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FATTY ACID OXIDATION IN MITOCHONDRIA ISOLATED FROM RAT SUBMANDIBULAR SALIVARY GLANDS

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SUMMARY

1. The ability of rat submandibular salivary gland mitochondria to utilize fatty acid as an energy source has been examined.

2. Mitochondria were isolated by a modification of a procedure developed by CHANCE AND HAGIHARA wherein a brief digestion of the tissue with a bacterial proteinase precedes homogenization. The isolated mitochondria exhibited respiratory control ratios of 5.0–5.5 for glutamate, α-ketoglutarate and pyruvate–malate; 4.5 for succinate; and 2.5 for β-hydroxybutyrate. The highest respiratory rate was obtained with succinate as substrate. β-hydroxybutyrate was oxidized relatively slowly in comparison to other NAD^+-linked substrates.

3. Palmityl-CoA was oxidized by the mitochondrial preparations at rates comparable to those reported for rat liver mitochondria. This oxidation was found to be very dependent on the presence of ADP, bovine serum albumin and L-carnitine in the medium. It was also demonstrated that palmitylcarnitine was formed during this oxidation and its level determined the rate of O_2 utilization. It is concluded that carnitine functions in submandibular gland mitochondria in a similar manner to that reported for other tissues, *i.e.* as a carrier of fatty acids across the mitochondrial membrane.

INTRODUCTION

It has been shown that the secretion process in exocrine glands is dependent upon energy production[1–4], however, very little is known about the relationship of energy production to secretion. GOLDMAN *et al.*[5] reported that in rat submandibular salivary glands glucose oxidation was mainly *via* Embden–Meyerhof and tricarboxylic acid pathways and he claimed that these processes supply the energy necessary for secretion. Other workers[6] showed that acetylcholine and norepinephrine, both powerful secretagogues, stimulated glucose oxidation (to CO_2) in submandibular gland slices. Recently, however, FEINSTEIN AND SCHRAMM[4] demonstrated that rat parotid gland slices had very low rates of glycolysis and, glucose utilization was not stimulated by epinephrine although it caused a rapid secretion of amylase from the slices and an increased O_2 consumption. BABAD *et al.*[2] have suggested that this increased O_2 uptake might be caused by increased oxidation of fatty acids.

Previous work of this laboratory demonstrated the presence of highly active palmityl-CoA–carnitine transferases in both mitochondrial and microsomal fractions of rat submandibular glands[7,8]. It is well established that acylcarnitines are intermediates in the carnitine-stimulated oxidation of fatty acids[9–12].

Although a recent report describes the isolation of phosphorylating parotid gland mitochondria[4], there are no reports in the literature on the isolation of submandibular gland mitochondria nor on the capacity of salivary gland mitochondria to oxidize fatty acids. The present study describes the preparation of coupled mitochondria from submandibular gland and reports on their capacity for fatty acid oxidation.

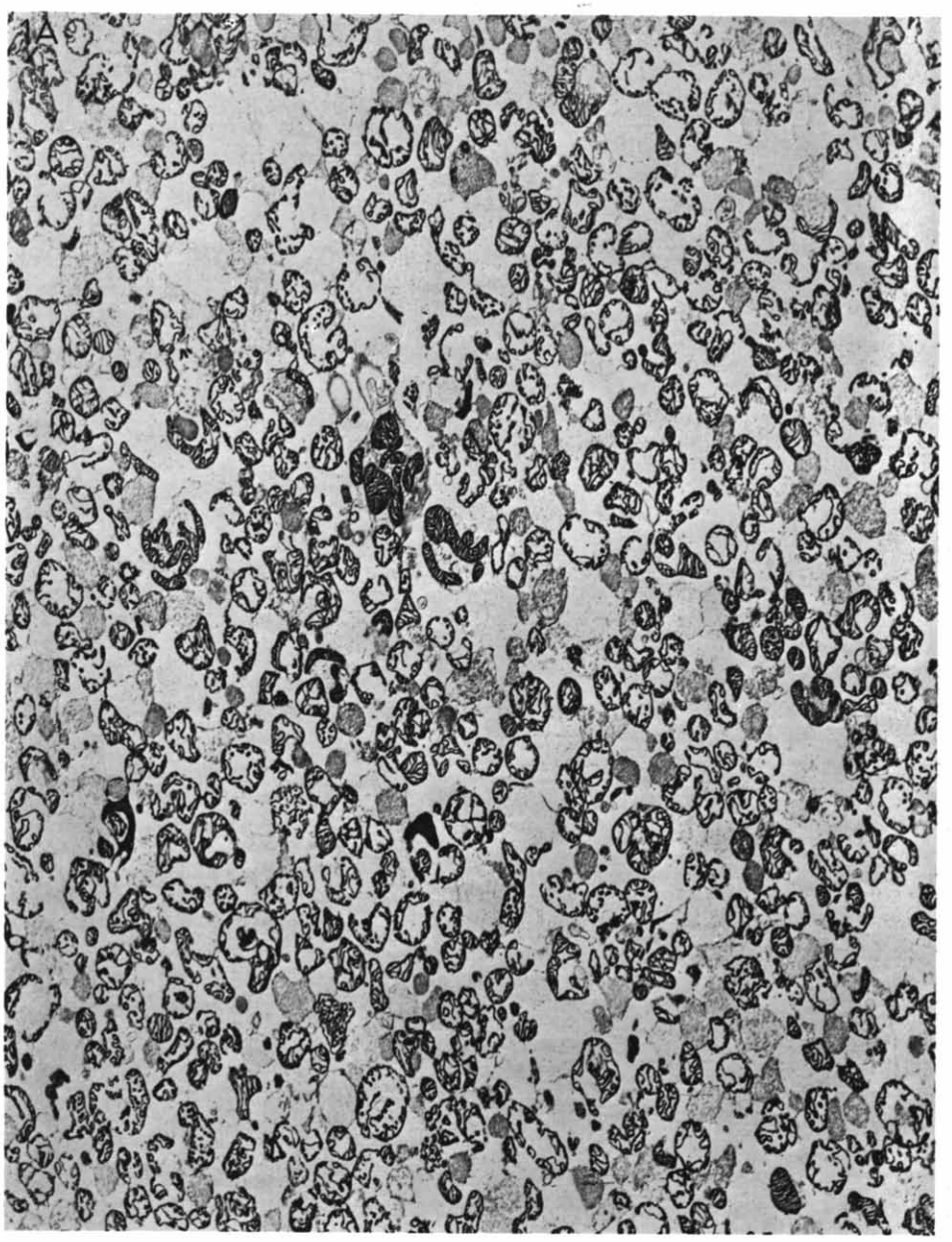
MATERIALS AND METHODS

Reagents

Nagarse was obtained from Nagase and Co., (Osaka, Japan). ADP, pyruvic acid, L-malic acid and succinic acid were from Calbiochem (Los Angeles, Calif.). L-Glutamic acid, α-ketoglutaric acid, DL-β-hydroxybutyric acid (sodium salt), NAD^+ and palmityl-CoA were from Sigma (St. Louis, Mo.). L-Carnitine was from Mann Research Labs. (New York) and bovine serum albumin (fatty acid poor, Fraction V) from Nutritional Biochemicals Corp., (Cleveland, Ohio). [*Me*-^{14}C]Carnitine was purchased from Tracerlab (Boston, Mass.).

Isolation of mitochondria from rat submandibular glands

Mitochondria were isolated by a modification of the procedure developed by CHANCE AND HAGIHARA[13]. Male rats of the Long–Evans strain (7–8 weeks old) were killed by decapitation under light diethyl ether anaesthesia, the glands quickly excised and placed in ice-cold medium consisting of 0.21 M mannitol, 0.07 M sucrose, 0.5 mM EDTA, 0.5 mM EGTA adjusted to pH 7.4 (Stock medium). The glands were cleaned, weighed and cut into small pieces with a razor blade. It was found to be of extreme importance to mince the tissue thoroughly in order to avoid any undue pressure during homogenization. The mince, weighing 1.5–2.0 g, was incubated for 3 min, on ice with 60 ml Stock medium buffered with 10 mM Tris–phosphate buffer (pH 7.6) and containing 0.5 mg/ml Nagarse. The mixture was stirred occasionally. The suspension was then homogenized for 1 min at 70 rev./min in a glass homogenizer fitted with a Teflon pestle (0.6-mm clearance). The resulting homogenate was kept at 0° for another 3 min, then diluted with 60 ml of Stock medium and homogenized at 150 rev./min for 1 min with a tighter-fitting Teflon pestle (0.2-mm clearance). After filtration through fine gauze, the homogenate was centrifuged for 5 min at 2000 × *g* and the supernatant carefully removed so as not to disturb the loosely-packed pellet of cellular debris, nuclei and zymogen granules. The supernatant was centrifuged for 10 min at 12000 × *g* and the resulting mitochondrial pellet rinsed with Stock medium to remove the loosely-packed white layer. The rinsed pellet was resuspended in 40 ml Stock medium buffered with 10 mM Tris–HCl buffer (pH 7.4) and centrifuged for 5 min at 8000 × *g* to produce the final mitochondrial pellet. Mitochondria were suspended in Tris–HCl–Stock medium to a concentration of 20 mg protein per ml. The yield was approx. 3 mg mitochondrial protein per g fresh tissue. All protein determinations were done by the method of LOWRY *et al.*[14]. An electron micrograph of a typical preparation is shown in Fig. 1.



Polarographic measurements

O_2 uptake by isolated mitochondria was monitored with a Teflon membrane coated Clark electrode[15] (Yellow Springs Instrument Co., polarizing voltage 0.8 V) attached to a KM Gilson Oxygraph (Gilson Medical Electronics, Middleton, Wisc.). Measurements were made at 28° in an air-saturated medium of final vol. 1.6 ml (ref. 16). The O_2 concentration in the reaction mixture (224 μM O_2 at 28°, 760 mm Hg) was

Fig. 1. Electron micrograph of isolated submandibular gland mitochondria. The mitochondrial pellet was processed immediately after isolation, hence they are not in any physiologically uniform state as can be seen from the presence of diverse structural forms. (A) 1704.3 ×. (B) 10374 ×.

calculated from the data for the solubility of O_2 in Ringer solution[17] and corrected for the prevailing barometric pressure.

The composition of the basic polarographic medium was 0.23 M mannitol, 0.07 M sucrose, 0.4 mM EGTA, 0.6% bovine serum albumin (when fatty acid oxidation was measured this was 0.15%; see RESULTS), 20 mM Tris–HCl, 5 mM potassium-

phosphate buffer (pH 7.2) and 0.4–1.0 mg mitochondrial protein. The final concentrations of added substrates were 5.0 mM pyruvate–1 mM malate, 4.5 mM succinate, 5 mM glutamate, 5 mM α-ketoglutarate, 9.5 mM β-hydroxybutyrate and, when present, 250 μM ADP. All substrates were added as sodium salts at pH 7.2. The respiratory control ratio (RCR) and ADP/O ratio were calculated from polarographic tracings by the method of CHANCE AND WILLIAMS[18]. Respiratory rates in State 3, State 4, ADP/O ratio and respiratory control rate were all calculated from data obtained from the second period of State 3–State 4 respiration[19].

For the measurement of fatty acid oxidation the final concentrations of added substrates were 0.05 mM palmityl-CoA, 0.3 mM L-carnitine, 1.0 mM ADP and 0.015 mM L-malate.

Measurement of palmitylcarnitine formation

In experiments designed to follow both rate of O_2 disappearance and palmitylcarnitine formation it was necessary to first measure O_2 uptake in the presence of unlabelled carnitine, then, in an identical experiment which followed immediately, labelled carnitine was present but O_2 uptake was not monitored[20]. This procedure was necessitated by the design of the Oxygraph cell which did not allow the withdrawal of sufficient volumes of samples while O_2 consumption was followed. In the latter experiments, 75 μl of suspension was removed from the incubation mixture at various time intervals and immediately placed in 100 μl of $CHCl_3$–CH_3OH (1:2, v/v). Lipids were treated as described by BLIGH AND DYER[21] resulting in a lower phase containing all the lipids including 95% of the palmitylcarnitine and an upper phase containng most of the [^{14}C]carnitine precursor. Lipids were separated by thin-layer chromatography on silica gel G using $CHCl_3$–CH_3OH–H_2O (14:6:1, by vol.) as solvent and the resulting chromatograms were treated with iodine vapours to visualize the lipids. A palmitylcarnitine standard, prepared as described by BØHMER AND BREMER[22], was run with all samples. Radioactive areas were located by radioautography and the radioactivity determined as previously described[7].

Electron microscopy

Mitochondrial pellets were fixed in chilled 2.5% glutaraldehyde solution containing 0.54% glucose and phosphate buffer. After washing, the pellets were fixed with 1% OsO_4, dehydrated with ethanol and embedded in methacrylate. Thin sections were viewed after double staining with uranyl acetate and lead citrate.

RESULTS

Properties of isolated mitochondria

The ability of rat submandibular gland mitochondria to oxidize various substrates is shown in Table I. Succinate was oxidized at the highest rate. O_2 uptake by NAD^+-linked substrates was 2–3 times higher than reported by FEINSTEIN AND SCHRAMM[4] for rat parotid gland and the present preparation exhibited higher respiratory control ratios. β-Hydroxybutyrate, however, was oxidized at only half the rate of other NAD^+-linked substrates.

A typical polarographic trace is shown in Fig. 2. After addition of ADP a slow rate of respiration was observed indicating the presence of endogenous sub-

strates. This rate of O_2 consumption, which did not decline even after 4 min, is referred to as the endogenous repiration (*cf.* Table I). Addition of an exogenous substrate (pyruvate–malate) resulted in a rapid increase in O_2 utilization which declined after the supply of ADP was exhausted. These cycles of State 3– State 4 transitions could be repeated several times during a single experiment.

As indicated in Table II, the use of 0.6% bovine serum albumin in the polarographic medium resulted in higher State 3 respiration and higher respiratory control

TABLE I

OXIDATION OF SUBSTRATES BY SUBMANDIBULAR GLAND MITOCHONDRIA

The endogenous respiration was 18.8 (range 16.5–23.8 in 6 experiments) and refers to the mitochondrial O_2 uptake in the presence of ADP but without any other added substrate. The basic medium (pH 7.2) was composed of 0.23 M mannitol, 0.07 M sucrose, 0.02 M Tris–HCl buffer, 5 mM potassium phosphate and 0.4 mM EGTA. For the experiments reported above this medium was supplemented with 0.6% bovine serum albumin and mitochondria (0.4–1.0 mg protein). Concentrations of substrates are as noted in methods. To commence the respiration 0.25 mM ADP was added. Figures in table are the mean values, figures in parentheses indicate the range.

Substrate	*Oxidation rate (μmoles O_2 per min per g protein)*		*Respiratory control ratio*	*ADP/O ratio*	*Number of experiments*
	State 3	*State 4*			
Pyruvate–malate	71.4 (59.2–79.0)	13.0 (11.2–15.1)	5.5 (5.0–6.7)	2.5 (2.4–2.6)	6
Succinate	139.5 (121.0–165.4)	31.1 (30.6–32.4)	4.5 (3.9–5.1)	1.6 (1.5–1.7)	3
L-Glutamate	58.0 (53.8–62.1)	10.9 (10.7–11.1)	5.3 (4.8–5.8)	2.25 (2.2–2.3)	2
DL-β-Hydroxybutyrate	35.1 (33.9–36.3)	14.6 (12.6–16.5)	2.5 (2.2–2.7)	2.1 (2.0–2.2)	2
α-Ketoglutarate	61.9	12.4	5.0	2.3	1

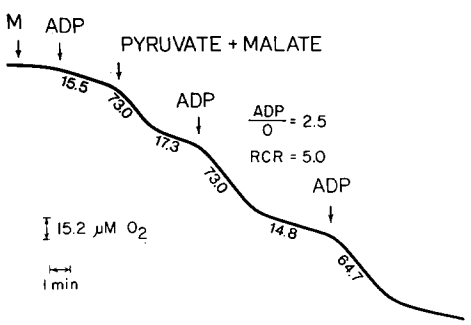


Fig. 2. Polarographic trace showing the oxidation of pyruvate–malate by submandibular gland mitochondria. Incubation vessels contained basic medium (Table I) and 0.6% bovine serum albumin to which 0.440 mg mitochondrial protein was added as shown (M). Other additions were calculated to have final concentrations in the medium of 0.25 mM ADP, 5 mM pyruvate–1 mM L-malate. Numbers below trace indicate the O_2 uptake in μmoles O_2 per min per g protein. RCR, respiratory control ratio. Arrows indicate time of additions.

and ADP/O ratios. Similar results have been reported for brain[23], pancreas[24] and liver[25] mitochondria.

Since rat parotid gland mitochondria have been reported to lose NAD^+ during preparation[4], the effect of added NAD^+ on the oxidation of NAD^+-linked substrates was examined in the present tissue preparation. It was found that NAD^+ (0.4–1.0 mM) had no effect on State 3 respiration, respiratory control and ADP/O ratios when pyruvate–malate, glutamate, α-ketoglutarate or β-hydroxybutyrate were employed as substrates.

Fatty acid oxidation by salivary gland mitochondria

The ability of submandibular gland mitochondria to oxidize long-chain fatty acids was measured with palmityl-CoA as substrate. DE JONG AND HÜLSMANN have shown that Nagarse treatment almost completely destroys rat heart and liver mito-

TABLE II

EFFECT OF BOVINE SERUM ALBUMIN ON OXIDATION OF SUBSTRATES BY SUBMANDIBULAR GLAND MITOCHONDRIA

Conditions were the same as those noted in Table I. With succinate 0.645 mg mitochondrial protein was used, with the others, 0.860. Bovine serum albumin concentration, when added, was 0.6%.

Substrate	*Bovine serum albumin*	*Rate of oxidation (μmoles O_2 per min per g protein)*		*Respiratory control ratio*	*ADP/O ratio*
		State 3	*State 4*		
5 mM pyruvate + 1 mM L-malate	0	58.9	14.9	3.9	2.4
	+	76.0	15.1	5.0	2.6
4.5 mM succinate	0	86.4	39.7	2.2	1.4
	+	132.1	30.2	4.4	1.7
5 mM L-glutamate	0	26.5	22.1	1.2	
	+	61.9	12.4	5.0	2.3

TABLE III

DEPENDENCE OF PALMITYL-CoA OXIDATION ON BOVINE SERUM ALBUMIN

Besides the basic medium (Table I) the vessels contained 1 mM ADP, 0.015 mM L-malate and 0.765 mg mitochondrial protein. The numbers indicate sequence of addition of palmityl-CoA (0.05 mM) and L-carnitine (0.3 mM).

Bovine serum albumin (%, w/v)	*Additions to medium*	*Oxidation rate (μmoles O_2 per min per g protein)*		
		(1) None	*(2) Palmityl-CoA*	*(3) L-Carnitine*
0.00		3.6	8.7	8.9
0.05		3.8	6.5	8.1
0.10		6.0	13.6	22.2
0.15		7.2	9.1	29.0
0.20		7.5	8.3	25.3
0.60		12.2	13.4	19.5

chondrial palmityl-CoA synthetase, and work in this laboratory has demonstrated that this is also true for rat submandibular gland mitochondria. The active form of palmitic acid, palmityl-CoA, had to be employed as substrate. It can be seen from Tables III and IV that under optimum conditions palmityl-CoA oxidation was dependent on the addition of L-carnitine. If carnitine was added to a medium free of palmityl-CoA no increase in O_2 utilization could be observed (unreported studies). Also tracer experiments[7,8] have shown that no palmitylcarnitine is formed from palmityl-CoA by submandibular mitochondria in the absence of L-carnitine, nor from L-carnitine in the absence of palmityl-CoA.

When palmityl-CoA oxidation was measured in a medium devoid of bovine serum albumin, the O_2-uptake was small and the addition of carnitine had no effect (Table III). If 0.6% bovine serum albumin was added to the medium (as used by KOERKER AND FRITZ[25]), a stimulation of palmityl-CoA oxidation by carnitine was observed, but the resulting oxidation rate was still very low (Table III). The importance of proper bovine serum albumin concentration for optimum oxidation is evident from the results described in Table III. A level of 0.15–0.20% appears to be best for carnitine-mediated palmityl-CoA oxidation.

The importance of added ADP to the carnitine-stimulated oxidation is shown by the data of Table IV. Palmityl-CoA was oxidized at a negligible rate (2.2 μmoles/min) in the absence of ADP and the addition of carnitine was without effect. The ad-

TABLE IV

EFFECT OF ADP ON PALMITYL-CoA OXIDATION

Besides the basic medium (Table I) the vessels contained 0.015 mM L-malate, 0.15% bovine serum albumin, 0.765 mg mitochondrial protein and, when added, 1 mM ADP. The numbers indicate the order of addition of palmityl-CoA (0.05 mM), L-carnitine (0.3 mM) and ADP.

Condition	*Additions to medium*	*Oxidation rate (μmoles O_2 per min per g protein)*			
		(1) None	*(2) Palmityl-CoA*	*(3) L-Carnitine*	*(4) ADP*
No ADP		2.8	5.0	5.0	21.0
+ ADP		7.2	9.1	29.0	—

TABLE V

EFFECT OF L-MALATE ON PALMITYL-CoA OXIDATION

Incubation vessels contained the basic medium (Table I) *plus* 1 mM ADP, 0.15% bovine serum albumin, 0.800 mg mitochondrial protein and, when present, 0.015 mM L-malate. Numbers indicate the sequence of addition of palmityl-CoA (0.05 mM), L-carnitine (0.3 mM), malate.

Condition	*Additions to medium*	*Oxidation rate (μmoles O_2 per min per g protein)*				
		(1) None	*(2) Palmityl-CoA*	*(3) L-Carnitine*	*(4) L-Malate*	*(5) Time after malate addition (min)*
No malate		5.6	8.2	18.3	41.4	28.7
+ malate		5.3	9.4	29.4	—	—

dition of ADP to this system greatly increased palmityl-CoA oxidation (18.2 μmoles/min). When ADP was present in the medium from the start, the addition of carnitine caused an 11-fold increase in palmityl-CoA oxidation (1.9–21.8 μmoles/min).

It was found that for optimum oxidation of palmityl-CoA in the presence of carnitine, a low concentration of L-malate was needed (Table V). In the absence of malate, carnitine stimulated palmityl-CoA oxidation but the rate after carnitine addition (12.7 μmoles/min) was about half of that given in the presence of malate (24.1 μmoles/min). The addition of malate to the malate-free medium resulted in a very rapid increase in O_2 uptake but this declined within 4 min to levels comparable to results wherein malate was present from the start of incubation (Table V). This latter observation suggested that a metabolite accumulates during palmityl-CoA

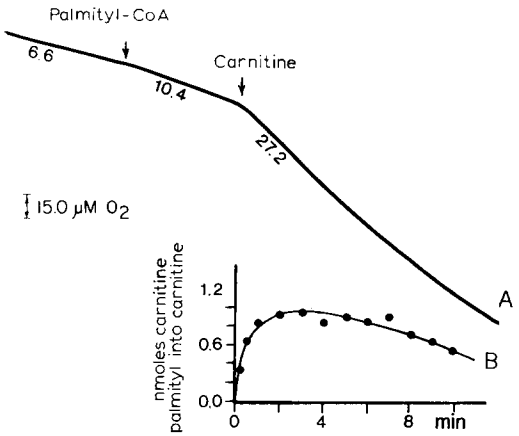


Fig. 3. Palmityl-CoA oxidation and palmitylcarnitine formation by submandibular gland mitochondria in the presence of L-malate. A. Polarographic trace showing rates of O_2 consumption. B. Palmitylcarnitine levels after adding [*Me*-^{14}C]carnitine to the medium. No incorporation of labelled carnitine occurred in the absence of palmityl-CoA. Composition of medium and additions were as described in legend to Table V (malate present). Mitochondrial protein content was 0.880 mg.

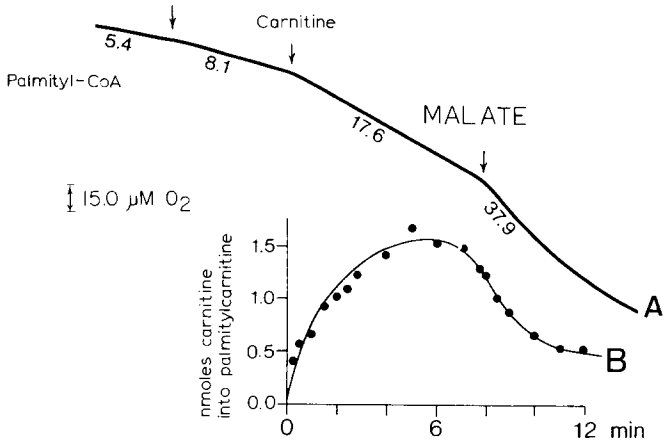


Fig. 4. Effect of L-malate on palmityl-CoA oxidation and palmitylcarnitine formation. All conditions as noted in Fig. 3 except 0.015 mM L-malate was added as shown instead of being present from the start of incubation.

oxidation in the absence of L-malate, then, after malate supplementation, this material becomes readily available for oxidation.

The experimental results depicted by Figs. 3 and 4 illustrate the correlation between palmitylcarnitine formation and the rate of O_2 uptake. When L-malate was present in the medium prior to the addition of palmityl-CoA and carnitine, the level of palmitylcarnitine increased rapidly after the addition of carnitine and then gradually decreased (Fig. 3). In the absence of L-malate, the amount of palmitylcarnitine formed was greater and, when L-malate was added, the level decreased rapidly (Fig. 4). These results suggest that the metabolite responsible for the increased O_2-uptake after L-malate addition (*cf.* Table V) is palmitylcarnitine and that the rate of palmityl-CoA oxidation in the presence of carnitine is dependent upon the effective concentration of palmitylcarnitine, as has been suggested for other tissues[20].

DISCUSSION

The study of salivary gland mitochondrial metabolism has received little attention to date although the importance of energy-yielding processes to secretory function of these glands is well-established[1–3]. Probably this has been due, in part, to the difficulty encountered in the preparation of salivary gland mitochondria. The presence of proteases and nucleases, which are released by homogenization[27], the toughness of the tissue and the innate high content of Ca^{2+}, which reportedly disrupts salivary mitochondria[4], makes the isolation of intact, functioning mitochondria rather difficult.

During the present investigation it was observed that in order to prepare phosphorylating mitochondria any pressure during homogenization had to be avoided. FEINSTEIN AND SCHRAMM[4] used a very loose-fitting pestle (0.8-mm clearance) for the preparation of rat parotid gland mitochondria. The direct application of their procedure to submandibular gland tissue was unsuccessful.

The modification of the method of CHANCE AND HAGIHARA[13], wherein a brief digestion of the tissue with a bacterial protease precedes homogenization, yielded the best results. These mitochondria showed respiratory rates for glutamate, α-ketoglutarate and β-hydroxybutyrate comparable to those reported for liver mitochondria, but for pyruvate–malate and succinate, the rates were 2–3 times greater than those of liver mitochondria[28,29].

BABAD *et al.*[2] found that β-hydroxybutyrate was the only substrate that, when added to a medium containing rat parotid gland slices, supported the secretion of amylase. These workers suggested that his material might be the endogenous substrate intimately associated with secretory processes. In more recent studies, however, with isolated parotid gland mitochondria[4] it was demonstrated that β-hydroxybutyrate was oxidized more slowly than other NAD^+-linked substrates. This suggested that the previous finding (*i.e.*, specific stimulation of secretion) might have been due to preferential penetration of this material into cells. In the present studies rat submandibular gland mitochondria were found to oxidize β-hydroxybutyrate at about half the rate obtained with other NAD^+-linked substrates, supporting the view that this material is not preferentially favoured by salivary gland mitochondria.

Palmityl-CoA was oxidized by salivary mitochondria at rates comparable to those reported for rat liver mitochondria[20,30], and this oxidation was found to be

dependent on the presence of ADP, bovine serum albumin and L-carnitine in the medium. It was further demonstrated that palmitylcarnitine was formed during the oxidation and its level determined the O_2 utilization rate. From these observations it can be concluded that carnitine functions as a carrier of fatty acid (*i.e.* as acylcarnitine) across mitochondrial membrane, a process that has been well-established for other tissues[9–12].

Palmityl-CoA oxidation in the absence of bovine serum albumin was found to be very low and it was not stimulated by carnitine. The albumin effect may be a result of its protection of mitochondrial integrity from surface-active compounds such as palmityl-CoA and palmitylcarnitine[9, 12]. The inhibition of oxidation at high bovine serum albumin concentrations could be caused by its binding palmityl-CoA, thereby decreasing the availability of oxidizable substrate[31]. GOODMANN[32] presented evidence that the binding of long-chain fatty acids to albumin is a function of the molar ratio of fatty acid to albumin. It is possible that this could also apply to fatty acid derivatives such as CoA esters.

In the present preparations of submandibular gland mitochondria a small amount of L-malate greatly stimulated palmitylcarnitine oxidation. One possible explanation of this effect is the increased availability of oxaloacetate for citrate formation after the oxidation of added malate by malate dehydrogenase. WILLIAMSON *et al.*[33] suggested that the conversion of acetyl-CoA, produced by oxidation of fatty acids, to either citrate or acetoacetate is determined by the availability of mitochondrial oxaloacetate. He provided experimental evidence[34] that addition of malate to liver mitochondria oxidizing palmitylcarnitine resulted in a switch of metabolism from acetoacetate to citrate formation. Thus the presence of malate favours complete palmitylcarnitine oxidation to CO_2 through the tricarboxylic acid cycle.

This paper has described the preparation of coupled mitochondria from rat submandibular salivary glands that were capable of rapid oxidation of palmitate by a carnitine-dependent pathway. In studies presently in progress it is hoped to assess the relationship of this pathway to secretion.

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