The Synthesis of Hippurate from Benzoate and Glycine by Rat Liver Mitochondria

SUBMITOCHONDRIAL LOCALIZATION AND KINETICS

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1. Rat liver mitochondria make hippurate at up to 4 nmol/min per mg of protein. The rate of synthesis supported by oxidation of glutamate with exogenous P_i present is identical with that supported by ATP plus oligomycin. Lower rates were obtained with other respiratory substrates, and when glutamate was used without P_i . 2. A matrix localization for hippurate synthesis is indicated by the latency of benzoyl-CoA synthetase and glycine *N*-acyltransferase to their extramitochondrial substrates, failure of exogenous benzoyl-CoA to inhibit incorporation of [^{14}C]benzoate into [^{14}C]hippurate and inhibition of hippurate synthesis supported by ATP, but not glutamate, by carboxyatractyloside. 3. The relative activities of the individual enzymes and the mitochondrial content of benzoyl-CoA in the presence and absence of glycine suggest that hippurate synthesis is rate-limited by formation of benzoyl-CoA. 4. The increases in rates of ATP hydrolysis and of O_2 consumption on the addition of benzoate and glycine were in good agreement with those required to support hippurate synthesis. The increase in respiration indicates that State-4 respiration [Chance & Williams (1957) *Adv. Enzymol.* 17, 65–134] is not used, with these conditions, for ATP synthesis.

Herbivores and omnivores consume significant amounts of benzoic acid, which occurs naturally in plant material, especially fruits and berries; it also finds its way into the human diet because of its widespread use as a food preservative. The major pathway of benzoate metabolism in most mammals is that of conjugation with glycine, yielding hippurate (for review, see Williams, 1971), and this involves formation of benzoyl-CoA, which is the co-substrate with glycine for acyl-CoA–glycine *N*-acyltransferase (EC 2.3.1.13) (Chantrenne, 1951; Schachter & Taggart, 1954). Humans excrete up to 1 g of hippurate daily.

The specificity of ox liver glycine *N*-acyltransferase for acyl-CoA is quite broad: Bartlett & Gompertz (1974) have studied the reaction of CoA esters of several carboxylic acids that are excreted in the urine of patients with some inborn errors of amino acid metabolism; Webster and his associates (Killenberg *et al.*, 1971; Forman *et al.*, 1971) have concluded that separate enzymes catalyse the formation of benzoyl-CoA and salicyl-CoA in ox liver, but that the thioesters are substrates for a common glycine *N*-acyltransferase.

Schachter & Taggart (1954) and Bartlett & Gompertz (1974) found that glycine *N*-acyltransferase is

associated with the mitochondrial fraction of ox liver. Gatley & Sherratt (1976) concluded that formation of hippurate probably occurs in the matrix compartment of rat liver mitochondria; synthesis of benzoyl-CoA from exogenous CoA was greatly increased by sonication, and mitochondria whose outer membrane had been removed by treatment with digitonin could synthesize hippurate. It was also shown that mitochondria concentrated benzoate from the suspending medium, and at equilibrium glycine was present in the matrix compartment at the same concentration as in the bulk phase (Gatley & Sherratt, 1976). We now report a more extensive investigation of the properties and submitochondrial localization of hippurate synthesis.

Materials and Methods

Materials

Benzoyl-CoA, CoA and ATP were from P-L Biochemicals (Milwaukee, WI, U.S.A.). [*carboxy*- ^{14}C]-Benzoic acid (55 mCi/mmol) was from Amersham/Searle (Arlington Heights, IL, U.S.A.). All other reagents were from commercial sources and were A.R. grade or better.

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Glycine *N*-acyltransferase was prepared from ox liver essentially as described by Bartlett & Gompertz (1974) and had a specific activity of 3.5 units/mg of protein. Other enzymes were from Boehringer Mannheim Corp., New York, NY, U.S.A.

Methods

Preparation of mitochondria. Liver mitochondria were prepared from male Sprague-Dawley rats (200–225 g) by the method of Johnson & Lardy (1967). The isolation medium contained 250 mM-sucrose, 5 mM-Hepes* and 0.1 mM-EGTA at pH 7.6. Protein was measured by the procedure of Gornall *et al.* (1949) after lysis of mitochondria with an equal volume of 10% (v/v) Triton X-100.

Analytical methods. P_i from ATP hydrolysis was determined as described by Sumner (1944), and the procedure of Parvin & Smith (1969) was used to measure the P_i content of mitochondria. O_2 consumption was recorded on a Gilson oxygraph equipped with a Clark electrode. Respiration in the absence of a phosphate acceptor is termed State 4 and in the presence of ADP as State 3 (Chance & Williams, 1957). CoA plus acetyl-CoA was determined by the catalytic assay of Michal & Bergmeyer (1974). Short-chain acyl-CoA was measured by addition of 0.1 unit of carnitine acetyltransferase (EC 2.3.1.7) plus 1 mM-L-carnitine to a cuvette containing sample, 0.1 M-Tris/HCl, pH 8.0, and 0.5 mM-5,5'-dithiobis-(2-nitrobenzoic acid), and measuring the increase in A_{412} . This was assumed to be mainly due to acetyl-CoA. Benzoyl-CoA was determined by subsequent addition to the same cuvette of 20 mM-glycine plus 0.1 unit of glycine *N*-acyltransferase.

Measurement of synthesis of hippurate. A 10 μ l portion of an incubation sample that had been deproteinized with 0.05 ml of 1.5 M-HClO₄, and which originally contained 0.2 μ Ci of [*carboxy*-¹⁴C]benzoic acid in 0.2 ml, was spotted on Whatman 3M paper (W. and R. Balston, London, U.K.). After drying, the spots were covered with 10 μ l of 50 mM-potassium hippurate. Ascending chromatograms were run in formic acid-saturated toluene to a height of 18 cm and allowed to dry overnight. Benzoic acid moves very near the solvent front in this system. Hippuric acid spots (which barely moved from the origin) were located under u.v. light and cut out. They were counted for radioactivity at 63% efficiency in 5 ml of toluene containing 4 g of diphenylloxazole/litre and 0.2 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre. This assay also detects some [*carboxy*-¹⁴C]benzoyl-CoA (R_F = 0); however, since in the presence of glycine the mitochondrial content of benzoyl-CoA is low (Table 5), the resulting overestimate is not large. At the 5 min

time-point in Fig. 1, it was less than 2%. There was good agreement between this technique, the chromatographic system used by Gatley & Sherratt (1976), and determination with ninhydrin of glycine released by acid hydrolysis of dried ethereal extracts (Chantrenne, 1951).

Results

Mitochondrial synthesis of hippurate

Hippurate synthesis from glycine plus benzoate by intact rat liver mitochondria can be supported by exogenous ATP or by an oxidizable substrate such as glutamate (Fig. 1). The rates were fairly linear with time up to 10 min, whereas in the absence of a substrate there was very little synthesis beyond 5 min.

Fig. 1. *Synthesis of hippurate by mitochondria, supported by ATP hydrolysis and by oxidation of glutamate* Mitochondria (0.5–0.75 mg of protein) were incubated with shaking (90 strokes/min) in 7 ml glass scintillation vials. The final volume was 0.2 ml and the temperature was 30°C. The medium was composed of 120 mM-KCl, 5 mM-glycine, 0.2 mM-benzoate, 0.1 μ Ci of [¹⁴C]benzoate and 10 mM-Hepes, pH 7.6 (■). Where indicated, 5 mM-ATP plus oligomycin (10 μ g/ml; ●) or 10 mM-potassium glutamate (▲) were also present. The reaction was initiated by addition of 0.02 ml of mitochondrial suspension and terminated with 0.05 ml of 1.5 M-HClO₄. Values for ATP- and glutamate-supported synthesis are means $\pm$ S.E.M. for seven experiments.

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Table 1. *Effects of metabolic inhibitors on the rates of hippurate synthesis by intact mitochondria expressed as a percentage of control value averaged over 0–5 min and 5–15 min*

Mitochondria were incubated as described in the legend to Fig. 1, with the indicated additions and omissions. Numbers of experiments are given in parentheses. The control rates of synthesis of hippurate are shown in Fig. 1.

Additions and omissions	Time range	...	Rate of hippurate synthesis (%)			
			5 mM-ATP+10 μ g of oligomycin/ml		10 mM-Potassium glutamate	
			0–5 min	5–15 min	0–5 min	5–15 min
None (7)			100	100	100	100
Minus oligomycin (3)			94	112	—	—
Minus oligomycin+rotenone (1 μ g/ml) (2)			81	88	—	—
+Carboxyatractyloside (2 nmol/mg of protein) (2)			39	3	92	—
+1 mM-Carbonylcyanide trifluoromethoxyphenylhydrazone (1)			76	47	—	—
+Antimycin A (1 μ g/ml) (2)			59	51	35	51
+Rotenone (1 μ g/ml) (3)			68	73	—	—
+10 μ M-Fluorocitrate (1)			122	97	102	40
+10 mM-Potassium malonate (1)			47	77	36	26
+10 μ M-Sodium arsenite (1)			54	70	30	16
+20 mM-KF (1)			42	17	—	—
+10 mM-Potassium glutamate (1)			96	112	—	—
+5 mM-ADP (2)			—	—	49	43
+0.3 mM-Benzoyl-CoA (1)			81	73	59	85
+2.5 mM-K ₃ PO ₄ (2)			—	—	131	128
+N-Ethylmaleimide (50 nmol/mg of protein) (1)			—	—	21	2
+N-Ethylmaleimide+2.5 mM-potassium phosphate (1)			—	—	73	5

Effects of metabolic inhibitors on the synthesis of hippurate

The influence of several compounds on hippurate formation is presented in Table 1. Results are tabulated as percentages of the control rates of glutamate- and ATP-supported synthesis over the first 5 min and between 5 and 15 min. The respiratory inhibitor antimycin A and the inhibitors of the tricarboxylic acid cycle, malonate, arsenite and fluorocitrate, all diminished glutamate-supported synthesis by more than 50%. Nearly 99% inhibition of oxidative metabolism may be necessary to inhibit hippurate formation by 50%, since the process requires only about 2.5% of the capacity of the mitochondria for making ATP. KF strongly decreased hippurate synthesis; this was expected because F[−] inhibits enzymic hydrolysis of pyrophosphate (formed by benzoyl-CoA synthetase) and thus causes product inhibition of the synthetase.

Carboxyatractyloside was without great effect on hippurate synthesis supported by glutamate; the mean amount in 5 min in one experiment ($\pm$ S.D., $n = 3$) was 8.93 ± 0.30 nmol/mg of protein in the control compared with 8.20 ± 0.29 in the presence of the translocase inhibitor. ATP-supported synthesis was, however, potentially (97%) inhibited by carboxyatractyloside between 5 and 15 min. Some caution must be exercised in interpreting the latter result as absolute proof of the matrix location of hippurate

synthesis. Skrede & Bremer (1970) have pointed out that atractyloside directly inhibits acyl-CoA synthetases. However, the lack of inhibition of glutamate-supported synthesis makes any other location unlikely. Additional evidence for this view is given by the lack of great effect of exogenous benzoyl-CoA, which would have substantially decreased incorporation of [*carboxy*-¹⁴C]benzoate into [¹⁴C]hippurate if it equilibrated with [*carboxy*-¹⁴C]benzoyl-CoA formed by the mitochondria.

Even in the presence of exogenous ADP, which is transported into the matrix in exchange for ATP (Pfaff *et al.*, 1965) and is thus expected to lower greatly the mitochondrial concentration of the latter, hippurate was formed at about one-half the control rate. The high activity of adenylate kinase (EC 2.7.4.3) in the intermembrane space of rat liver mitochondria makes interpretation of this observation difficult. The apparent equilibration constant for the adenylate kinase reaction is about 0.8, so that at equilibrium slightly less than one-third of the added adenine nucleotide (1 μ mol) will be present as ATP. Schnaitman & Greenawalt (1968) give the activity of adenylate kinase as 300 nmol/min per mg of protein, so that the reaction should approach equilibrium after about 10 min. Since exogenous ADP is actively concentrated in the matrix relative to ATP (see Klingenberg, 1970), it can be concluded that the affinity of benzoyl-CoA synthetase for ATP must be

Fig. 2. Dependence of hippurate synthesis by mitochondria on the concentrations of glycine and benzoate. Mitochondria (0.56 mg of protein) were incubated for 5 min as described in the legend to Fig. 1, except that the concentrations of glycine and benzoate were varied as shown. In (a), 0.2 mM-benzoate was present; there was 5 mM-glycine in (b). ▲, Control; ●, +rotenone (1 μg/ml).

high relative to that of the translocate. Addition of 2.5 mM-phosphate stimulated synthesis supported by glutamate to the rate observed with ATP plus oligomycin. Presumably, resynthesis of ATP is limited by a low free P_i concentration in the matrix, even though a standard preparation of mitochondria contained 5.7 nmol of P_i /mg of protein. This value is in fair agreement with other determinations (Pfeiffer *et al.*, 1976) and would represent a matrix concentration of about 10 mM if it were all in free solution. The effect of phosphate was also observed on 3-hydroxybutyrate-supported hippurate synthesis; the rate (0.5 min) was increased from 0.91 to 1.60 nmol/min per mg of protein, but this was less than the rate with ATP plus oligomycin (3.34). *N*-Ethylmaleimide (which inhibits phosphate transport into the matrix compartment) inhibited hippurate synthesis almost completely; phosphate partially protected against this effect.

Antimycin, rotenone, carbonyl cyanide trifluoromethoxyphenylhydrazone, malonate and arsenite, but not fluorocitrate, each inhibited synthesis of hippurate supported by ATP plus oligomycin by 25–50% in separate experiments (Table 1). The effects of antimycin and rotenone were equal in one experiment where they were directly compared. Rotenone inhibited less in the absence than in the presence of oligomycin. Increasing the concentration

of glycine or of benzoate did not decrease the inhibition caused by rotenone (Fig. 2). These observations are discussed below.

Comparison of hippurate synthesis and citrulline synthesis

It was decided to compare hippurate synthesis with that of citrulline, which also takes place in the mitochondrial matrix (Charles *et al.*, 1967). However, 1 mol of citrulline is synthesized at the expense of 2 mol of ATP hydrolysed to ADP, whereas hippurate synthesis results in formation of 1 mol of AMP. The major difference apparent is that 2,4-dinitrophenol stimulates citrulline synthesis supported by ATP plus oligomycin, but inhibits hippurate formation (Table 2). Graafmans *et al.* (1968), who first recorded this effect of an uncoupler on citrulline synthesis, suggested that stimulation resulted from facilitation of transport across the inner membrane of ATP (for discussion, see Klingenberg, 1970), for which the synthesis of citrulline had a relatively low affinity.

Whereas all the respiratory substrates tested were approximately equal in their abilities to support citrulline synthesis, glutamate was clearly the best substrate for formation of hippurate. In other experiments, glycerol 3-phosphate, 2-oxoglutarate, pyruvate

and citrate were also shown to support lower rates of hippurate synthesis than did glutamate.

Activities of enzymes involved in hippurate synthesis

In agreement with the results of Gatley & Sherratt (1976), mitochondrial benzoyl-CoA synthetase activity was greatly increased by sonication. A value of 11.4 nmol/min per mg of protein for sonicated mitochondria incubated at pH 8, compared with 0.8 nmol/min per mg of protein for intact mitochondria at pH 7.6. These values are reported for completeness, and because Sprague-Dawley rather than Wistar rats were used in the present study.

Glycine *N*-acyltransferase and a benzoyl-CoA hydrolase activity were also increased by disruption of the mitochondria (Tables 3 and 4). The activity of glycine *N*-acyltransferase was several times that of benzoyl-CoA synthetase. Carnitine was without effect on the rate of glycine-dependent benzoyl-CoA disappearance, showing that this thioester is not a substrate for any carnitine acyltransferase. Table 3 shows that although freeze-thawing makes mitochondrial glycine *N*-acyltransferase accessible to exogenous benzoyl-CoA, it does not release all the enzyme, since when the broken mitochondria were sedimented by centrifugation less than 10% of the activity remained in the supernatant, compared with over one-half of the total protein.

Mitochondrial content of benzoyl-CoA

Benzoate acylated much of the mitochondrial CoA (Table 5); the extent of benzylation was much

lower (about 30%) when glycine was also present. Glycine alone caused a small increase in free CoA. Slightly more benzoyl-CoA was formed when mitochondria were energized with ATP plus oligomycin

Table 2. *Comparison of the rates of synthesis of hippurate and of citrulline by intact mitochondria with different conditions*

Hippurate synthesis was measured as described in Fig. 1, except that the protein concentration was 5 mg/ml and that 2.5 mM-K₃PO₄ was present. The medium and conditions for measurement of citrulline synthesis were identical except that benzoate and glycine were replaced by 10 mM-L-ornithine, 10 mM-NH₄Cl and 10 mM-KHCO₃, and the final volume was 1 ml in standard scintillation vials previously equilibrated with O₂/CO₂ (19:1). Rates were calculated from duplicate 2 min and 4 min time points for citrulline synthesis and duplicate 3 min and 6 min points for hippurate synthesis.

Additions	Rates of synthesis (nmol/min per mg of protein) of:	
	Hippurate	Citrulline
10 mM-Glutamate	3.6	6.3
10 mM-Succinate	2.9	5.3
10 mM-3-Hydroxybutyrate	2.1	6.4
1.5 mM-Ascorbic acid + 1.5 mM- NNN'-tetramethylphenylene- diamine	2.9	6.3
5 mM-ATP	3.5	2.8
5 mM-ATP + 10 µg of oligomycin/ ml / + 100 µM-2,4-dinitrophenol	1.7	14.1

Table 3. *Hydrolysis of benzoyl-CoA by intact and by freeze-thawed mitochondria*

In Expt. I, mitochondria (1.5 mg of protein) were incubated with 60 nmol of benzoyl-CoA in a final volume of 0.5 ml. The medium was composed of 120 mM-KCl and 10 mM-Hepes, pH 7.6. For Expt. II, the same volume was used containing 85 nmol of benzoyl-CoA and 1.95 mg of protein. At the indicated times the suspension was spun for 2 min in an Eppendorf 3200 centrifuge; a 0.5 ml portion of the supernatant was removed immediately and kept on ice until assayed for benzoyl-CoA. For Expt. III, mitochondria were added to an equal volume of water and freeze-thawed five times. The incubation volume was 0.4 ml, containing 69 nmol of benzoyl-CoA and 2.0 mg of protein. Reaction was stopped by addition of 0.1 ml of 1.5 M-HClO₄. After removal of precipitated protein, 0.2 ml was added to 0.8 ml of 0.1 M-Tris/HCl; the mixture was carefully adjusted to pH 8.0 and kept on ice until assayed for benzoyl-CoA.

Additions	Benzoyl-CoA hydrolysis (nmol/mg of protein)			
	0 min	5 min	10 min	15 min
Expt. I; intact				
Control	0	—	—	2.9
+5 mM-Glycine	—	5.7	7.2	10.1
Expt. II*; intact				
Control	0.7	—	—	2.3
+5 mM-Glycine	—	—	—	16.9
+5 mM-Glycine + 1 mM-L-carnitine	—	—	—	15.1
Expt. III*; freeze-thawed				
Control	0	—	13	—

* Values are the average of duplicate incubations.

Table 4. *Hydrolysis of benzoyl-CoA by freeze-thawed and by Triton-treated mitochondria*

Benzoyl-CoA hydrolysis was assayed by recording the decrease in A_{280} (Schachter & Taggart, 1954). Mitochondria (30mg of protein/ml) were treated with 1% Triton X-100 (v/v, final concn.) or by freeze-thawing five times after addition to an equal volume of either isolation medium (iso-osmotic) or water (hypo-osmotic). Freeze-thawed mitochondria were centrifuged for 4min in an Eppendorf 3200 centrifuge. The supernatants were slightly turbid. Assays were done in 1 ml of 120mM-KCl/10mM-Hepes, pH 7.6, with 24 μ M-benzoyl-CoA and 5mM-glycine; 0.6mg of protein was used for Triton-treated mitochondria and 0.15mg of protein for freeze-thawed mitochondria. Rates are corrected for the rates of decrease of A_{280} in the absence of glycine.

Treatment	Rate of benzoyl-CoA hydrolysis (nmol/min per mg of mitochondrial protein)	Percentage of protein in supernatant
1% Triton X-100	37	—
Freeze-thawed (iso-osmotic)	23	—
Freeze-thawed (hypo-osmotic)	21	—
Supernatant from freeze-thawed preparation (iso-osmotic)	<0.1	54
Supernatant from freeze-thawed preparation (hypo-osmotic)	2	61

Table 5. *Mitochondrial content of free CoA, acetyl-CoA and benzoyl-CoA*

Mitochondria (12.5mg of protein) were incubated under the conditions of Fig. 1 for 2min in a final volume of 1.0ml and the reaction was then stopped with 0.17 ml of 1.5M-HClO₄. Benzoyl-CoA was assayed as substrate for glycine *N*-acyltransferase, and acetyl-CoA (measured as short-chain acyl-CoA) as substrate for pigeon liver carnitine acetyltransferase. Values are the mean $\pm$ S.E.M. for three preparations of mitochondria.

Substrate	Additions	Mitochondrial content of CoA species (nmol/mg of protein)		
		CoA	Benzoyl-CoA	Acetyl-CoA
10mM-Glutamate	None	0.54 $\pm$ 0.10	0.19 $\pm$ 0.05	0.33 $\pm$ 0.04
	0.2mM-Benzoate	0.20 $\pm$ 0.05	1.37 $\pm$ 0.04	0.28 $\pm$ 0.04
	5mM-Glycine	0.62 $\pm$ 0.12	0.12 $\pm$ 0.05	0.28 $\pm$ 0.08
	0.2mM-Benzoate + 5mM-glycine	0.63 $\pm$ 0.09	0.47 $\pm$ 0.07	0.21 $\pm$ 0.07
5mM-ATP plus 10 μ g of oligomycin/ml	None	0.76 $\pm$ 0.07	0.04 $\pm$ 0.01	<0.06
	0.2mM-Benzoate	0.30 $\pm$ 0.04	1.55 $\pm$ 0.04	<0.08
	5mM-Glycine	0.90 $\pm$ 0.07	<0.06	<0.05
	0.2mM-Benzoate + 5mM-glycine	0.63 $\pm$ 0.06	0.58 $\pm$ 0.05	<0.06

rather than with glutamate. In the latter case, especially, some 'benzoyl-CoA' was apparent in the absence of benzoate. This is presumed to represent another acyl-CoA, which is a substrate for glycine *N*-acyltransferase. The total (free CoA plus acetyl-CoA plus benzoyl-CoA) detected was considerably higher in the presence of benzoate. The only other source of CoA in mitochondrial preparations appears to be long-chain acyl-CoA derivatives (see Holland & Sherratt, 1973). Table 5 also shows that free CoA was greater and acetyl-CoA less with ATP plus oligomycin rather than glutamate. Neither glycine nor benzoate greatly affected the amount of acetyl-CoA.

ATP hydrolysis and O₂ consumption owing to hippurate synthesis

The hydrolysis of ATP by intact mitochondria in the presence of oligomycin, benzoate and glycine reflected the energy requirements for hippurate

formation, when corrected for hydrolysis in the absence of benzoate (Table 6). The corrected rate of 53nmol of ATP hydrolysed/min per mg of protein would represent formation of 26.5nmol of hippuric acid/min per mg of protein, perhaps 20% higher than that observed (Fig. 1). There was, however, about one-half this rate of hydrolysis in the absence of glycine; this was presumably due to simultaneous operation of benzoyl-CoA hydrolase (Table 3) and benzoyl-CoA synthetase.

O₂ uptake owing to hippurate synthesis was also measured (Fig. 3). These data have been replotted in differential form from polarographic recordings of O₂ concentration, since the changes in rate were small. A small increase was observed on addition of benzoate alone, but when glycine was also present the increase in rate was sufficient to account for observed rates of hippurate formation. In another experiment (done in the absence of P_i), the increase in the rate of O₂ uptake was 1.30 $\pm$ 0.17 ng-atoms/min per mg of

Table 6. *ATP hydrolysis by intact rat liver mitochondria in the presence of glycine and benzoate*

Mitochondria were incubated with 5mM-ATP plus 10 µg of oligomycin/ml as described in the legend to Fig. 1, except that 2.0mg of mitochondrial protein was used in a final volume of 0.4ml. Reaction was stopped with 0.1ml of 1.5M-HClO₄. Values are means of two incubations with different preparations of mitochondria.

Additions	Hydrolysis of ATP (nmol of P _i /mg of protein)		
	5min	10min	15min
None	1.3	10.6	24.2
0.2mM-Benzoate	27.3	58.5	69
5mM-Glycine	7.6	16.8	21.2
0.2mM-Benzoate+5mM-glycine	61	91	126

Fig. 3. *O₂ uptake by mitochondria in the presence of glycine and benzoate*

Mitochondria (3mg of protein/ml) were incubated at 30°C in 3.8ml of the medium described in the legend to Fig. 1; 2.5mM-K₃PO₄ was also present. Additions of 10mM-glutamate, 0.25mM-benzoate (BzO⁻) and 5mM-glycine were made as indicated. The rates of O₂ consumption at 50s intervals are shown. Two separate incubations are shown (●, additions shown by ↓; ▲, additions shown by ↑).

protein, whereas the required increase calculated from hippurate synthesis was 1.19 ± 0.04 (means $\pm$ S.D., $n = 3$). Since this gives a P/O ratio of 2.75, close to the expected value of 3 for oxidation of glutamate, it seems unlikely that the increase in respiration is due to, for instance, a synergistic uncoupling effect of glycine plus benzoate. Neither 5mM-glycine nor 1mM-hippurate (results not shown) caused any measurable extra O₂ uptake.

There was also an increase in mitochondrial O₂ uptake in the combined presence of pyruvate and

Fig. 4. *Inhibition by benzoate of mitochondrial uptake of O₂ caused by pyruvate plus HCO₃⁻*

Mitochondria were incubated as described in the legend to Fig. 3. ▲, +P_i; ●, -P_i. Additions of 5mM-pyruvate (Pyr), 10mM-HCO₃⁻, 0.25mM-benzoate (BzO⁻) and 5mM-glycine were made as indicated. The final arrow marks the exhaustion of available O₂.

HCO₃⁻, provided that the medium also contained P_i (Fig. 4). This confirms the conclusion of Bottger *et al.* (1969) that pyruvate carboxylase (EC 6.4.1.1) is located in the matrix compartment, where ADP produced by the enzyme can be rephosphorylated at the expense of respiration. That benzoate inhibited this extra consumption of O₂ (Fig. 4) further demonstrates that the compound did not increase respiration when added with glycine by acting as a classical uncoupler. S. J. Gatley & H. A. Lardy (unpublished work) have shown that mitochondrial carboxylation of pyruvate depends on exogenous P_i and is strongly inhibited by 0.2mM-benzoate.

Discussion

Proof of matrix location of synthesis of hippurate

The latency of benzoyl-CoA synthetase and of glycine *N*-acyltransferase to procedures which disrupt the mitochondrial inner membrane (sonication, freeze-thawing and lysis with Triton X-100) supports the conclusion of Gatley & Sherratt (1976) that hippurate is synthesized in the matrix. Additional evidence is given by: the absence of great effect of exogenous benzoyl-CoA on incorporation of

[carboxy- ^{14}C]benzoate into [carboxy- ^{14}C]hippurate; the observations that synthesis is supported by respiration (Fig. 1), even in the presence of carboxy-atractyloside (Table 1); and by the effect of the latter compound on synthesis supported by exogenous ATP.

Rate-limiting step of hippurate synthesis

The activity of glycine *N*-acyltransferase under the conditions used (Table 4) was severalfold greater than that of benzoyl-CoA synthetase, or the maximum rate of hippurate synthesis (Fig. 1). Although the physiological significance of enzyme activities measured in disrupted mitochondria may be uncertain because of ignorance about conditions in the matrix, it seems likely that benzoyl-CoA synthesis limits the rate of formation of hippurate. This is supported by the considerably lower concentration of benzoyl-CoA in mitochondria incubated with benzoate plus glycine compared with benzoate alone (Table 5). A consequence of the decrease in matrix benzoyl-CoA is the fairly good agreement between measured and calculated ATP hydrolysis rates (Table 6), without correction for the rate in the absence of glycine; the rate of the benzoyl-CoA hydrolase reaction presumably decreases markedly with the concentration of benzoyl-CoA, indicating that the enzyme has a high K_m for this substrate.

The K_m of ox liver glycine *N*-acyltransferase for glycine is about 15 mM (Schachter & Taggart, 1954; Killenberg *et al.*, 1971). This value, if assumed for the rat liver enzyme, contrasts with the results given in Fig. 2, where hippurate synthesis reached a plateau with 5 mM-glycine. Presumably this is because benzoyl-CoA is already being removed as rapidly as it can be synthesized. This is probably also true *in vivo*, since liver of starved rats contains 4.8 mmol of glycine/100 g wet wt. of tissue [Leung, (1965), cited by Young (1970)], indicating a glycine concentration somewhat higher than 5 mM if it is distributed evenly.

Rephosphorylation of AMP produced during hippurate synthesis

The agreement between the measured rates of ATP hydrolysis (Table 4) and of O_2 consumption (Fig. 3) and the values calculated from observed rates of hippurate synthesis demonstrate that AMP is rephosphorylated at the expense of ATP, rather than by GTP generated by substrate-level phosphorylation. Schnaitman & Greenawalt (1968) showed that the inner-membrane plus matrix fraction prepared by treatment with digitonin contained no adenylate kinase, but some 6% of the total nucleoside diphosphate kinase. The latter enzyme, in concert with GTP-AMP phosphotransferase (EC 2.7.4.10) may recycle AMP.

Cause of State-4 respiration

In the presence of benzoate and glycine mitochondria consumed an additional quantity of O_2 , which was in good agreement with that necessary to support the observed rate of hippurate synthesis (Fig. 3). This is unlikely to be an artifact, since benzoate alone caused very little O_2 uptake (Fig. 3), since neither 5 mM-glycine nor 1 mM-hippurate stimulated respiration, and since benzoate strongly inhibited increased respiration caused by pyruvate plus HCO_3^- (Fig. 4). These results strongly suggest that State-4 respiration is not used, at least under our conditions, for ATP synthesis. This does not contradict the suggestion of Nicholls (1974) that State-4 respiration is caused by dielectric breakdown of the inner mitochondrial membrane. According to this view, which is discussed in terms of the chemiosmotic hypothesis (Mitchell, 1966), the membrane can be compared to a zener diode across which the respiratory chain, which behaves as an electrical cell, and adenosine triphosphatase (EC 3.6.1.4), which can be likened to an electrical load, are arranged in parallel. When the demand for ATP (i.e. the load) is high, as in State-3 respiration, the membrane potential (the voltage across the diode) will decrease, as shown experimentally by Nichols (1974) and by Mitchell & Moyle (1969). However, with this model at small loads the resistance represented by adenosine triphosphatase will be high and the voltage across the diode will not decrease significantly, whereas extra current will be taken from the cell. In other words, respiration will increase in response to small demands for ATP, even though a large part of the O_2 consumption is 'wasted' because of dielectric breakdown of the inner membrane.

Remaining problems

The mechanism of inhibition of ATP-supported hippurate synthesis by inhibitors of the respiratory chain and of the tricarboxylic acid cycle remains to be explained. However, the effect of rotenone was much less in the absence of oligomycin, which itself was not inhibitory (Table 1). One possible explanation is that the membrane potential (or other high-energy intermediate of oxidative phosphorylation), which can be generated either by respiration or by ATP hydrolysis is necessary for maximum synthesis of hippurate. If this is so, the effect appears not to be on transport of glycine or of benzoate, since increased concentrations of these did not stimulate synthesis in the presence of rotenone plus oligomycin (Fig. 2). Another possibility is that the intramitochondrial pH is lowered because of inhibition of respiration, and that this decreases the activity of benzoyl-CoA synthetase, which limits the rate of synthesis of hippurate (see above).

Scheme 1. Reactions for synthesis of hippurate

A further question is why glutamate supports hippurate synthesis better than any other respiratory substrate tested. The answer cannot involve generation of GTP (to transphosphorylate AMP) by succinate-CoA ligase (EC 6.2.1.4), since oxidation of pyruvate or of 2-oxoglutarate should also provide flux through that portion of the tricarboxylic acid cycle. Further, if that were so it would be hard to explain why ATP plus oligomycin can support the maximum rate of synthesis.

Summary of reactions involved in the synthesis of hippurate by mitochondria

The present work, with that of Gatley & Sherratt (1976), supports the suggestion that hippurate is synthesized by the set of reactions shown in Scheme 1. Because of the pH gradient across the inner membrane, benzoate accumulates (Gatley & Sherratt, 1976), and acylates CoA, forming benzoyl-CoA while ATP is hydrolysed to AMP and PP_i. Inorganic pyrophosphatase (EC 3.6.1.1) hydrolyses the PP_i to P_i. AMP is rephosphorylated to ADP at the expense of GTP, and the resulting GDP is rephosphorylated by ATP. Thus for every molecule of benzoyl-CoA synthesized, two molecules of ATP are hydrolysed to ADP, which can exchange for exogenous ATP, if this is present, or be phosphorylated by the respiratory-chain-linked adenosine triphosphatase (EC 3.6.1.3). Benzoyl-CoA reacts with glycine, which freely crosses the inner membrane (Gatley & Sherratt, 1976), to form hippurate, which probably leaves the mitochondrion as the undissociated acid. In addition, but not shown in Scheme 1, there is a benzoyl-CoA hydrolase of high *K_m*, the function of which is probably to prevent acylation of the total mitochondrial CoA if insufficient glycine is available for conjugation.

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