The Availability of Carnitine Acetyltransferase in Mitochondria from Guinea-Pig Liver and other Tissues

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The carnitine acetyltransferase and glutamate dehydrogenase activities of guinea-pig liver and other tissues were estimated. Both enzymes are wholly mitochondrial, and can only be fully observed after disruption of the mitochondrion. Triton X-100 (0.1%) or freeze-drying revealed more activity than other methods tried. In mitochondria prepared and suspended in 0.25M-sucrose and in cell cytoplasm only small fractions of the total enzymic activity could be observed in guinea-pig liver: on average 7.5% of carnitine acetyltransferase and 5.5% of glutamate dehydrogenase. It is concluded that, in liver or mammary gland of goat, guinea pig or rat, little or no carnitine acetyltransferase is available *in vivo* to acetyl-CoA outside the mitochondrion.

The normal function of CAT† is as yet unclear, though several hypotheses have been advanced. Fritz & Yue (1964) showed that carnitine increased the oxidation rate of external acetyl-CoA in isolated rat heart mitochondria, and Bressler & Katz (1965) presented evidence that the enzyme transports acetyl groups from the inside to the outside of guinea-pig liver mitochondria. It has also been suggested that acetylcarnitine may act as a store of readily available 'active' acetyl groups (Pearson & Tubbs, 1967), or a means of increasing available CoA, or that the transferase may be involved in intramitochondrial transport of such groups (Lowenstein, 1965).

If acetyl groups are transported from inside to outside mitochondria as acetylcarnitine, some means of reconversion into acetyl-CoA must be operative. It now appears likely (Beenackers & Henderson, 1967; Norum & Bremer, 1967) that the major part and probably the whole of CAT activity is associated with the mitochondrial fraction, so that any reconversion of acetylcarnitine into acetyl-CoA must occur on the mitochondria outside the barrier to acetyl-CoA, and not in the cytoplasm. The acetyl-CoA-binding site of the enzyme (Chase & Tubbs, 1966a) would thus be available to external acetyl-CoA and the carnitine-binding site (Schemes 1a and 1b) available to external carnitine. Schemes

1(b) and 1(c) allow for the observation by Yates & Garland (1966) that the CoA barrier may also be a carnitine barrier. In Scheme 1(c), although the system would transport acetyl-CoA, the carnitine-binding sites would not be available to external carnitine, and the enzyme would not be measurable in intact mitochondria unless sufficient carnitine were present inside the mitochondrion and internal acetyl-CoA were deacylated. In the present work we measured the activity in 'intact' mitochondria as a fraction of that in disrupted mitochondria, testing the possibility of the situations in Schemes 1(a) and 1(b). A few experimental observations suggesting that Scheme 1(c) does not apply to guinea-pig liver are also reported.

For these experiments, the following must be considered: (1) a suitable method for measuring CAT, especially in preparations of 'intact' mitochondria; (2) a suitable method for revealing the total amount of enzyme present; (3) a measurement of the increased permeability of isolated 'intact' mitochondria compared with their state *in vivo*.

(1) Assay of CAT. Measurement of the change in thiol ester bond concentration at 232m μ (Chase & Tubbs, 1966a) is the simplest method and can be used in either direction. However, 'intact' mitochondria have a high extinction at this wavelength and it has not proved feasible to use this assay. The coupled assay, with citrate synthase and malate dehydrogenase (Stern, Shapiro, Stadtman & Ochoa, 1951; Beinert *et al.* 1953; Fritz, Schultz & Srere, 1963), is at first sight more sensitive than the assay at 232m μ , but as shown by Buckel & Eggerer

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† Abbreviations: CAT, carnitine acetyltransferase (EC 2.3.1.7); GDH, glutamate dehydrogenase (EC 1.4.1.2); DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

Scheme 1. Transfer of acetyl-CoA across a mitochondrial barrier. (a) Barrier permeable to carnitine and acetylcarnitine. (b) and (c) Barrier impermeable to carnitine and acetylcarnitine: (b) left-hand side outside and right-hand side inside mitochondrion; (c) right-hand side outside and left-hand side inside mitochondrion.

(1965) and Pearson (1965), values must be corrected (see the Appendix). Nevertheless the coupled assay is better because mitochondria have a lower extinction at $340\text{m}\mu$ than at $232\text{m}\mu$.

The assay with DTNB (Fritz *et al.* 1963) is more sensitive than either assay, but DTNB reacts with protein thiol groups and may inhibit the enzyme or affect mitochondrial permeability. In spite of these disadvantages, the bulk of the evidence was obtained by this method because of its sensitivity and the lower absorption of 'intact' mitochondria at this wavelength; comparison with the other methods, where possible, was made in a few instances.

(2) Release of total enzyme. A variety of physical and chemical procedures can affect the integrity of mitochondria; whether, with a particular enzyme, a procedure reveals the whole of the activity can only be assumed by showing that (a) at least one other process reveals the same maximum activity and (b) the procedures used do not inhibit or destroy the enzyme. In the present work we used hypotonic conditions, detergents, freeze-drying and mechanical treatment.

(3) Mitochondrial integrity. It is necessary to know what proportion of mitochondria are broken or sufficiently permeable to acetyl-CoA to reveal CAT activity. We have assumed that GDH could not be observed at all in completely intact mitochondria (Hogeboom & Schneider, 1953; Bendall & de Duve, 1960; Pette, 1966); the GDH activity observed in 'intact' mitochondria as a fraction of

the maximum activity in suitably disintegrated mitochondria measures the fraction of mitochondria that is permeable to the rate-determining substance (NADH or, possibly, 2-oxoglutarate; Robinson & Chappell, 1967) or that have released GDH.

It is assumed that permeability to acetyl-CoA and CoA or uncovering of CAT would change to a similar extent to the corresponding factors affecting GDH and its substrates. If a mitochondrion is completely broken this is clearly true, but, if activity is revealed because the mitochondrial membrane becomes more permeable to a rate-limiting substrate, it is possible that the permeability of different substances might be changed to different extents. The results (see Table 2) suggest that CAT and GDH activities are similarly affected.

METHODS

Preparation of subcellular fractions. Guinea pigs and rats were killed by cervical dislocation and bleeding; goats and sheep were anaesthetized with Nembutal and bled; tissues were placed in cold 0.25M -sucrose and their volumes measured. After being minced briefly with scissors, liver was homogenized in 3 vol. of 0.25M -sucrose containing 5mM -tris-EDTA, pH 7.5, with six strokes of a TenBroek or Dounce all-glass hand-operated homogenizer (clearance $0.005\text{--}0.01\text{in.}$). Mammary glands were minced for 5 min. with scissors, and the minced tissue was suspended in 3 vol. of homogenizing medium and centrifuged for $2.4 \times 10^6\text{g-min.}$ before being homogenized in fresh medium in a loose-fitting homogenizer (clearance $0.01\text{--}0.015\text{in.}$).

Bremer (1962) reported that only by using 10mM -EDTA

in the homogenizing medium could CAT activity be demonstrated. We have always used 5mM-EDTA in the homogenizing medium. With guinea-pig liver, omission of EDTA made no difference to the CAT and GDH activities found in 'intact' or Triton X-100-treated mitochondria or in high-speed supernatant.

Successive centrifugation for about 6×10^3 g-min., 2×10^5 g-min. and 4×10^6 g-min. (measured at the middle of the tube) in a Spinco model L preparative ultracentrifuge sedimented the debris, mitochondrial and microsomal fractions respectively. These fractions were not washed; though clearly impure the last two are referred to below as mitochondrial and microsomal fractions. Pellets were resuspended in 0.25M-sucrose or water in a volume 25% of the original homogenate. It was assumed that the high-speed supernatant fraction represented 0.25g. of original tissue/ml. and the particulate fractions 1g./ml.; this ignores particulate material lost in the debris fraction.

Assay methods. All assays were done at room temperature (22–25°) with a Hilger-Gilford recording spectrophotometer or Unicam SP.800 spectrophotometer. In all assays with 'intact' mitochondria, 180mM-sucrose was also present. The rate was measured over a two- or four-fold range of protein concentration to show that rate was proportional to protein.

(a) Assay of CAT. In all assays the final concentrations in the spectrophotometer cell were: tris-HCl buffer, pH 7.8, 100mM; CoA or acetyl-CoA, 0.3mM; L-carnitine or acetyl-DL-carnitine, 10mM; the final pH was 7.6–8.0. For the DTNB assay 1.0mM-DTNB was used, added from a stock of 30mM-DTNB in 100mM-K₂HPO₄. For the coupled assay 30mM-L-malate, 1mM-NAD⁺, 3mM-KCN, dialysed malate dehydrogenase (50 μ g./ml.) and citrate synthase (17 μ g./ml.) were also present. The reactions were initiated by adding acetyl-CoA (DTNB assay), carnitine or acetyl-carnitine (232m μ assay) and CoA or acetyl-carnitine (coupled assay). In the DTNB assay, blanks without L-carnitine were always done at the same time.

(b) Assay of GDH. Concentrations in the assay medium were: tris-HCl buffer, pH 7.7, 100mM; 2-oxoglutarate, 5mM; NH₄Cl, 50mM; NADH, 0.2mM; ADP, 8 μ M (this concentration was subsequently found to give submaximal activity; M. Prosenjak, personal communication); KCN, 3mM; EDTA, 0.3mM; the final pH was 7.5–7.8. The reaction was initiated by NH₄Cl. Activity in whole mitochondria was also measured in the absence of 2-oxoglutarate or against a blank without NADH to ensure that any non-specific changes in extinction were allowed for; 'intact' mitochondria showed little extinction change under these circumstances.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Total protein in guinea-pig liver homogenate, excluding debris, was calculated to be 120–175mg./g. of fresh tissue; the mitochondrial fraction contained about 25% of this and the high-speed supernatant 60%.

(c) Assay of carnitine. Mitochondrial suspensions were treated with an equal volume of 1M-HClO₄ and, after removal of the precipitated protein by centrifugation, the supernatant was neutralized to pH 6–8 with 3.5M-KOH. The liquid was chilled and the KClO₄ precipitate removed by centrifugation. Carnitine was measured in the supernatant by CoA release from acetyl-CoA in the presence of CAT. Tris buffer, DTNB and acetyl-CoA were added in the same

concentration used for CAT estimation, and the extinction change at 412m μ on adding CAT was measured; a second addition of CAT was made to estimate a blank value.

MATERIALS

Acetyl-CoA and bromoacetyl-CoA were synthesized by treating CoA, dissolved in KHCO₃ solution, with acetic anhydride and bromoacetyl bromide respectively. Acetyl-CoA was estimated as described by Chase (1967), and bromoacetyl-CoA by reaction with hydroxylamine (Lipmann & Tuttle, 1945); the yields were about 70% for acetyl-CoA and 50% for bromoacetyl-CoA. Acetyl-DL-carnitine was synthesized by the method of Fraenkel & Friedman (1957); assay as hydroxamate indicated 85% purity and the m.p. (uncorrected) was 188–189°. Malate dehydrogenase, citrate synthase, CoA, NAD⁺, NADH and purified CAT were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), L-carnitine was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.), DTNB was from Aldrich Chemicals Inc. (Milwaukee, Wis., U.S.A.) and Triton X-100 was from C. Lennig and Co. Ltd. (London, W.C. 1).

The following molar extinction coefficients were assumed (cm.²/mole at pH 7.5–8 and 25°): NADH, 6.2×10^6 at 340m μ ; acetyl-CoA minus CoA, 4.5×10^6 at 232m μ ; DTNB, 13.6×10^6 at 412m μ ; acetylhydroxamate and bromoacetylhydroxamate (as ferric complexes), 0.72×10^6 at 490m μ .

RESULTS

Method of CAT assay. The three methods of assay were compared with purified CAT and the results are summarized in Table 1.

(a) DTNB assay. In preliminary experiments DTNB was added 4min. before the reaction was started with acetyl-CoA. Clearly both with purified enzyme and with extracts it is markedly inhibitory and we observed that the inhibition was progressive. Unlike Fritz *et al.* (1963), we observed that even when DTNB was added with acetyl-CoA the initial rate was low. Similar results were obtained with lysed mitochondria (Table 1), but 'intact' mitochondria showed no clear inhibitory effects, possibly because DTNB caused enzyme activity to be revealed as well as inhibited. Unless otherwise stated, all subsequent assays were done by adding DTNB immediately after acetyl-CoA and measuring the initial rate, because much of the data had been obtained before the inhibition by DTNB was observed. The fraction of activity in 'intact' mitochondria might appear higher as a result.

(b) Assay at 232m μ . This can be used in either direction. The observed ratio of forward to backward reaction rates (equilibrium constant) was 0.4 for the purified enzyme and 0.6–0.9 for mitochondrial activity, compared with the value 0.585 given by Chase & Tubbs (1966a). Values agreed tolerably well with those obtained by measuring CoA production by adding DTNB after 5min. (to

Table 1. *Comparison of methods of measuring CAT activity*

Method	Direction	Purified enzyme (units/mg. of protein)	Guinea-pig liver mitochondria treated with 0.1% Triton X-100 (units/g. of tissue)		
(a) DTNB assay	Acetyl-CoA → CoA				
DTNB added 3 min. before acetyl-CoA		8	0.47	0.37	—
DTNB added just after acetyl-CoA		49	1.57	1.40	1.30
DTNB added 5 min. after acetyl-CoA		70	2.82	2.8	—
(b) Assay at 232 mμ	Acetyl-CoA → CoA	80	1.7	1.3	1.9
	CoA → Acetyl-CoA	33	1.3	1.8	1.2
(c) Coupled assay	CoA → Acetyl-CoA	46	1.45	1.9	2.0
Ratio $\frac{\text{CoA} \rightarrow \text{acetyl-CoA rate}}{\text{Acetyl-CoA} \rightarrow \text{CoA rate}}$		0.41	0.7	1.35	0.62

minimize inhibition) in the one direction and maximal values observed in the coupled assay in the other.

(c) Coupled assay. The initial rate in this assay is expected to be only 50% of the true value (see the Appendix) because the equilibrium constant for malate dehydrogenase is towards malate plus NAD⁺; this is quite distinct from the general problem of coupled assays (Bergmeyer, 1965), and increasing the citrate synthase concentration tenfold did not alter the initial rate or the form of the progress curve. In general, when purified enzyme or lysed mitochondria were assayed, maximum rates were in reasonable agreement with those observed in the 232 mμ assay; however, when 'intact' mitochondria were used it was not possible to obtain consistent results with the coupled assay.

Comparison of methods of mitochondrial disruption.

Hypo-osmotic conditions, detergents, freeze-drying and mechanical treatment (Ultra-Turrax blender; Janke und Kunkel A.-G., Staufen i. Br., Germany) were used. Not all methods were used on any one preparation, and values are given in Table 2 as percentages of those observed in mitochondria treated with water and Triton X-100 at 0° [to give 25 mM-sucrose, 0.1% Triton X-100 and 2–3 mg. of protein/ml. (equivalent to 0.1 g. of tissue/ml.)] for at least 30 min. before assay ('lysed mitochondria'). Values did not increase when such lysed mitochondria were stored overnight at 0°. Triton X-100 at 0.05% did not clarify mitochondrial suspensions completely, but no differences were observed in the activities of CAT and GDH revealed by Triton X-100 in the concentration range 0.05–0.8%.

CAT seemed more readily destroyed by mechanical treatment than GDH. Maximum CAT activity was observed after 2 min. Ultra-Turrax treatment (at 0° in 15 sec. periods with 15 sec. intervals) and after 4 min. less than 10% of the total observable CAT activity was left, compared with about 50% of the GDH. It seemed reasonable to suppose that,

Table 2. *Enzyme activities in guinea-pig liver mitochondria treated in various ways as a percentage of that observed in mitochondria suspended in 0.1% Triton X-100–25 mM-sucrose (lysed mitochondria)*

Treatment	CAT activity (%)	GDH activity (%)
25 mM-Sucrose	38	35
5 mM-Sucrose	42	54
0.1% Triton X-100 and incubation at 37° for 1 hr.	32	100
Ultra-Turrax (2 min.)	57	100
0.025% Sodium dodecyl sulphate	70	84
0.1% Triton X-100 in spectrophotometer cell	73	48
Mitochondria in 0.25 M-sucrose, freeze-dried and resuspended in 25 mM-sucrose	116	106

in 'intact' mitochondria, uncovering and destruction of CAT activity occurred simultaneously because CAT rates decreased when lysed mitochondria were subjected to Ultra-Turrax treatment. Destruction of CAT activity also appeared to occur when lysed mitochondria were incubated for 1 hr. at 37°, but GDH activity did not decrease.

When the solution in the spectrophotometer cell contained 0.1% Triton X-100 and activities in 'intact' mitochondria were measured, the fraction of GDH activity observed was less than that of CAT activity. This concentration of Triton X-100 inhibited GDH activity in high-speed supernatant fractions by about 20% (5–30%) and purified CAT by 8%.

Sodium dodecyl sulphate also disrupts mitochondria; 250 μg./ml. in the spectrophotometer cell completely inhibited GDH activity, but had no effect on CAT activity. When the mitochondrial suspension was treated with sodium dodecyl sulphate before measurement (Table 2), so that the

Table 3. *Enzyme activities observed in 'intact' guinea-pig liver mitochondria, microsomal fraction and high-speed supernatant as percentages of those in disrupted mitochondria*

Abbreviation: HSS, high-speed supernatant.

Enzyme	Fraction	Expt. no. ...	Enzyme activity (%)					
			1	2	3	4	5	6
CAT	Mitochondrial		9	11.5	3.7	6.5	3.5	5.5
	Microsomal		<1*	<1*	<1.5*	3.8	4.6	4
	HSS		<1	<1	2.6	0.6	<1	<1
	Total (mitochondrial + HSS)		10	12	6.3	7.0	4.5	5.5
GDH	Mitochondrial		6.7	3.7	3.2	3	2	5
	Microsomal		1.4*	2.4*	2.7*	3	2	2
	HSS		2.3	2.5	0.5	1.3	1.6	1.2
	Total (mitochondrial + HSS)		9.0	6.2	3.7	4.3	3.6	6.2

* Centrifuged for 2.5×10^5 g.-min., not 2×10^5 g.-min.

concentration in the spectrophotometer cell was only about $1 \mu\text{g./ml.}$, some 84% of the GDH activity was observed. Thus, except when destruction or inhibition would be expected, the methods used revealed GDH and CAT activities to similar extents and no procedure revealed significantly more than did 0.1% Triton X-100 or freeze-drying.

Activity in 'intact' mitochondria. Table 3 shows the activities of CAT and GDH observed in 'intact' mitochondria as a percentage of the values obtained in lysed mitochondria. Values for microsomal and high-speed supernatant fractions are also given. It was assumed that the low activity in the microsomal fractions was due to contamination with mitochondria, an impression strengthened by the fact that a slight decrease in centrifugation at the mitochondrial stage increased the CAT activity in the microsomal fractions (but not, however, the GDH activity). It seemed probable that most of the mitochondria in the microsomal fraction were no longer intact because treatment with Triton X-100 did not greatly increase GDH activity.

The fractional activity of GDH in high-speed supernatant was notably higher than that of CAT. GDH is a soluble mitochondrial enzyme, whereas CAT may be largely or wholly membrane-bound, so that breakdown of mitochondria during homogenization and centrifugation might release GDH into the high-speed supernatant but only reveal CAT still attached to mitochondrial material. Breakdown after the mitochondria have been separated will increase the observable CAT in this fraction and reveal or release further GDH. Only occasionally was noteworthy CAT activity observed in high-speed supernatant. Addition of inactive high-speed supernatant to lysed mitochondria showed that it had no inhibitory properties. The large amount of thiol in liver high-speed supernatant and the low

CAT activity made assay by DTNB difficult; the CAT activity was a small difference between two large quantities.

Table 4 shows that GDH is more readily released from mitochondria than is CAT. When suspensions of lysed mitochondria were centrifuged for 4×10^6 g.-min., most of the GDH, but only about half of the CAT, was present in the supernatant. Pellets were resuspended in 0.25M-sucrose. The pellet from mitochondria treated with Triton X-100 was very small and contained 20% of the mitochondrial protein, whereas in 25mM-sucrose the pellet contained 65% of the mitochondrial protein.

The total activities observed in fractions from mitochondria in 25mM-sucrose were always greater than those of the sample before centrifugation, presumably because more enzyme had been revealed. Total recovery of activities in fractions from lysed mitochondria was variable, but averaged 90% (CAT) and 100% (GDH). In any one experiment the totals for CAT and GDH were similar.

Availability of CAT in other tissues. Table 5 shows the results of estimations in liver and mammary gland of guinea pig, goat and rat and in sheep liver. CAT was measured by adding DTNB immediately after acetyl-CoA and the values may therefore be low. In all species but the rat the fractions of GDH activity observed in 'intact' mitochondria plus high-speed supernatant were similar to those of CAT activity. In tissues from larger animals, with more connective tissue, it would appear that mitochondria are more damaged during preparation. In the goat and the guinea pig the total activities of both CAT and GDH were lower in mammary gland than in liver.

CAT values in the rat liver and mammary gland were low and measurement in 'intact' mitochondria was unreliable for this reason. Values for rat liver

Table 4. *Distribution of activities between pellet and supernatant after treatment of guinea-pig liver mitochondria with 25 mM-sucrose with or without 0.1% Triton X-100 and centrifuging for 4×10^6 g·min.*

Pellets were resuspended in 0.25 M-sucrose. Values are given as percentages of those observed in the appropriately treated mitochondrial suspension before centrifuging.

Treatment	Expt. no.	CAT				GDH			
		(% of value of lysed mitochondria)	(% of activity observed in suspension before centrifuging)			(% of value of lysed mitochondria)	(% of activity observed in suspension before centrifuging)		
			Supernatant	Pellet	Total		Supernatant	Pellet	Total
0.1% Triton X-100 25 mM-sucrose	1		46	15*	61		80	10*	90
	2		43	29	72		95	1	96
	3		104	25	129		110	7	117
	4		51	46	97		94	5	99
25 mM-sucrose	1	23	59	170*	229	40	65	85*	150
	2	32	55	106	161	36	108	22	130
	3	35	27	103	130	30	73	37	110
	4	48	40	93	133	40	97	27	124

* Pellet resuspended in water.

Table 5. *Activities in lysed mitochondria and the sum of the activities in high-speed supernatant and 'intact' mitochondria as a percentage of this*

One unit of activity is defined as 1 μ mole of substrate converted/min. under the assay conditions given in the text. Abbreviation: HSS, high-speed supernatant.

Species	Tissue	CAT		GDH		Mitochondrial protein (mg./g. of tissue)
		Activity (units/g. of tissue)	Activity in HSS plus 'intact' mitochondria (%)	Activity (units/g. of tissue)	Activity in HSS plus 'intact' mitochondria (%)	
Guinea pig	Liver	1.4	5	22	3.6	25
	Mammary gland	0.25	1.5	1.6	2.5	9
Goat	Liver	1.4	13.5	60	8	27
		1.2	12	26	10	22.4
	Mammary gland	0.4	15	0.4	13	8.2
Sheep	Liver	1.9	15	28	21	31.5
Rat	Liver	0.05	30	3.5	10	12
	Mammary gland	0.1	11	0.33	27	4.8

were much lower than those reported by Norum (1963) and Marquis & Fritz (1965), but the reasons for the difference are unknown.

Evidence on CAT inaccessible to external carnitine (Scheme 1c). For such an enzyme to be active, carnitine must be present inside the mitochondrion. In rat liver, free carnitine is about 170 μ mole/g. of tissue (Pearson & Tubbs, 1967); we estimated that our unwashed guinea-pig liver mitochondria contained 30–50 μ mole of L-carnitine/ml. of preparation. On washing the mitochondria once, by centrifuging the original suspension and resuspending in the same volume of 0.25 M-sucrose,

the carnitine concentration fell to 5–10 μ mole/ml. and a second wash lowered the concentration to less than 5 μ mole/ml.

In 'intact' mitochondria, we have observed release of CoA from acetyl-CoA in the absence of added carnitine and the rate has generally been 10–20% of the CAT activity of disrupted mitochondria. The carnitine-independent activity in disrupted mitochondria was only two to three times that in 'intact' mitochondria. In both cases, the activity was observable (though gradually diminishing) after 5 min., whereas the carnitine available would last for only 10–20 sec. in unwashed and

2–5 sec. in washed mitochondria. Some other acetyl acceptor may have been present, but rates were not increased in the presence of 30 mM-L-malate.

The rate of carnitine-independent CoA release from acetyl-CoA was decreased in twice-washed mitochondria to about 25% of the value in unwashed 'intact' mitochondria. Washing did not change carnitine-dependent CAT or GDH activities significantly in mitochondria, when these were assayed either 'intact' or after lysis. In measurements with lysed mitochondria, carnitine concentrations would be decreased 10^2 – 10^3 -fold, and none of the activity observed could reasonably be ascribed to CAT.

Bromoacetyl-CoA has been used by Chase & Tubbs (1966b) to inhibit CAT available to acetyl-CoA; the initial binding is presumably to the CoA-binding site (Chase & Tubbs, 1966a). It seemed reasonable to assume that bromoacetyl-CoA would inhibit CAT if Scheme 1(c) were correct. However, the carnitine-independent CoA release from acetyl-CoA was not inhibited by $10\text{ }\mu\text{M}$ -bromoacetyl-CoA, though $0.1\text{ }\mu\text{M}$ inhibited purified CAT and CAT activity in lysed mitochondria almost completely. This indicated that the possibility indicated in Scheme 1(c) is unlikely. At most, the activity of such an enzyme would be much less than the total CAT activity; there is clearly acetyl-CoA-deacylating activity present in mitochondria quite unrelated to carnitine, so that the fraction of CAT placed in the membrane as in Scheme 1(c) is certainly less than 10–20%.

DISCUSSION

The present results show that, in liver and mammary gland of guinea pig and goat, at most only a small proportion of mitochondrial CAT activity is available to external acetyl-CoA. In goat mammary gland we have shown (Hardwick, 1966) that only 0.5–2.5% of acetyl-CoA formed intramitochondrially from glucose appears in fatty acids of milk triglycerides. The fraction of CAT observable in 'intact' mitochondria is similar in each tissue studied to the fraction of GDH, so in the other tissues too extramitochondrial acetyl-CoA can be provided only slowly, if at all, by CAT.

Bressler, Katz & Wittels (1965) showed, in effect, that less than 10% of CAT in ox heart mitochondria is available to external acetyl-CoA. Using pyruvate as a source of intramitochondrial acetyl-CoA, and fatty acid synthesis to detect extramitochondrial acetyl-CoA, they found that acetyl transport was dependent on added CAT, without which the incorporation of pyruvate carbon into fat was only 10% of that with the complete system. The incorporation was also dependent on added

carnitine, but in its absence the value was 50% of that with the complete system.

The possibility exists, in some tissues, that acetyl-CoA could be transported by CAT if it is located as indicated in Scheme 1(c). Deacylation of acetyl-CoA occurred in the absence of added carnitine, and in 'intact' mitochondria this represented less than 20% of the total CAT activity. This carnitine-independent activity was not stimulated by malate, which might have provided oxaloacetate to remove acetyl-CoA, nor was it inhibited by bromoacetyl-CoA (Chase & Tubbs, 1966b). It would seem unlikely that any great part of the carnitine-independent activity was due to a system like Scheme 1(c). Again, the occurrence of carnitine-independent deacylation of acetyl-CoA in goat mammary gland, where acetyl transport is minimal, supports this view.

Mammary gland, where fatty acid synthesis rates are high, has lower total CAT activity than liver in the guinea pig and goat. Rat mammary gland, synthesizing fatty acids predominantly from glucose carbon, has much lower CAT activity/g. of tissue than goat mammary gland, which synthesizes fatty acids only from acetate. In rat mammary gland, fatty acid synthesis is about $1\text{ }\mu\text{mole}$ of acetyl group/min./g. of tissue (Howaritz & Levy, 1965), much higher than the total CAT activity. In tissues and species where acetate or fatty acids are oxidized in preference to glucose, CAT activity is higher than in those where glucose is freely oxidized (see also Beenakkers & Klingenberg, 1964). Such a correlation does not suggest that carnitine is important for transporting acetyl-CoA out of mitochondria, but suggests a role connected with acetyl-CoA within the mitochondria.

Yates & Garland (1966) found that 15% of carnitine palmitoyl-CoA transferase found in ultrasonically treated rat liver mitochondria could be observed in 'intact' mitochondria, and considered this was an external enzyme. We find that, with CAT, such an external enzyme must represent only about 2% of the total activity of CAT. In the absence of quantitative evidence about the extent of breakdown of mitochondrial permeability, it is, however, difficult to assess the various claims in the literature about the occurrence of CAT on the outside of the mitochondrion or in cell cytoplasm (McCaman, McCaman & Stafford, 1966). Childress, Sacktor & Traynor (1967) found no evidence that CAT mediated the transfer of acetyl-CoA in blowfly muscle mitochondria.

The small percentage of the total CAT activity that is available to acetyl-CoA in guinea-pig liver may be significant in the experiments of Bressler & Katz (1965). They reported that injected carnitine stimulated fatty acid synthesis rates *in vivo* from 0.03 to 0.08 μmole of acetyl group/hr./g. of liver

with pyruvate and from 0.005 to 0.02 $\mu\text{mole/hr./g.}$ of liver with acetate as substrate. Using $^3\text{H}_2\text{O}$ *in vivo*, Hardwick & Lowenstein (1966) observed fatty acid synthesis rates of 2–8 μmoles of acetyl group/hr./g. of liver in normal guinea pigs, some 100 times the values obtained by Bressler & Katz (1965). 'Intact' guinea-pig liver mitochondria have a CAT activity of 3–6 $\mu\text{moles/hr./g.}$ of liver (data of Tables 3 and 5) and, even if 80% of the activity in 'intact' mitochondria is due to damage during preparation (and allowing a temperature coefficient of about 2), there would still be enough activity for the increase in fatty acid synthesis observed by Bressler & Katz (1965).

The possibility that acetyl groups may be transported into mitochondria of heart muscle was suggested by the experiments of Fritz & Yue (1964). They showed that 1 mM-DL-carnitine stimulated the oxidation of $[^{14}\text{C}]$ acetyl-CoA to $^{14}\text{CO}_2$ by 'intact' rat heart mitochondria, though acetate oxidation was not increased. In these experiments nicotinamide nucleotides were clearly retained, the cytochrome chain remained intact and the mitochondria were largely impermeable to acetyl-CoA. Nevertheless, experiments from the same Laboratory (Marquis & Fritz, 1965) showed that 15% of the CAT activity in mitochondrial lysed with deoxycholate was observable in 'intact' mitochondria. This activity would be ample to explain the observations of Fritz & Yue (1964), where the rate of oxidation of acetyl-CoA would require only about 1% of the total CAT found by Marquis & Fritz (1965). Another interpretation of the increased oxidation of acetyl-CoA by carnitine is that carnitine may stimulate some other rate-limiting process than CAT.

The present results confirm that CAT is wholly associated with the mitochondrial fraction (Beenakkers & Henderson, 1967; Norum & Bremer, 1967). The distribution of enzyme activity between high-speed supernatant and residue after treatment of mitochondria with hypo-osmotic sucrose with or without Triton X-100 (Table 4) suggests that CAT is normally partially or wholly bound, though it is possible that it is more readily adsorbed by membrane than is GDH under these conditions. The virtual absence of CAT from high-speed supernatant when 3–9% of GDH activity was present in this fraction supports the idea that CAT is normally membrane-bound. This conclusion was also reached by Norum & Bremer (1967), but not by D. Pette (quoted by Beenakkers & Klingenberg, 1964).

If CAT is membrane-bound, it is probably bound to the inner mitochondrial membrane, because it seems generally agreed that, in mitochondria prepared in 0.25 M-sucrose, the outer membrane is permeable to CoA and acetyl-CoA (Yates & Garland, 1966; Beenakkers & Henderson, 1967).

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