetate is formed to permit the oxidation of acetyl-CoA. In these circumstances oxygen uptake is depressed and ketone-body formation increased. Some workers have added catalytic quantities of dicarboxylic acid to the incubation medium to 'spark' the oxidation of pyruvate by mitochondria. Such additions are unnecessary unless a low ATP:ADP ratio is maintained with dinitrophenol or hexokinase.

In the experiment recorded in Table 8 over 18 μmoles of ADP were present in the medium when the incubation began, but within 10 min. the actual concentration of ADP fell below 1.5 mM and remained below this level throughout the incubation. Thus a concentration of ADP within the physiological range found in rat liver (Hohorst, Kreuz & Bücher, 1959) has been shown to inhibit pyruvate carboxylation. These results are entirely consistent with the studies of Keech & Utter (1963) on partially purified pyruvate carboxylase, and indicate that the sensitivity of this enzyme to ADP can be demonstrated with organized systems such as isolated mitochondria or liver cells.

Whether a high ATP concentration can overcome the inhibitory action of ADP on pyruvate carboxylation remains uncertain. Tables 8 and 9 show that an ATP concentration of between 1 and 2 mM was not effective, but the ATP:ADP ratio in this experiment was only about 1:1, whereas in the liver cell *in vivo* the ratio is over 3:1 (Hohorst *et al.* 1959).

This work was supported by grants from the Rockefeller Foundation and U.S. Public Health Service and was carried out during the tenure of a Nuffield Dominions Demonstratorship. The author thanks Professor Sir Hans Krebs, F.R.S., for his interest in this work, and Mr R. Hems and Mr D. H. Williamson for valuable advice.

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