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Phosphorylation of Adenosine Monophosphate in the Mitochondrial Matrix

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The origin of the GTP needed for the phosphorylation of AMP in the mitochondrial matrix was investigated. When short-chain fatty acids are metabolized by hepatocytes, AMP is readily formed within the matrix by the butyryl-CoA ligase (AMP-forming) reaction (EC 6.2.1.2). The rate of matrix AMP formation in rat hepatocytes was calculated from the rate of ketone-body formation. The rate of the reconversion of matrix AMP into ADP by GTP–AMP transphosphorylase is limited by the rate of supply of GTP. GTP can be formed either by succinic thiokinase (EC 6.2.1.4) or by nucleoside diphosphokinase (EC 2.7.4.6). The rate of the succinic thiokinase reaction was calculated from turnover of the tricarboxylic acid cycle and this was calculated from the rates of O_2 consumption and ketone-body formation. The results show that nucleoside diphosphokinase can make a major contribution (up to 80%) to the supply of GTP under the test conditions.

The present paper is on the nature of the reactions that rephosphorylate AMP arising within the hepatic mitochondrial matrix. AMP is rapidly formed in the matrix when short-chain fatty acids are converted into their acyl-CoA derivatives by the medium-chain fatty acid thiokinase (butyrate–CoA ligase, AMP-forming; EC 6.2.1.2):

$$\text{Fatty acid } (C_4\text{–}C_8) + \text{CoA} + \text{ATP} \rightarrow \text{acyl-CoA} + \text{AMP} + PP_i$$

(Mahler *et al.*, 1954; Jencks & Lipmann, 1957).

Information on the rephosphorylation of AMP is as follows: AMP does not readily diffuse out of the matrix into the cytosol and no translocating system has so far been found (Klingenberg & Pfaff, 1966; Klingenberg, 1970*a*; Pfaff *et al.*, 1965). A matrix enzyme that can phosphorylate AMP to ADP is the GTP–AMP transphosphorylase described by Gibson *et al.* (1956) and by Heldt & Schwalbach (1967), which catalyses the reaction:

$$\text{GTP} + \text{AMP} \rightleftharpoons \text{GDP} + \text{ADP}$$

The extent of this reaction, however, is limited by the rate of supply of GTP which arises in the matrix by the succinic thiokinase reaction [succinyl (3-carboxypropionyl)-CoA synthetase; succinate–CoA ligase (GDP-forming); EC 6.2.1.4] (Sanadi *et al.*, 1954, 1956) at the rate of 1 GTP for each turn of the tricarboxylic acid cycle:

$$\text{Succinyl-CoA} + \text{GDP} + P_i \rightarrow \text{succinate} + \text{CoA} + \text{GTP}$$

A second reaction generating GTP is the nucleoside diphosphokinase reaction (EC 2.7.4.6):

$$\text{GDP} + \text{ATP} \rightarrow \text{GTP} + \text{ADP}$$

Heldt & Schwalbach (1967) expressed doubts as to whether the capacity of this enzyme within the hepatic mitochondrial matrix is sufficient to make a major contribution to the regeneration of GTP, because they believed that the assayed activity is located between the inner and outer mitochondrial membranes. As ATP is readily available in the matrix through oxidative phosphorylation, the amounts of GTP that potentially could be generated by this reaction would be large, provided that sufficient enzyme activity is located within the matrix.

To obtain further relevant information, the rates of the succinic thiokinase reaction and of the matrix AMP formation were measured under conditions where the latter is high, i.e. when butyrate or other short-chain fatty acids were added to hepatocytes. The rate of AMP formation was calculated from the rate of fatty acid metabolism and the succinic thiokinase reaction was ascertained from measurements of the rate of the tricarboxylic acid cycle. Under the test conditions the rate of AMP formation was far greater, by factors approaching 4, than the generation of GTP by succinate thiokinase. Thus GTP must have been readily regenerated in the matrix by nucleoside diphosphokinase.

Materials and Methods

Liver cells and metabolite assay

Isolated rat liver hepatocytes from 48h-starved female rats, prepared and handled as previously described (Cornell *et al.*, 1974; Krebs *et al.*, 1974), were incubated with 5mM- or 10mM-short-chain fatty acid in manometer vessels containing $NaHCO_3$/

CO_2 buffer plus carbonic anhydrase, so that the O_2 consumption could be measured in the presence of 5% CO_2. At the end of the incubation the suspension was acidified with $HClO_4$ (1ml; 10%, w/v), and acetoacetate and β-hydroxybutyrate were determined, after neutralization with KOH, by the enzymic method of Williamson *et al.* (1962).

The concentration of butyrate was measured by the micro diffusion method of Conway (1962). Water (1ml) was placed in the centre compartment of a Conway dish and the acidified sample to be analysed was placed in the ring containing anhydrous Na_2SO_4. Tests showed that after 16h at room temperature (22°C) the butyric acid had distilled into the centre compartment, the contents of which were quantitatively transferred to a graduated tube and made up to 2ml. The amount of acid in the solution was measured manometrically in small conical Warburg vessels (8ml total volume) by adding 0.2ml of 0.2M-$NaHCO_3$ solution from the side arm to 1ml of the unknown solution in the main compartment. The gas space contained 5% CO_2 and the temperature was 37°C. A control containing 1ml of water instead of acid solution is required because CO_2 is absorbed by the carbonate present in any 'bicarbonate' solution. On dilution, the pH changes and CO_3^{2-} reacts with CO_2.

Calculation of the rate of AMP formation

The rate of intramitochondrial AMP formation was taken to be stoicheiometric with short-chain fatty acid removal, as other sources of AMP were negligible. A first approximation to the rate of short-chain fatty acid removal and AMP formation is the rate of production of ketone bodies. However, two corrections of this first approximation are to be considered. (1) Short-chain fatty acids do not necessarily suppress the endogenous ketone-body production in the starved liver (see below). Endogenous ketogenesis is mainly due to oxidation of long-chain fatty acids which may not be inhibited at all, or only partially, by the addition of 5mM- or 10mM-short-chain fatty acids, as discussed below. For the calculation of the rate of AMP formation the unfavourable assumption was made that endogenous ketogenesis was not affected by the addition of short-chain fatty acids. The mean rate of endogenous ketone-body formation was 28μmol/h per g in hepatocytes from 48h-starved rats, 90% of which was acetoacetate. The rate of AMP production was calculated by correcting the total ketone-body production in the presence of short-chain fatty acids on the basis of this endogenous rate. AMP formation arising in connection with endogenous ketogenesis was neglected because long-chain fatty acids are converted into their acyl-CoA derivatives in the cytosol. The calculated rate of AMP formation thus represented a minimum value. (2) Measurements of the rate of butyrate removal indicated that the removal was significantly greater than the rate of ketone-body formation. This was to be expected because some butyrate may undergo complete oxidation. No correction in the calculation of the rate of AMP formation was made for the oxidation of short-chain fatty acids and this adds to the underestimate of the rate of removal of short-chain fatty acid. The extent of the underestimate was assessed and is discussed below. The underestimates are not of importance to the main conclusion that matrix nucleoside diphosphokinase is required for the rephosphorylation of AMP.

When butyrate or crotonate was the added fatty acid the uncorrected rate of AMP formation was taken to be equal to the rate of ketone-body formation. When hexanoate was added the uncorrected rate was taken to be two-thirds of the rate of ketone-body production, because hexanoate forms 1.5 molecules of ketone bodies per molecule.

Results and Discussion

The results of four sets of experiments (Table 1) show that the succinyl-CoA synthetase reaction, under the test conditions, supplied between 19 and 60% of the total GTP needed for the rephosphorylation of AMP. The percentage depended on the nature of the added fatty acid. (+)-Decanoylcarnitine, which inhibits the oxidation of long-chain fatty acids (Williamson *et al.*, 1968), decreased the formation of β-hydroxybutyrate and somewhat increased the GTP supply by the tricarboxylic acid cycle.

It is evident that an additional GTP-generating mechanism within the mitochondrial matrix must be postulated. Nucleoside diphosphokinase has long been known to occur in hepatocytes, though its presence within the mitochondrial matrix has been questioned. According to Söling & Kleineke (1976) the rat liver mitochondria contain only 5% of the total rat liver nucleoside diphosphokinase. In the experiments of Söling & Kleineke (1976) the assayed mitochondrial activity at 30°C was 3.7μmol/min per g of liver. If the mitochondrial activity is all located within the matrix the activity would be sufficient for the rephosphorylation of AMP as the rate of AMP formation in the experiments of Table 1 was about 2.2μmol/min per g at 38°C. The cytosolic nucleoside diphosphokinase is needed in rat liver for the rephosphorylation of GDP arising in the synthesis of phosphoenolpyruvate from oxaloacetate, in the formation of AMP from IMP, as well as for protein and RNA synthesis.

For the interpretation of the data in Table 1, as already mentioned, it is important to know whether the addition of short-chain fatty acids affects the

Table 1. *Metabolism of butyrate, crotonate and hexanoate in isolated rat hepatocytes*

Hepatocytes from 48h-starved rats were incubated at 38°C as described in the Materials and Methods section. The period of incubation was 60min in Expt. 1 and 40min in Expts. 2, 3 and 4. The data are given as μmol/h per g fresh wt. The values for β-hydroxybutyrate and acetoacetate were corrected for the zero-time value. For the calculation of the formation of AMP see the text. The amount of GTP formed from the tricarboxylic acid cycle was calculated on the assumption that the O_2 used per turn of the cycle was 2.5 when butyrate, 2.25 when crotonate and 2.66 when hexanoate was the substrate, and that 1 GTP is formed for each turn of the cycle.

Expt. no.	Cells wet wt. (mg)	Substrates added	Aceto-acetate formed	β-Hydroxy-butyrate formed	O_2 uptake	O_2 needed for formation of: Aceto-acetate	O_2 needed for formation of: β-Hydroxy-butyrate	O_2 needed for formation of: Total ketones	O_2 uptake not accounted for by ketone bodies	AMP formed	GTP formed by tricarboxylic acid cycle	% of total supplied by tricarboxylic acid cycle
1	78.5	5mM-Butyrate	87.0	30.6	175	87.0	15.3	102	72.6	89.6	29.3	33
		5mM-Hexanoate	92.4	53.0	220	92.4	26.5	119	102	78.2	38.2	43
2	112	10mM-Butyrate	121	27.9	204	121	13.9	135	68.3	121	26.8	22
		5mM-Butyrate	115	32.5	189	115	16.3	132	57.6	120	22.8	19
		10mM-Crotonate	107	8.04	163	53.3	nil	53.3	110	87	52.2	60
		5mM-Crotonate	101	9.24	146	50.6	nil	50.6	95.1	82.4	42.9	52
3	102	10mM-Butyrate	121	26.6	223	121	13.4	134	89.7	120	35.3	30
		10mM-Butyrate; 0.5mM-(+)-decanoylcarnitine	114	10.4	216	114	5.3	112	104	96	41.2	43
4	97	10mM-Butyrate	133	24.9	227	133	12.5	146	82.0	130	32.5	25
		10mM-Butyrate; 1mM-(+)-decanoylcarnitine	119	7.11	206	119	3.56	123	83.5	98	34.0	35
		10mM-Butyrate; 2mM-(+)-decanoylcarnitine	112	3.71	209	112	1.86	114	94.3	88	37.1	42

Table 2. *Competition between fatty acids*

Hepatocytes from 48h-starved rats (87mg of cells in 4ml of suspension) were incubated for 1h with fatty acids, singly and in mixtures. The O_2 uptake was measured manometrically.

Substrates added	Metabolic changes: Total ketone bodies formed (μmol/h per g)	Metabolic changes: [β-Hydroxybutyrate]/[Acetoacetate]	Metabolic changes: O_2 used (μmol/min per g)
None	23.5	0.09	1.94
Butyrate (10mM)	121	0.14	3.00
Oleate (1mM)	120	1.55	3.12
Butyrate (10mM); oleate (1mM)	165	1.34	3.66
Butyrate (5mM)	127	0.23	2.98
Butyrate (5mM); oleate (1mM)	165	1.32	3.63
Linoleate (1mM)	125	1.12	3.20
Linoleate (1mM); oleate (1mM)	131	1.32	3.25

endogenous ketogenesis in liver from starved rats. Table 2 shows that ketogenesis from two long-chain fatty acids (oleate and linoleate) is not additive, but ketogenesis from butyrate and oleate is partially additive. This suggests that the endogenous ketone-body formation may not be fully suppressed by the addition of short-chain fatty acids.

To assess the magnitude of errors arising from the assumption that butyrate does not suppress the endogenous ketone-body formation, the rates of butyrate removal and total ketone-body production were compared with initial concentrations of 10mM-butyrate. The experimental conditions were as described in the legend of Table 1. The rates (±S.E.M., $n = 5$) were 134 ± 10.5 and 136 ± 11 μmol/h per g respectively, i.e. about equal. This equality does not indicate that each molecule of butyrate removed was converted into ketone bodies. It is very probable that endogenous ketone-body production was partially suppressed and that some butyrate served as a substrate of oxidation. If the total O_2 consumption represented butyrate oxidation, the rate of butyrate removal by oxidation would have been about 40μmol/h per g.

The findings indicate that, as expected, the rate of AMP formation was underestimated. This strengthens the conclusion that much GTP was formed by matrix nucleoside diphosphokinase.

This conclusion, to sum up, depends on the validity of the following four assumptions, which are substantiated by the references quoted. (1) That the internal mitochondrial membrane is impermeable to AMP (Klingenberg, 1970*a*,*b*). (2) That adenylate kinase, which converts cytosolic ATP into AMP, is absent from the matrix (Sottocasa *et al.*, 1967*a*,*b*; Brdiczka *et al.*, 1968). (3) That short-chain acyl-CoA synthetase is located within the matrix (Aas & Bremer, 1968). (4) That sufficient nucleoside diphosphokinase is located in the matrix (Söling & Kleineke, 1976).

It should be emphasized that these assumptions apply to rat liver. There are species differences (Söling & Kleineke, 1976; Garber & Ballard, 1970).

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