THE UNIVERSITY OF MANITOBA

ACYLCARNITINE FORMATION AND FATTY ACID OXIDATION IN SUBCELLULAR PARTICULATES ISOLATED FROM RAT SUBMANDIBULAR SALIVARY GLANDS

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LIST OF ABBREVIATIONS

ADP ..... adenosine diphosphate

AMP ..... adenosine monophosphate

ANSA ..... aminonaphthol sulfonic acid

ATP ..... adenosine triphosphate

BSA ..... bovine serum albumin

CoA ..... coenzyme A

CL ..... carnitine lipid

DNA ..... deoxyribonucleic acid

EDTA ..... ethylene-diamine-tetraacetic acid

EGTA ..... ethylene glycol-bis (ß-amino ethyl ether) N, N$^{'}$-tetraacetic acid

FFA ..... free fatty acid

INT ..... 2-(p-Iodophenyl)-3(p-nitrophenyl)-5-phenyltetrazolium chloride

LEC ..... lecithin

MES ..... 2 N-morpholino-ethane-sulfonic acid

NAD ..... nicotinamide adenine dinucleotide

NADH ..... reduced nicotinamide adenine dinucleotide

NADPH .... reduced nicotinamide adenine dinucleotide phosphate

PBD ..... 2-(4-biphenyl)-5-phenyl-1,3,4 oxadiazole

PC ..... palmitoyl-carnitine

PE ..... phosphatidylethanolamine

POPOP .... 1,4 bis (2-(5-phenyloxazolyl)) benzene

PPO ..... 2,5 diphenyloxazole

RCR ..... respiratory control ratio

RNA ..... ribonucleic acid

SMSG ..... submandibular salivary gland

TCA ..... trichloroacetic acid

TES ..... N-tris (hydroxymethyl) methyl-2-amino-ethane sulfonic acid

TG ..... triglyceride

TLC ..... thin layer chromatography

TRICINE .. N-tris (hydroxy methyl) methylglycine

TRIS ..... tris hydroxymethylaminomethane

ABSTRACT

Palmitic-1-^{14}C acid in the presence of ATP, CoA and Mg^{++} was readily incorporated into the phospholipids and triglycerides of all subcellular fractions prepared from whole homogenates of rat submandibular salivary glands by differential centrifugation. The presence of carnitine in the incubation mixture decreased palmitic-1-^{14}C acid incorporation into lecithin, phosphatidylethanolamine and triglyceride. All subcellular fractions very effectively incorporated palmitic-1-^{14}C acid into palmitoyl-carnitine and, with the exception of the 75000 x g - 30 min. pellet and the final high speed supernatant, into another carnitine containing lipid (referred to as carnitine lipid). The highest palmitic-1-^{14}C acid incorporation into palmitoyl-carnitine (per mg protein) was associated with microsomal fractions, while mitochondria were the major site of the carnitine lipid formation. Both, palmitoyl-carnitine and carnitine lipid were also formed on incubation of the mitochondrial fraction with carnitine-^{14}C in the presence of palmitoyl-CoA.

Experiments in which the effect of the respiratory inhibitors, cyanide and rotenone, on carnitine lipid formation from carnitine-^{14}C and palmitoyl-CoA was studied, suggested that carnitine lipid might be β-hydroxy-palmitoyl-carnitine. The identity of carnitine lipid with a known standard sample of β-hydroxy-palmitoyl-carnitine was demonstrated by their behaviour on thin layer and gas chromatography. It is suggested that β-hydroxy-palmitoyl-carnitine was formed enzymatically from carnitine and β-hydroxy-palmitoyl-CoA derived by β-oxidation of palmitoyl-

CoA. The accumulation of β-hydroxy-palmitoyl-CoA could be caused by a lack of NAD which would limit its further oxidation.

Phosphorylating submandibular salivary gland mitochondria were prepared by a modification of a procedure developed by Chance and Hagihara. These isolated mitochondria were characterized morphologically by electron microscopy and biochemically by their respiratory control and ADP/O ratios associated with oxidation of various substrates. The mitochondria exhibited respiratory control ratios of 5.0 to 5.5 for glutamate, α-keto-glutarate and pyruvate-malate; 4.5 for succinate and 2.5 for β-hydroxy-butyrate. ADP/O ratios of 2.1 to 2.5 were associated with oxidation of NAD linked substrates, value of 1.6 was obtained for succinate. Palmitoyl-CoA oxidation by mitochondria 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 palmitoyl-carnitine was formed during this oxidation and its level determined the rate of O_2 utilization. Thus 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. It is concluded that, under resting conditions, submandibular salivary gland mitochondria can readily oxidize fatty acids through a carnitine dependent pathway at rates comparable to those of liver mitochondria.

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INTRODUCTION

Salivary glands have been extensively used to study secretory processes of exocrine glands, due, in part, to the accessibility of these glands to operative manipulations and also to the relative ease of collecting secretions (1). Although the mechanisms associated with salivary secretion may not be exactly identical to those of other glandular tissues, it can be assumed that experimental findings on salivary glands will aid in studying general secretory mechanisms.

It has been shown that the secretion processes of exocrine glands are critically dependent upon energy production (2, 3, 4, 5). However, very little information is available on the relative contribution of the major metabolic pathways to the energy production in resting or stimulated exocrine glands. Goldman et al. (6) reported that in rat submandibular salivary glands glucose oxidation was mainly via the Embden-Meyerhof and tricarboxylic acid pathways. Other workers (7) showed that acetylcholine and norepinephrine, both powerful secretagogues, stimulated oxidation of glucose (to CO_2) in submandibular gland slices. Recently, however, Feinstein and Schramm (5) demonstrated that resting rat parotid gland slices utilized glucose very slowly and glucose uptake was not stimulated by epinephrine although it caused a rapid secretion of amylase and an increased O_2 consumption.

While studying the lipid metabolism of rat submandibular salivary gland, Pritchard (8) demonstrated the presence of a highly active carnitine palmitoyl transferase in submandibular gland homogenates. It is well established now that in all mammalian tissues, thus far inves-

tigated, this enzyme functions to transport fatty acids (as acyl-carnitines) across the mitochondrial membrane to the sites of fatty acid oxidation (9, 10, 11). Norum (12) found a positive correlation between the amount of carnitine palmitoyl transferase present in an organ and the rate of fatty acid oxidation in that organ. Therefore, the finding of very active carnitine palmitoyl transferase activity in submandibular glands suggested that these glands might readily oxidize fatty acids and focused attention on the possibility that fatty acids play an important role in supplying energy for both resting and stimulated salivary glands.

The purpose of the present work was to study the lipid metabolism in resting rat submandibular salivary glands, particularly acylcarnitine formation in subcellular particulates and the relation of acylcarnitine formation to fatty acid oxidation in mitochondria.

LITERATURE REVIEW

The salivary glands secrete water, electrolytes, and proteins in response to stimulation of either parasympathetic or sympathetic nerves (13). Numerous workers have demonstrated that the transmitter of the parasympathetic nerve fibres, which causes salivary secretion is acetylcholine (14). Since the investigations by Cattell, Wolff and Clark (15) the sympathetic fibres of salivary glands have been supposed to be adrenergic. Agents which imitate the action of parasympathetic or sympathetic nerve fibres have been commonly used to activate the secretory mechanism of salivary glands.

It is known that the true secretion of both proteins and electrolytes is an active, energy requiring process, however the present knowledge of the energy-yielding metabolic processes associated with salivary gland secretion is very limited. Salivary glands are very active metabolically. Unstimulated salivary gland, under both *in vivo* (dog submandibular (16)) and *in vitro* (submandibular and parotid gland of dog, cat and rabbit investigated (17)) conditions, has a high rate of 0_2 consumption, e.g., higher than that of heart, pancreas or liver (18). Seventy years ago Barcroft (19) demonstrated that increased secretion of saliva in response to parasympathetic stimulation is accompanied by greatly increased 0_2 utilization and carbon dioxide production by dog submandibular gland. The observations of Barcroft were later confirmed by Deutsch and Raper (17, 20). By using slices of cat or rabbit submandibular or parotid gland, they found increased 0_2 uptake in the presence of acetylcholine or pilocarpine. The increase in respiration caused by these drugs

could be inhibited by atropine (atropine is known to block salivary secretion caused by parasympathetic impulses or parasympathomimetic drugs (21)). Similarly, Barcroft and Müller (22) observed stimulation of salivary secretion, and an increase in the O_2 consumption of the glands when the sympathetic nerve supply to the submandibular gland in the intact cat was stimulated or when adrenaline was injected. Experiments in vitro confirmed this observation. Addition of adrenaline to the cat submandibular gland slices (17) or rabbit parotid slices (23) increased O_2 uptake.

The increased O_2 uptake upon parasympathomimetic or sympathomimetic stimulation indicates the involvement of aerobic metabolic processes, however, several workers have reported that anaerobic glycolysis may also be involved in supplying the energy for secretion. Northrup (24) reported that stimulation of sympathetic or parasympathetic nerves to the submandibular gland of the dog caused a decrease in gland glycogen and creatine phosphate and an increase in lactic acid. Wills (25) found increased concentration of lactic acid in cat submandibular saliva following pilocarpine injection. On the other hand Deutsch and Raper (20) were not able to find any evidence that anaerobic glycolysis can supply energy for the secretion processes, because acetylcholine had no effect on lactic acid production by submandibular gland slices. More recently, in vivo experiments of Strömblad (26) showed that parasympathetic stimulation did not increase lactic acid production by cat submandibular gland at low secretory rates, however, at higher secretory rates lactic acid output from the gland was increased. He

concluded that the lactic acid mechanism is not preferentially used by the gland for energy supply during secretion, but seems to be used only when the demand for energy is high and when, most likely, the oxidative metabolism is unable to supply enough energy. In 1957 Hokin and Sherwin (23) demonstrated that acetylcholine and adrenaline stimulated mucin secretion in submandibular slices and amylase secretion in parotid slices. By examining the rate of amylase release into the medium from the slices of rat parotid gland, Bdolah and Schramm (27, 28) were able to show that enhanced secretion caused by addition of adrenaline was dependent on the presence of O_2 and was completely suppressed by cyanide or dinitrophenol but not iodoacetate, which has been shown to completely block glycolysis (5). Babad et al (3) found that in rat parotid slices oligomycin inhibited both the increased O_2 uptake caused by epinephrine as well as the amylase secretion. Results reported by these workers indicate that energy for amylase secretion by parotid gland is supplied mainly by oxidative phosphorylation and not by glycolysis.

Only limited information is available on the substrates utilized by salivary glands. Deutsch and Raper (20) measured the respiratory quotient in both resting and active (acetylcholine added) cat submandibular gland slices in the absence of exogenous substrate, and found it varied between 0.59-0.80. When exogenous glucose was added to the active slices, it was rapidly oxidized and the respiratory quotient rose to 1.02. These results suggest that the endogenous substrate

serving the oxidation occurring both in the resting and active states, is not solely carbohydrate, but when a carbohydrate (glucose) is present, it is preferentially used. Goldman et al (6) studied the metabolism of glucose in rat submandibular gland slices. From tracer studies with glucose labelled in various positions, they concluded that glucose was metabolized mainly through the Embden-Meyerhof-Krebs cycle pathway. Sandhu et al (7) found increased production of $^{14}CO_2$ from glucose $-^{14}C$ after addition of adrenaline or acetylcholine to rat submandibular gland slices. The effect was dependent on the presence of exogenous Ca^{++} in the system. On the other hand, Feinstein and Schramm (5) recently demonstrated that resting rat parotid gland slices utilized glucose very slowly and epinephrine did not stimulate the uptake of glucose although it caused a rapid secretion of amylase and an increased 0_2 consumption (3). This suggests that at least in rat parotid gland, glucose does not serve as an efficient energy source. Babad and coworkers (3) have suggested that the increased 0_2 uptake caused by epinephrine might reflect an increase in the oxidation of fatty acids, however they presented no supporting experimental evidence.

Pritchard (8) demonstrated the presence of highly active carnitine palmitoyl transferase in rat submandibular gland homogenates. It is well established that in all mammalian tissues thus far investigated this enzyme functions to transport fatty acids across the mitochondrial membrane to the sites of fatty acid oxidation. Therefore the finding of an active carnitine palmitoyl transferase in submandibular glands

suggested that these glands might readily oxidize fatty acids and focused attention on the possibility that fatty acids play an important role in supplying energy in resting and stimulated salivary glands.

Although discovered in 1905 (29, 30), carnitine's physiological role remained obscure for over fifty years. Despite its close structural resemblance to choline, carnitine (3-hydroxy-4-trimethylammonium butyrobetain, Fig. 1) as well as its acetyl derivative, has negligible activity on neuromuscular preparations (31). The attempts to establish carnitine as a methyl donor in biochemical reactions involving transmethylation have been unsuccessful (32,33). In 1952 (34) carnitine was

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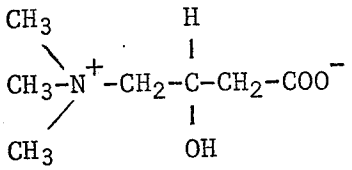
CARNITINE



ACYL CARNITINE

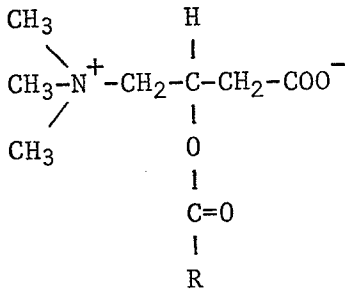


Fig. 1. Structural formulae of carnitine and acyl carnitine.

identified as an insect growth factor and on the basis of this biological activity a sensitive method was elaborated for estimating the levels of carnitine in different tissues. Carnitine was found to be present almost universally in biological materials. The distribution of carnitine in rat tissues as measured by Broekhuysen is shown in Table 1 (35).

However, no physiological role for this compound was apparent until 1955, when two significant observations were reported. Friedman and Fraenkel (36) discovered an enzyme in pigeon liver which catalyzed the reversible reaction:

acetyl-CoA + carnitine $\rightleftharpoons$ CoA + acetyl-carnitine; and Fritz (37) reported that the action of muscle extracts on fatty acid oxidation by liver homogenates could be simulated by the addition of carnitine. Carnitine augmented the conversion of palmitate-1-^{14}C to ketones by both liver homogenates and slices (37). Subsequently it was found that many tissues responded similarly to the addition of carnitine. Carnitine greatly increased the rate of long chain fatty acid oxidation by heart muscle, skeletal muscle, intestine, kidney and brain (38). The effects of carnitine on fatty acid oxidation could be readily obtained with particulate preparations, but in a solubilized fatty acid oxidase system, carnitine's action could not be elicited (39), suggesting no direct effect on these enzymes. Observations of Fritz (40) suggested that carnitine did not act upon the fatty acyl synthetase enzyme system, which converts free fatty acid to the corresponding

TABLE 1

CONCENTRATIONS OF CARNITINE IN VARIOUS RAT TISSUES(†)

Tissue	Free Carnitine Concentration (mg/g dry weight)
Muscle (striated)	0.53
Testes	0.67
Heart	1.00
Adrenals	6.40
Kidney	0.15
Spleen	< 0.16
Intestine (small)	< 0.16
Brain	0.18
Liver	0.21
Lung	0.13

(†) According to Broekhuysen (35).

thiol ester according to the following net reaction (39):

$$\text{free fatty acid} + \text{ATP} + \text{CoA} \overset{Mg^{++}}{\rightleftharpoons} \text{fatty-acyl-CoA} + \text{AMP} + \text{P-P}.$$

Studying the activity of this enzyme system in the presence of hydroxylamine as a trapping agent, Fritz (40) reported that carnitine had no effect on the production of acyl-hydroxamate. However, the CoA derivative seemed to be a required intermediate in carnitine-stimulated fatty acid oxidation. Addition of carnitine did not appreciably enhance palmitate oxidation by heart muscle mitochondria, unless ATP and CoA were provided (11).

Although acyl-CoA thiol esters are the immediate substrates for the isolated enzymes of β-oxidation, such compounds were not readily oxidized when added to intact mitochondria (11). This observation implied that a permeability barrier exists between extramitochondrial acyl-CoA and intramitochondrial sites for β-oxidation. Addition of carnitine to intact mitochondrial preparations resulted in a ready oxidation of palmitoyl-CoA (11), indicating that carnitine could in some way aid in the transport of palmitoyl-CoA across the membrane to the sites of β-oxidation. Fritz (11) and Bremer (10) reported that both heart and liver preparations had the ability to incorporate carnitine-^{3}H and palmitate-^{14}C into a compound having the characteristics of palmitoyl-carnitine. This compound had the same R_F value (as obtained by paper chromatography (11)) in several solvent systems as synthetic palmitoyl-carnitine. Carnitine-^{3}H was incorporated into

this compound when only palmitoyl-CoA and tissue preparations were added. When free palmitic acid was the substrate, ATP, CoA and tissue preparation were required to form palmitoyl-carnitine (41). Fatty acid esters of carnitine were rapidly oxidized by mitochondria isolated from rat heart muscle (11) or liver (9). From these observations and those concerning carnitine acetyltransferase action (36) it was suggested that carnitine enhances fatty acid oxidation by acylcarnitine formation (10, 11), and the presence of an enzyme (carnitine palmitoyl transferase) catalyzing the reaction:

palmitoyl-CoA + carnitine $\rightleftharpoons$ palmitoyl-carnitine + CoA, was postulated. The reversibility of the reaction was demonstrated by the rapid incorporation of carnitine-^{14}C into palmitoyl-carnitine in the presence of CoA and palmitoyl-carnitine (10). Thus the enzyme functions to transport fatty acids across the mitochondrial membrane in the form of acylcarnitine esters. However, once the acylcarnitine is at the inner side of the mitochondrial membrane it must be reconverted to its CoA ester which serves as the immediate substrate for β-oxidation. Therefore, it was necessary to hypothesize the presence of palmitoyl-carnitine transferase on both sides of the mitochondrial barrier impermeable to acyl-CoA ester, but not to acyl carnitine ester. A scheme suggested by Fritz and Yue (11) is presented in Fig. 2.

Carnitine palmitoyl transferase was purified by Norum (42) from calf liver mitochondria. By determining the equilibrium constant (toward palmitoyl-carnitine) of the reaction palmitoyl-CoA + carnitine

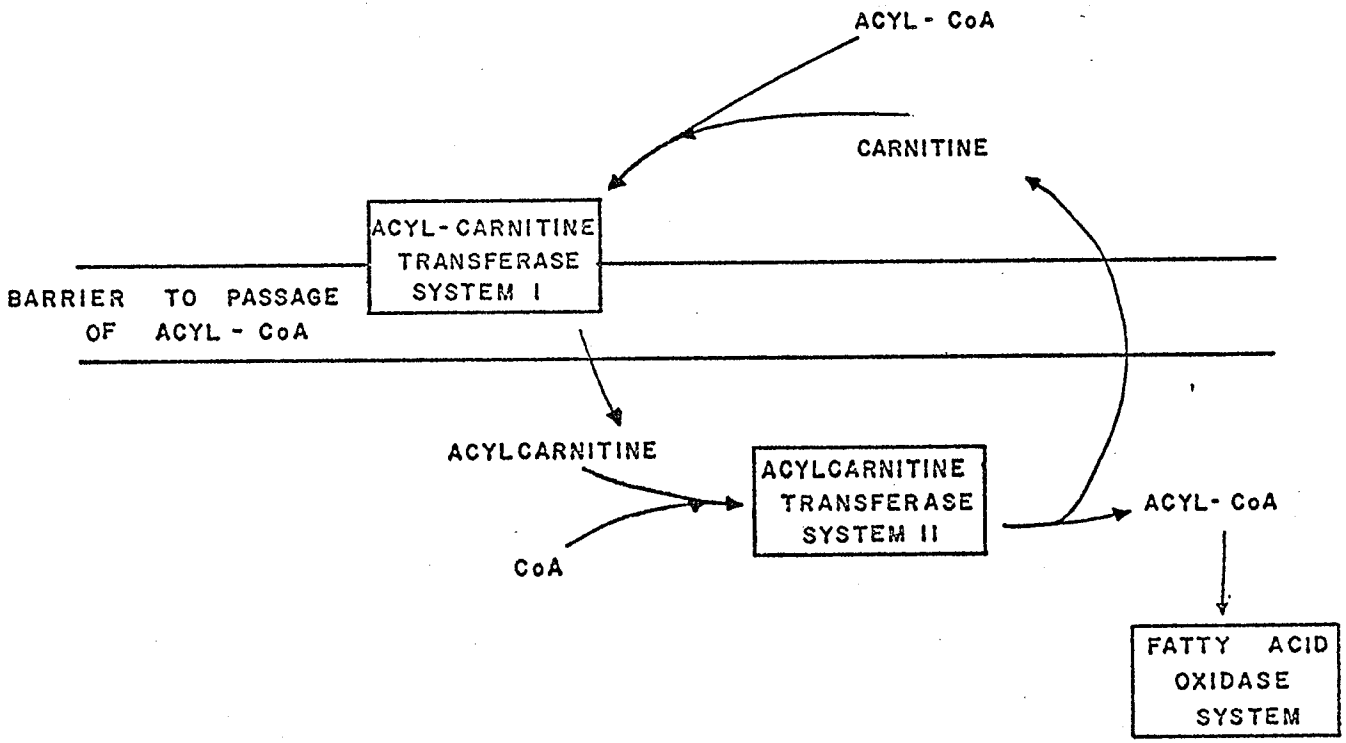


Fig. 2. Scheme of a carnitine mediated fatty acid transport across the mitochondrial membrane (Fritz and Yue (11)).

$\rightleftharpoons$ palmitoyl-carnitine + CoA, and calculating the change in standard free energy, he found that palmitoyl-carnitine has about 0.5 kcal higher free energy than palmitoyl-CoA. This implied that palmitoyl-carnitine possesses high group potential and explained the reversibility of the palmitoyl transfer. Norum (42) also studied acyl group specificity of the enzyme. He found that it exhibits broad specificity toward acyl chain lengths. The activity of the enzyme increased with increasing chain length, reaching a maximum at palmitate. The acyl group specificity was completely different from that of acetyl transferase, thus proving that at least two carnitine acyl transferases exist.

Hollis and Blecher (43) demonstrated that carnitine added to liver or heart mitochondria stimulated the oxidation not only of palmitate, but also of the products of β-oxidation of palmitate, namely trans α,β-unsaturated palmitic acid and β-OH-palmitic acid. The finding was later confirmed by Mahadevan and coworkers (44) who also reported that oxidation of β-keto-palmitic acid was greatly increased by carnitine. When studying the acyl transferase activity in sonically disrupted mitochondria with palmitoyl-carnitine and carnitine esters of β-oxidation intermediates as substrates, they found that β-keto-palmitate was as efficient a substrate for the transferase as palmitoyl-carnitine. The rates of the reaction with carnitine esters of β-OH-palmitic and trans α,β-unsaturated palmitic acid were about 70% and 20% (respectively)that obtained with palmitoyl-carnitine. However, whether there is only one long chain carnitine acyl transferase with

broad substrate specificity, or whether there exist specific transferases for β-oxidation derivatives of long chain fatty acids remains to be established.

Norum (12) has reported that carnitine palmitoyl transferase is present in all human organs and tissues tested, including liver, kidney, heart, muscle, brain, placenta, lung, adipose tissue, leucocytes and platelets. He found a positive correlation between the amount of carnitine palmitoyl transferase present in an organ and the rate of fatty acid oxidation in that organ. The enzyme was absent from red blood cells and serum.

There does not seem to be complete agreement among investigators concerning the subcellular localization of carnitine palmitoyl transferases. Bremer (10) reported that both rat liver microsomes and mitochondria can synthesize palmitoyl-carnitine from carnitine and palmitate in the presence of ATP, CoA and Mg^{++}. In a later publication, however, Norum and Bremer (45) reinvestigated the subcellular localization of carnitine palmitoyl transferase by measuring the CoA dependent incorporation of labelled carnitine into palmitoyl-carnitine, and reported that the enzyme is solely mitochondrial. Recently van Tol and Hűlsmann (46) studied the subcellular distribution of carnitine palmitoyl transferase in rat liver by measuring both the CoA dependent incorporation of labelled carnitine into palmitoyl-carnitine and the formation of palmitoyl-carnitine from palmitate, ATP, CoA and carnitine in the presence of an excess of fatty acyl-CoA synthetase. They reported that the sub-

cellular distribution of carnitine palmitoyl transferase activity is strongly dependent on the method of estimation used. When the exchange reaction was used for activity estimation, the results suggested that the enzyme is solely mitochondrial. However, when the synthetic reaction was used, the distribution pattern suggested a dual localization of the enzyme with activity both in mitochondria and microsomes. The reason for this discrepancy is not known, and thus the presence of carnitine palmitoyl transferase in microsomes remains controversial. On the other hand, the presence of the enzyme in mitochondria is well-established and findings from different laboratories agree on the intramitochondrial localization of this enzyme, i.e., the inner mitochondrial membrane (46, 47, 48).

Carnitine palmitoyl transferase was shown to be very powerfully inhibited by α-bromoacyl analogues of normal acyl substrates provided that the acceptor substrate (free carnitine or CoA) was also present (49). With intact mitochondria α-bromoacyl-CoA (in the presence of carnitine) inhibits the oxidation of acyl-CoA but not acylcarnitine. On the other hand, bromoacylcarnitine inhibits the utilization of both fatty acyl derivatives (50). The results indicate that mitochondria contain two pools of carnitine palmitoyl transferase, the inner one accessible only to acylcarnitine derivatives, and the outer one, accessible to acyl-CoA derivatives as well. Yates and Garland (51) have presented experimental evidence in favour of this suggestion. They reported that mitochondria contain non-latent, and latent carnitine

palmitoyl transferase activity. The non-latent is inhibited by bromostearoyl-CoA, whereas the latent transferase is unaffected by it. The latent enzyme, which constitutes a major part of total mitochondrial carnitine palmitoyl transferase activity, is membrane bound, most probably located in the inner mitochondrial membrane. The position of the non-latent enzyme is not clear. It could be associated with the outer membrane, the outer aspect of the inner membrane or the intermembrane space. Recently, West et al (52) reported the separation of two forms of carnitine palmitoyl transferase from ox liver mitochondria. Thus the hypothetical feature of the scheme presented by Fritz and Yue (Fig. 2), in which the presence of two carnitine palmitoyl transferases is postulated, one on each side of the barrier impermeable to acyl-CoA esters, has now been supported by experimental results.

MATERIALS

Bacterial proteinase Nagarse was obtained from Nagase and Co. Ltd., Osaka, Japan.

Cytochrome c (equine heart), p-nitrophenylphosphate (disodium salt), succinate (disodium salt), pyruvic acid, L-malic acid, AMP (sodium salt) and ADP (sodium salt) were from Calbiochem, Los Angeles, California.

Glucose-6-phosphate (dipotassium salt), ATP (disodium salt), coenzyme A (trilithium salt) , palmitoyl-CoA (free acid), L-glutamic acid, α-ketoglutaric acid, DL-β-hydroxybutyrate (sodium salt) and NAD were from Sigma, St. Louis, Mo.

2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), phenolphthalein glucuronide (cinchonidine derivative), indole and L-carnitine were from Mann Research Laboratories, New York, New York.

Palmitic acid (1-^{14}C) and palmitic acid (16-^{14}C) were purchased from New England Nuclear Corporation, Boston, Massachusetts.

DL-carnitine-(^{14}C-methyl) and DL-carnitine (^{14}C-carboxyl) were from Tracerlab, Boston, Massachusetts.

Bovine serum albumin (fatty acid poor, Fraction V) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Rotenone was from K & K Laboratories Incorporated, Plainview, New York, and sodium cyanide from Baker Chemical Company, Phillipsburg, New Jersey. Standard lipid mixture (sphingomyelin, lecithin, phosphatidylethanolamine and cholesterol) was from Applied Science Laboratories Incorporated, State College, Pennsylvania.

Standard sample of palmitoylcarnitine was prepared according to Bremer (9).

β -hydroxy-palmitoylcarnitine was a gift from Dr. F. Sauer, Animal Research Institute, Canada Department of Agriculture, Ottawa, Ontario.

METHODS

A. Preparation of Subcellular Particles from Rat Submandibular Salivary Gland (SMSG) Homogenate

1. Preparation of subcellular fractions

Male rats of the Long-Evans strain (4-5 weeks old) were killed by decapitation under light ether anaesthesia, the glands quickly excised and placed in an ice-cold medium containing 0.32 M sucrose-1mM TES (N-Tris (hydroxymethyl) methyl-2-amino ethane sulphonic acid) buffer 7.4. The glands were cleaned, weighed and cut into small pieces with a razor blade. The mince was transferred into a Potter-Elvehjem homogenizer containing 0.32 M sucrose-1mM TES medium and disintegrated using ten hand strokes with a Teflon pestle. The homogenate was filtered through fine mesh bolting cloth to remove connective tissue and large undisintegrated particles and the volume adjusted to 10% w/v based on the original tissue wet weight. The resulting suspension is referred to as the whole homogenate (WH). Separation of six subcellular fractions from the WH was effected by differential centrifugation, an outline of which is given in the Fig. 3. R_1 fraction was obtained by use of an International clinical centrifuge, R_2 to R_5 fractions by an International centrifuge Model B-20, (Rotor Type A-321) and R_6 fraction by an International centrifuge Model B-60, (Rotor Type SB-283).

After the initial centrifugation, each fraction was resuspended in 1 ml of sucrose-TES medium, resedimented and the supernatants were added to the appropriate original supernatant. This means the

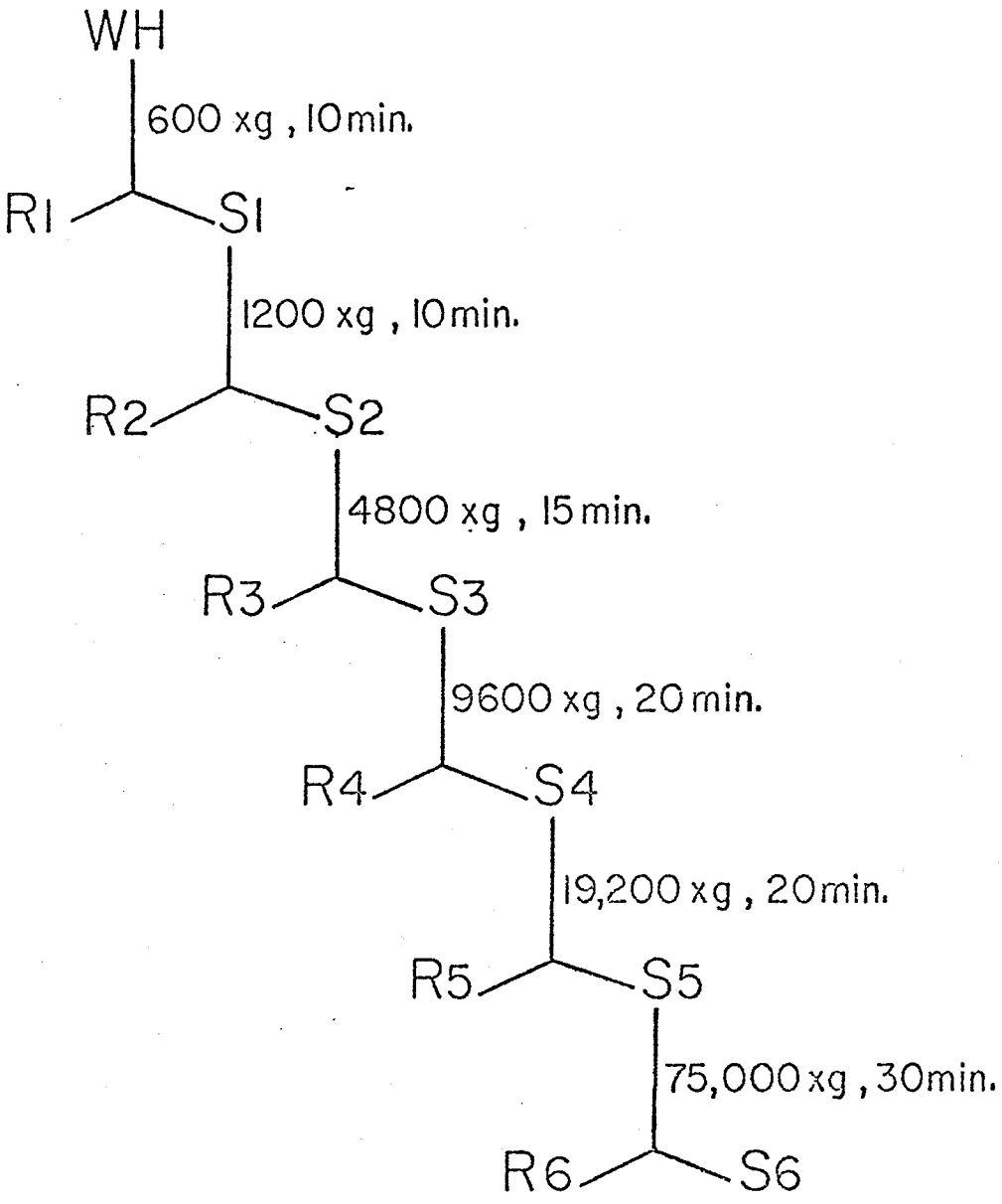


Fig. 3. Flow diagram illustrating the six centrifugation steps to obtain seven fractions from rat SMSG homogenate.

original homogenate volume was increased by approximately 6 mls by the termination of centrifugation. Before use, the washed pellets were suspended in 50 mM TES-KOH buffer, pH 7.4, and the whole homogenate and final supernatant, when required, were diluted 1:1 with 100 mM TES-KOH buffer, pH 7.4.

2. Preparation of the mitochondrial fraction

In the procedure described below the composition of the medium and centrifugal forces used to sediment subcellular particles were similar to those described by Chance and Hagihara (53) for the isolation of beef heart mitochondria.

Male rats of the Long-Evans strain (7-8 weeks old) were killed by decapitation, excised glands were placed in an ice cold medium containing 0.21 M mannitol, 0.07 M sucrose, 0.1 mM EDTA, 0.01 M TES-KOH, pH 7.4 (MSE Medium). After the tissue was cleaned and minced, it was transferred into a Potter-Elvehjem homogenizer containing MSE Medium and homogenized for 1 min at 70 rev./min. using a Teflon pestle (0.15 mm clearance). The homogenate was filtered through gauze and the filtrate adjusted with MSE Medium to 5% w/v based on the original tissue wet weight. The resulting suspension is referred to as the whole homogenate (WH). The homogenate was then centrifuged for 10 min at 500 x g to produce the R_1 fraction. The supernatant, after careful removal, was centrifuged for 10 min at 12000 x g. The resulting mitochondrial pellet was resuspended in 20 ml of MSE Medium and centrifuged for 5 min at

8000 x g to yield the final mitochondrial fraction (M). The supernatants obtained after the second and third centrifugation were combined to yield final supernatant (S). -All sedimentations were done on the International centrifuge Model B 20 (Rotor Model A 211). For use the mitochondrial fraction was suspended in MSE Medium. Although the mitochondria prepared by this method oxidized pyruvate-malate (as measured polarographically), addition of ADP had no effect on the respiration. Thus these mitochondria failed to exhibit a respiratory control ratio (RCR).

3. Preparation of coupled mitochondria

Phosphorylating mitochondria were obtained by modification of Chance and Hagihara's method (53), wherein a brief digestion of the tissue with bacterial proteinase precedes homogenization.

Male rats of the Long-Evans strain (7-8 weeks old) were killed by decapitation. The excised glands were placed in an 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 with occasional stirring, for 3 min on ice with 60 ml Stock Medium buffered with 10 mM Tris-phosphate, pH 7.6, and containing 0.5 mg/ml Nagarse. 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^{o} 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 x 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 x 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 10mM Tris-HCl (pH 7.4) and centrifuged for 5 min. at 8000 x g to produce the final mitochondrial pellet. Finally, mitochondria were suspended in Tris-HCl Stock Medium. The yield was approximately 3 mg mitochondrial protein/g fresh tissue.

B. Protein Determinations

Protein was determined by the method of Lowry *et al* (54). Aliquots of the tissue suspension was solubilized in 1 N NaOH overnight, diluted to 0.5 N NaOH and 1 ml portions removed for analysis. To 1 ml of the sample 5 ml of copper carbonate reagent was added, and the solution was mixed immediately. Copper carbonate reagent was prepared immediately before use, by mixing 50 parts of 2% Na_2CO_3 with one part of copper tartrate reagent (1% Na-tartrate, 0.5% $CuSO_4$, in 0.025 N NaOH).

After 10 min., 0.5 ml of 1 N Folin phenol reagent was added to each test tube, mixed, and allowed to stand for 30 min. Absorbance was measured at 750 nm in a Unicam SP-600 spectrophotometer against a blank sample (prepared by mixing 0.5 N NaOH with copper carbonate and Folin reagent).

Standard protein samples were prepared by mixing 10-100 µg of bovine serum albumin in 0.5 N NaOH with copper carbonate and Folin reagent.

C. Electron Microscopy

Mitochondrial pellets were fixed for 2 hours in chilled 2.5% glutaraldehyde solution containing 0.54% glucose and phosphate buffer, pH 7.2. After washing, the pellets were fixed for 1 hr. with 1% OsO_4, dehydrated with ethanol and embedded in methacrylate. Thin sections were viewed, after double staining with uranyl acetate and lead citrate, with a Philips 300 electron microscope. Electron microscopic pictures of the isolated coupled mitochondria are presented in the Result section.

D. Characterization of the Isolated Subcellular Fractions

1. Deoxyribonucleic acid (DNA) determination

Nucleic acids were separated from other tissue compounds as described by Schneider (55). The isolated nucleic acids were quantitatively determined by means of the indole reaction (56, 57).

i. Separation of acid soluble compounds An aliquot of the whole homogenate, or subcellular fraction, (containing 0.5-2.00 mg of protein) was mixed with 2.5 ml of cold 10% TCA, centrifuged, and sediment was washed once with 2.5 ml of cold 10% TCA.

ii. Separation of lipoidal compounds The final sediment remaining after removal of the acid-soluble compounds was extracted twice with 5 ml of 95% ethanol and recovered by centrifugation.

iii. Removal of nucleic acids The lipid free residue was suspended in 2.6 ml of 5% TCA and the mixture heated for 15 min. at 90°C with occasional stirring. This treatment quantitatively extracts both DNA and RNA from the tissue proteins and leaves the latter as an insoluble residue which was centrifuged off and washed once with 2.5 ml of 5% TCA (supernatants were combined).

iv. Estimation of DNA Two ml of the nucleic acid extract were mixed thoroughly with 1 ml of 0.04% aqueous indole and 1 ml of conc.HCl in a glass stoppered test tube. The mixture was heated for 10 min. in a boiling water bath, than cooled in cold water. Cold solutions were extracted twice with 4 ml of chloroform, and absorbance of the water layer was read at 490 nm in a Unicam SP-600 spectrophotometer against a sample blank. Blank samples were treated in the same way as the experimental samples, except that 2 ml of 5% TCA were used instead of the nucleic acid extract. Standard samples were treated in

the same way as the experimental samples, except that 2 ml of 5% TCA containing 10-80 µg of calf thymus DNA (Na salt) were used instead of the nucleic acid extract. Results are expressed as µg DNA/mg protein.

2. Succinate - INT - reductase

Succinate-INT-reductase was estimated by the method described by Pennington (58) using the artificial substrate 2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT).

The incubation was performed for 15 min. at 37°C in a final volume of 1.0 ml. The incubation mixture contained 0.05 M potassium phosphate buffer, pH 7.4; 0.1% INT; 0.05 M sodium succinate, 0.05 - 0.50 mg of tissue protein. The reaction was stopped by addition of 1 ml 10% TCA and the formazan was extracted into 4 ml of ethylacetate by vigorous shaking. After centrifugation, the absorbance of the organic layer was measured at 490 nm in a Unicam SP-600 spectrophotometer against an appropriate control. Control samples were treated in the same way as the experimental ones, except that TCA was added at the same time as the tissue suspension. When a medium lacking in succinate was incubated with tissue, no INT reduction occurred as compared to the TCA treated control, indicating that INT is being reduced specifically by succinate dehydrogenase. A molar extinction coefficient (490 nm) of 20.1×10^3 (55) for the formazan dissolved in ethylacetate was used to transform absorbance units into µ moles of formazan released. The enzymic activity is expressed as µ moles of formazan/mg protein/15 min.

3. Cytochrome - c - oxidase

Cytochrome-c-oxidase was assayed by the spectrophotometric method of Smith (59). However, the rate of oxidation of ferrocytochrome-c was measured by following the decrease in absorbance at 385 nm instead of 500 nm as recommended by Smith, as the maximum in the differential spectrum between reduced and oxidized cytochrome c was found at 385 nm.

The reaction rate was followed directly in a spectrophotometric cuvette (1 ml cuvette, 1 cm light path, dual beam differential Unicam SP-800 spectrophotometer) at 38°C. The incubation mixture (final volume 1 ml) contained 0.01 M potassium phosphate buffer, pH 7.0; 0.07% ferrocytochrome c (reduced with ascorbic acid). The reaction was initiated by addition of 10-50 µg of tissue protein and the absorbance at 385 nm was read every 15 seconds against the control. Control sample contained 0.01 M potassium phosphate buffer, pH 7.0; 0.07% ferrocytochrome-c and 0.001 M potassium ferricyanide. The activity is expressed as change in absorbance (A_{385nm})/min/mg protein.

4. β - Glucuronidase

β-glucuronidase was estimated by the procedure of Gianetto and de Duve (60).

Incubations were performed for 30 min. at 37°C in a final volume of 1.0 ml. The incubation mixture contained 0.150 M sodium acetate buffer, pH 5.0; 0.25 mM phenolphthalein glucuronide (prepared from the cinchonidine derivative (60)); and 0.1 - 1.0 mg of tissue protein

(treated for 20 min. with 0.2% Triton X-100 before use). The reaction was stopped by addition of 5 ml of alkaline reagent (0.133 M glycine, 0.067 M NaCl and 0.083 M Na_2CO_3, pH 10.7). The mixture was centrifuged and the liberated phenolphthalein was determined by measuring the absorbance at 540 nm in a Unicam SP-600 spectrophotometer against the blank sample (containing Na acetate buffer and alkaline reagent). Control samples were treated in the same way as the experimental ones, except that water was added instead of the tissue suspension. No difference in optical density was found between this control and the control containing boiled tissue suspension. Standard samples contained 5-20 µg of phenolphthalein. The activity of the enzyme is expressed in µmoles phenolphthalein released/mg protein/30 min.

5. Glucose - 6 - Phosphatase

Glucose-6-phosphatase was determined according to de Duve et al. (61).

The incubation was for 30 min. at 37°C in a final volume of 0.5 ml. The incubation mixture contained 0.007 M histidine, pH 6.5; 0.001 M EDTA; 0.020 M glucose-6-phosphate, and 0.05-0.50 mg of tissue protein. The reactions were stopped by the addition of 0.5 ml 10% TCA and, after centrifugation, released inorganic phosphate was estimated in the supernatants as described below. Control samples were treated in the same way as the experimental ones, except that TCA was added at the same time as the tissue suspension.

Inorganic phosphate determination:

i. Phosphate was estimated by a modified Fiske and Subbarow's method (62) on the supernatants after TCA precipitation.

To each supernatant were added 2 ml of 1.25% ammonium molybdate and 4 ml of aminonaphthol sulfonic acid (ANSA) reagent containing 0.006% ANSA in 1.5% Na HSO_3 and 0.05% Na_2SO_3. The samples were mixed well, allowed to stand for 45 to 60 min. before measuring the absorbance at 650 nm in a Unicam SP-600 spectrophotometer against the blank samples (containing TCA, ammonium molybdate and ANSA reagent). Standard samples contained 5-20 µg of phosphorus.

ii. Occasionally, phosphate was determined by a modified method of Mozersky et al (63), where the concentration of the unreduced phosphomolybdate complex is measured after extraction into isobutanol-benzene. This method was found to be approximately 20 times more sensitive than the Fiske-Subbarow procedure.

After the reaction was stopped by TCA and samples centrifuged, the supernatants were transferred into tubes kept in an ice bath and diluted to 2 ml volume with water. Two ml of 1.5% ammonium molybdate in 0.5 N H_2SO_4 and 4 ml of isobutanol-benzene (1:1), saturated with water) were added to the diluted supernatant and the suspension was mixed with a Vortex for 30 seconds, centrifuged, and the upper organic phase was transferred into tubes at room temperature (extraction of the phosphomolybdate complex into isobutanol-benzene at $0^{o}C$ and

subsequent warming of the organic phase prevented the formation of opaqueness in the organic phase). The absorbance was measured at 313 nm in a Unicam SP-800 spectrophotometer against appropriate blanks. The blank samples were prepared by shaking a solution of histidine-EDTA buffer, glucose-6-phosphate, TCA and ammonium molybdate with ethylacetate. Standard samples contained 1 to 4 µg of phosphorus. The activity of glucose-6-phosphatase is expressed as µmoles of phosphate released/mg protein/30 min.

6. 5' - nucleotidase

5'-nucleotidase was estimated by the method of Michell and Hawthorne (64).

The incubation was performed for 15 min. at 37°C in a final volume of 1.0 ml. The incubation mixture contained 0.100 M KCl: 0.010 M $MgCl_2$; 0.050 M Tris-HCl buffer, pH 7.4; 0.002 M AMP; 0.010 M Na-K tartrate and 0.1-1.0 mg of tissue protein. The reaction was stopped by the addition of 0.5 ml of cold 25% TCA. Control samples were treated in the same way as the experimental ones, except that TCA was added at the same time as the tissue suspension. Inorganic phosphate was determined by the modified Fiske and Subbarow method as described previously (glucose-6-phosphatase). The activity of 5'-nucleotidase is expressed as µmoles of phosphate released/mg protein/15 min.

7. Alkaline phosphatase

Alkaline phosphatase activity was estimated by using p-nitrophenyl phosphate as substrate essentially as described by Bessey et al (65).

The incubation was performed for 30 min. at 37°C in a final volume of 0.5 ml. The incubation mixture contained 0.05 M glycine buffer, pH 10.5; 0.5 mM $MgCl_2$; 0.0055 M p-nitro-phenylphosphate (Na salt). The reaction was stopped by the addition of 5 ml 0.02 N NaOH, centrifuged, and absorbance of the clear supernatant was read at 405 nm in a Unicam SP-600 spectrophotometer against the control. Control samples were treated in the same manner as the experimental ones, except that the tissue suspension was added after the addition of NaOH. The measured absorbance was converted to µmoles of product released (A_{405nm} x 200 = m µmoles p-nitrophenol formed).* The activity of the enzyme is expressed in µmole p-nitrophenol formed/mg protein/min.

E. Incorporation of Radioactive Precursors into Lipids of Cell Free Preparations from Rat SMSG

1. Purification of fatty acids - ^{14}C

^{14}C - fatty acids (namely palmitic-1-^{14}C and palmitic-16-^{14}C) were purified by thin layer chromatography (TLC) prior to every experiment, as it was noted that the compounds demonstrated extensive alterations even when stored in a sealed vial at -10° under N_2. The

* Boehringer (1967), Colorimetric method sheet No. 15987-T-AAF.

purification procedure was previously described by Pritchard (8).

^{14}C - fatty acids were run on Silica Gel G - coated plates with petroleum ether-ethyl ether - acetic acid (90:10:1) as developing solvent (for preparation of plates, see Section E (4)). Radioactive areas were detected with a Packard Radiochromatogram Scanner, Model 385, or visualized by autoradiography. Fatty acid area was scraped from TLC plate, fatty acid extracted with acetone and the pooled extracts concentrated to an appropriate activity. Fatty acids were added to the incubation medium in acetone, final concentration of which did not exceed 2.5% v/v. No observable effect of acetone on fatty acid incorporation has been noted.

2. Incubation media

i. Free fatty acids - ^{14}C

The incubations were performed as described by Pritchard et al. (66) in a medium (final volume 0.2 ml) containing: 0.025-0.035 mM palmitic acid-^{14}C; 12.5 mM ATP; 0.5 mM CoA; 25 mM $MgCl_2$; 40 mM KF; 0.1-1.0 mg of tissue protein, in 50 mM TES-KOH buffer, pH 7.4. When L-carnitine was present, its final concentration was 10 mM. Unless noted otherwise, incubations were for 60 min. at 37°C. The composition of this medium has been discussed by Pritchard (8) who showed that it gave maximum uptake of free fatty acids into lipids of SMSG whole homogenate. Incubations were terminated by the addition of 0.75 ml

of chloroform-methanol 1:2 v/v and the lipids were extracted and purified according to Bligh and Dyer (67) as described below (Section E (3)). Control samples were treated in the same way as the experimental ones, except that chloroform-methanol was added at the same time as the tissue suspension.

ii. Carnitine - ^{14}C

When the incorporation of carnitine-^{14}C into carnitine containing lipids was studied, the incubation mixture (final volume 0.2 ml) contained: palmitoyl-CoA; L-carnitine (DL carnitine-^{14}C was diluted 5-10 times with non-radioactive L-carnitine to give the appropriate final concentration); mitochondrial protein (preparation of mitochondrial fraction is given in Section A (2)), and in some experiments KF, in 50 mM TES-KOH buffer, pH 7.4. The incubations were performed at 37^{o}C in a final volume of 0.2 ml. Concentrations of substrates, cofactors, mitochondrial protein as well as the time of incubations are given in legends to appropriate figures and tables. Palmitoyl-CoA was employed as substrate in order to overcome the possible interference of fatty acyl-CoA synthetase (see Literature Review) in the rate of lipid formation. Under these conditions the presence of ATP and CoA was unnecessary for good incorporation (66). Incubations were terminated by the addition of 0.75 ml of chloroform-methanol 1:2 v/v and the lipids were extracted and purified according to Bligh and Dyer (67). Control samples were treated in the same way

as the experimental ones, except that chloroform-methanol was added at the same time as mitochondrial fraction.

3. Lipid extraction (Bligh and Dyer (67))

After the addition of chloroform-methanol, the samples were mixed thoroughly for 10 sec. on Vortex mixer. To produce a biphasic system, 0.25 ml of chloroform and 0.25 ml of water was added, and the suspension mixed for 10 sec. after each addition. The samples were centrifuged, aliquots of the lower chloroform phase containing purified lipid extract withdrawn and evaporated to dryness under a stream of nitrogen. The residue was dissolved in a small volume of chloroform-methanol (2:1) for TLC.

4. Thin layer chromatography

Silica Gel G (containing 5% $CaSO_4$, obtained from Camag, Switzerland) coated plates were prepared by spreading a Silica Gel G suspension in water onto the glass plates (thickness of the layer 0.6 mm), air drying and activating immediately before use at 110°C for 45 min. The lipid extract was applied 3 cm from bottom edge of the plates. After the samples had dried, the plates were developed by ascending TLC with chloroform-methanol-water (14:6:1) as developing solvent (68). The solvent was allowed to run approximately 14 cm (to within 2-3 cm of the plate top). In some cases a different solvent system was used (chloroform-methanol-acetic acid-water, 50:25:8:4, or

chloroform-methanol-ammonia, 50:30:8) as is noted in appropriate figures. For preparation of radioactive palmitoyl-carnitine and carnitine lipid two dimensional TLC on Adsorbosil-5 plates was used, as described below in Section F. Mixtures of known lipid standards were run along with the samples on most plates. The resulting chromatograms were treated with iodine vapours to visualize the lipids. Radioactive areas were located by autoradiography with Ilflex 25 EP X-ray film.

An example of a chromatogram showing both iodine positive and radioactive areas is presented in Fig. 4 . It could be seen that, when palmitic-1-C^{14} acid was used as a precursor in the absence of carnitine, the major radioactive spots visualized by autoradiography were lecithin (A), phosphatidylethanolamine (B), free palmitic acid (C) and triglyceride (8) (D). In the presence of carnitine two more radioactive spots were detected: palmitoyl-carnitine (F) and an unknown lipid (E). This lipid has recently been identified as β-hydroxy-palmitoyl-carnitine (see Results). Meanwhile it will be referred to as carnitine lipid (CL) (8). When carnitine-^{14}C was used as a radioactive precursor, only two radioactive spots appeared, carnitine lipid and palmitoyl-carnitine.

5. Radioactivity determination

Radioactivity was determined as described by Pritchard (8). Silica Gel containing the radioactive spots was scraped from TLC plates and transferred to counting vials. After addition of 10 ml of

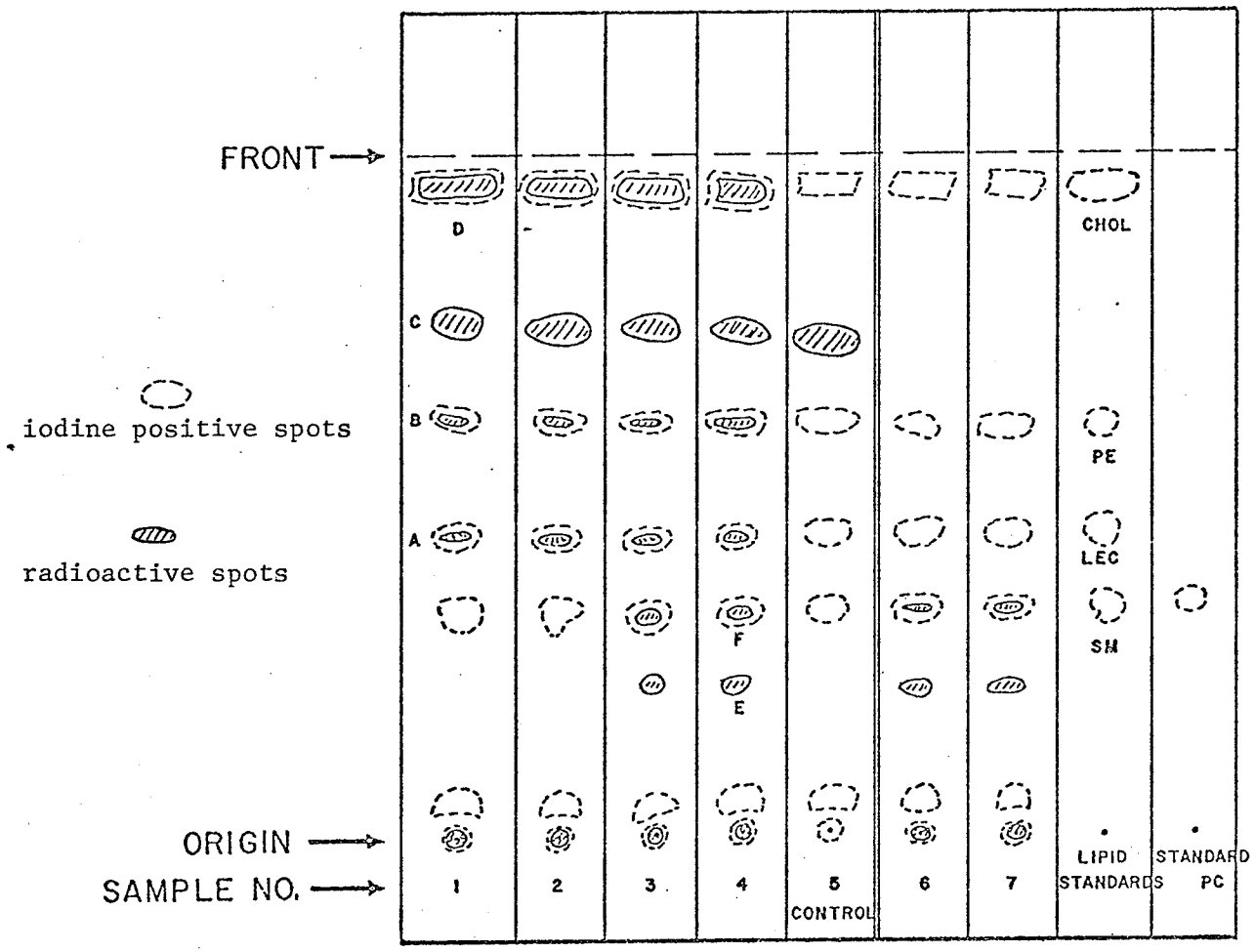


Fig. 4. Thin layer chromatogram of SMSG lipids. Solvent: chloroform-methanol-water (14:6:1). Samples 1 to 5 contained palmitic-1-C^{14} acid as a precursor. Carnitine was not added to samples 1 and 2, while it was present in samples 3, 4. Incubation conditions are described in the Section E (2). Samples 6, 7 contained 50 mM TES-KOH buffer, pH 7.4; 0.25 mM L-carnitine (^{14}COOH-labelled); 0.05 mM palmitoyl CoA, 40 mM KF and 0.250 mg of mitochondrial protein. Incubations were performed for 15 min. in a final volume of 0.2 ml. A- lecithin (LEC); B- phosphatidylethanolamine (PE); C- free fatty acid (FFA); D- triglyceride (TG); E- carnitine lipid (CL); F- palmitoyl-carnitine (PC); SM- sphingomyelin; CHOL- cholesterol.

scintillation fluid (Spectrafluor PPO-POPOP or butyl-PBD in toluene; Nuclear Chicago), the radioactivity was measured in a Nuclear Chicago Scintillation Counter Unilux II. All counts were corrected for quenching using the channel ratio method (69).

F. Enzymatic Preparation of Palmitoyl-carnitine and Carnitine Lipid

Palmitoyl-carnitine and carnitine lipid labelled on the fatty acid moiety were prepared by incubating palmitic-1-C^{14} acid in the presence of L-carnitine for 60 min. with approximately 0.25 mg of mitochondrial protein (preparation of mitochondrial fraction, see Section A (2)). Composition of the incubation mixture is described in Section E (2).

Palmitoyl-carnitine and carnitine lipid labelled on the carnitine moiety were prepared by incubating 0.25 mM L-carnitine (^{14}COOH-labelled) and 0.05 mM palmitoyl-CoA for 15 min. with 0.25-0.50 mg of mitochondrial protein in 50 mM TES-KOH buffer, pH 7.4 (final volume 0.2 ml).

Extraction of lipids for both palmitic-1-C^{14} and carnitine-^{14}C samples was done as described above (E (3)). The extracted lipids were separated by two dimensional thin layer chromatography on Adsorbosil-5 (Applied Science Lab.) coated plates. The first solvent system contained chloroform-methanol-water (14/6/1). This system did not separate palmitoyl-carnitine from sphingomyelin (see Fig. 4). Therefore,after a shortdrying period the plate was turned 90 degrees

and developed in a solvent system consisting of chloroform-methanol-acetic acid-water (50:25:8:4) (70). This effectively separated palmitoyl-carnitine and carnitine lipid from all other lipids. After visualization by autoradiography, the areas containing palmitoyl-carnitine and carnitine lipid were scraped off the plates, and the lipids were extracted from the Silica Gel by chloroform-methanol-water (14:6:1). The extraction procedure was repeated at least three times.

G. Hydrolysis of Radioactive Carnitine Lipid

Mild alkaline hydrolysis described below is a part of sequential hydrolysis procedure originally developed by Dawson (71, 72) for the identification of individual phospholipids in a mixed phospholipid sample obtained from biological materials. Under the conditions of mild alkaline hydrolysis, lipid ester bonds are being selectively hydrolysed, thus giving rise to water soluble phosphate esters from the diacylphosphoglycerides, while the plasmalogens and alkyl ether phospholipids give rise to chloroform soluble lyso compounds, and sphingomyelins are unchanged (73).

As in the early stages of the work on identification of carnitine lipid, it was thought that this lipid might be a phospholipid containing carnitine, the hydrolysis procedure was used to aid in identification. Together with carnitine lipid, palmitoyl-carnitine or sometimes lecithin was hydrolysed under the same conditions to compare hydrolysis products of known simple carnitine ester (palmitoyl-carnitine) or diacylphospho-

glyceride (lecithin) to those of carnitine lipid. Palmitic-1-^{14}C acid and carnitine-^{14}COOH were taken through all the hydrolysis steps for comparative purposes and to partially eliminate possible artifacts.

1. Procedure

The procedure of Dittmer and Wells was followed (73). Solutions of ^{14}C-labelled carnitine lipid and palmitoyl-carnitine (or in some cases lecithin) containing approximately 40000 dpm were dried under a stream of N_2 and the residue was re-dissolved in 0.2 ml of chloroform methanol (1:4 v/v). To the solutions 0.02 ml of 1.2 N NaOH in methanol-water (1:1 v/v) were added, samples were mixed well and incubated for 25 min. at 37°C. At the end of the incubation period, the mixture was neutralized with 0.03 ml of 1 N acetic acid. Subsequently, 0.4 ml of chloroform-methanol (9:1v/v) was added, followed by 0.2 ml of isobutanol and 0.4 ml of water. The mixture was shaken, centrifuged for 10 min., and the upper aqueous phase was drawn off. The lower phase was re-extracted with 0.2 ml of methanol-water (1:2 v/v), upper phases were combined. The distribution of radioactivity between the organic and aqueous phases was measured after hydrolysis. With fatty acid-1-^{14}C labelled lipids all the radioactivity was recovered in the organic phase. With carnitine-^{14}COOH labelled lipids all the radioactivity was recovered in the aqueous phase. Unhydrolysed compounds (carnitine lipid, palmitoyl-carnitine or lecithin) exhibited 95% of the total radioactivity in the organic phase.

2. Separation of radioactive products obtained after mild alkaline hydrolysis of fatty acid-1-^{14}C labelled lipids

Aliquots of the organic phase containing hydrolysis products of fatty acid-1-^{14}C labelled compounds, a standard sample of palmitic-1-^{14}C acid and of the methyl ester of palmitic-1-^{14}C acid were applied on Silica Gel G coated plates and developed in chloroform-methanol-water (14:6:1). Radioactive spots were visualized by autoradiography.

3. Paper chromatography of radioactive products obtained after mild alkaline hydrolysis of carnitine-^{14}COOH labelled lipids

Aliquots of the water phase containing hydrolysis products of carnitine-^{14}COOH labelled compounds and a standard sample of carnitine-^{14}COOH were applied on Whatman No. 1 or No. 4 paper and chromatographed in 3 different solvent systems: 1) propanol-ammonia-water, 85:5:10, (74) (run on Whatman No. 1 paper); 2) ethanol-ammonia-water, 90:5:5, (9) (run on Whatman No. 4 paper); 3) phenol-dioxane-water-potassium chloride, (75) (run on Whatman No. 1 paper). Solvent was prepared by mixing 50 g. of phenol with 25 ml of dioxane and 10 ml of water saturated with KCl. Paper was pretreated with 0.5 M KCl and dried (75).

Radioactive areas were visualized by autoradiography.

H. Polarographic Experiments

Oxygen uptake by isolated mitochondria was monitored with a Teflon membrane coated Clark electrode (76) (Yellow Springs Instrument

Co., polarizing voltage 0.8 volts) attached to a KM Gilson Oxygraph (Gilson Medical Electronics, Middleton, Wisconsin). Measurements were made at 28°C in an air-saturated medium of final volume 1.6 ml (77). The oxygen concentration in the reaction mixture (224 μM O_2 at 28°C, 760 mM Hg) was calculated from the data for the solubility of O_2 in Ringer solution (78) and corrected for the prevailing barometric pressure.

1. Incubation media

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 for discussion), 20 mM Tris-HCl, 5 mM K-phosphate (pH 7.2) and 0.4 to 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 Na salts at pH 7.2.

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

2. Calculation of respiratory rates, ADP/O and respiratory control ratios

Fig. 5 shows an example of a polarographic trace and the method

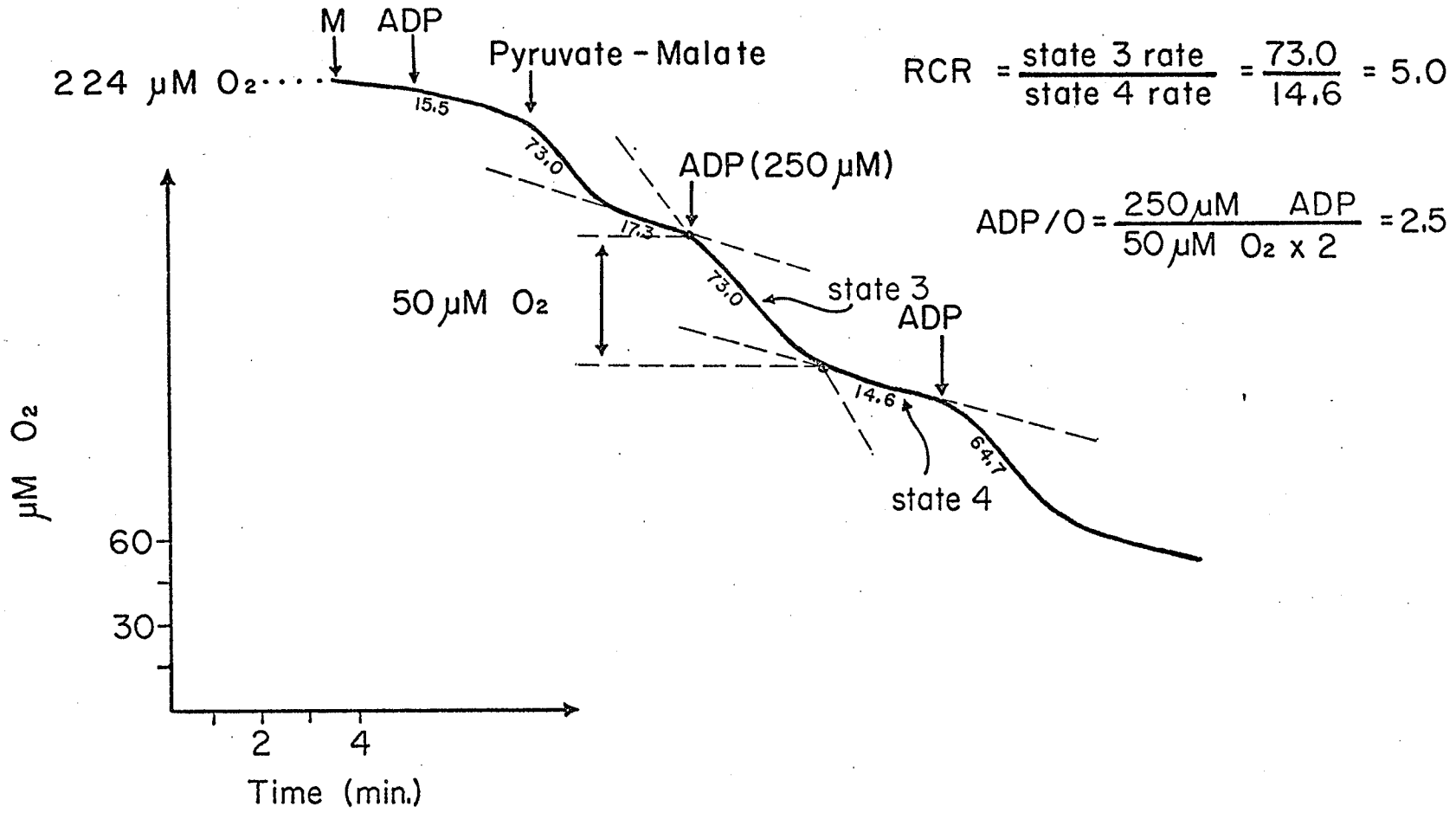


Fig. 5. Schematic representation of a polarographic experiment showing the calculation of ADP/O and respiratory control ratios. Mitochondria (M), substrate and ADP were added at points indicated by arrows. Numbers below the tracing indicate the rate of O_2 uptake in μmoles/min./g protein.

of calculating the ADP/O and respiratory control ratios (RCR).

It is seen that after addition of ADP to the basic polarographic medium containing mitochondria, a slow rate of respiration was observed indicating the presence of endogenous oxidizable substrates. This rate of O_2 consumption, which did not decline even after 4 min., is referred to as the endogenous respiration (Results, Table 11). Addition of an exogenous substrate (pyruvate-malate) resulted in a rapid increase in O_2 utilization (State 3) which declined after the supply of ADP was exhausted (State 4). These cycles of State 3-State 4 transitions could be repeated several times during a single experiment by repeated additions of limiting amounts of ADP. The concentration of oxygen utilized during the State 3 period is proportional to the amount of ADP phosphorylated to ATP (79,80). The ADP/O ratio, which is a measure of the efficiency of phosphorylation with regard to O_2 uptake, can be directly calculated from the polarographic tracing (81) as presented in Fig. 5. RCR was calculated according to Chance and Williams (81) as the ratio of State 3 respiration over State 4 respiration. This ratio is considered to be one of the most sensitive criteria for the intactness of mitochondria (81). Respiratory rates in State 3 and State 4 (expressed in μmoles O_2 utilized/min./g protein), ADP/O ratios and RCR were all calculated from data obtained from the second period of State 3-State 4 transitions (82).

3. Oxygen uptake and palmitoyl-carnitine formation

In experiments designed to follow both the rate of oxygen disappearance and palmitoyl-carnitine formation, it was found necessary to first measure 0_2 uptake in the presence of unlabelled carnitine, then in an identical experiment (using the same tissue and performed immediately afterwards) labelled carnitine was present but 0_2 uptake was not monitored (83). This procedure was necessitated by the design of the Oxygraph cell which did not allow the withdrawal of sufficient sample volume while oxygen consumption was being measured. In these latter experiments, 75 µl of suspension was removed from the incubation mixture at various time intervals and immediately placed in 280 µl of chloroform-methanol 1/2 v:v. Lipids were extracted and purified by the Bligh and Dyer procedure as described above. Separation of purified lipids and radioactivity determinations were done as described in Sections E (4), E (5).

I. Gas-Liquid Chromatography

Fatty acid esters were prepared from lipids by a direct transesterification reaction in 5% v/v methanolic H_2SO_4 (84).

The direct transfer of the silica gel area containing the lipid to centrifuge tubes without prior extraction was preferred, as it produced a much higher yield than prior elution from the gel.

To the dried lipid samples or silica gel powders 1-2 ml of

methanolic H_2SO_4 were added, the mixtures were heated at 70°C for 3 hours. Two ml of water was added to each tube and the methyl esters were extracted with 1 ml portions of petroleum ether. The combined petroleum ether extracts were washed with water, dried over Na_2SO_4, and taken to dryness under a stream of nitrogen. The residue containing methyl esters was redissolved in 2, 2, 4, trimethylpentane and appropriate aliquots used for gas liquid chromatography analysis in a Pye "Series 104" Gas Chromatograph, Model 64 (5 feet x 1/4 inch stainless steel column packed with 3% SE-30 on 100-200 mesh of Gas Chrom Q).

RESULTS AND DISCUSSION

A. Studies with Subcellular Particulates Isolated from Rat SMSG

Subcellular fractions dealt with in this chapter were isolated as described in Methods, Section A (1).

1. Characterization of isolated subcellular fractions by specific enzyme markers

Fig. 6 shows the distribution of characteristic marker enzymes and DNA among the isolated subcellular fractions. The following enzyme activities were estimated: succinate-INT-reductase for mitochondria (85,50), β-glucuronidase for lysosomes (86, 87), glucose-6-phosphatase for endoplasmic reticulum (88), 5'-nucleotidase (89, 90) and alkaline phosphatase (89, 91) for plasma membranes (however, as reported by other workers, alkaline phosphatase may also be indicative of nuclei (92)). DNA was estimated in order to determine the distribution of nuclei among the isolated fractions (93). The average per cent distribution of protein in these fractions is shown in Table 2. Fractions R_1 and R_2 were found to be predominantly composed of unbroken cells, cellular debris and nuclei. This was also visualized by phase contrast microscopy. Most mitochondria were concentrated in the R_3 fraction, with some lighter ones in R_4 along with lysosomes and endoplasmic reticulum. The endoplasmic reticulum appeared to be distributed among the R_4 to R_6 fractions, with R_4 and R_5 fractions enriched in endoplasmic reticulum, while R_6 fraction was enriched in plasma membrane.

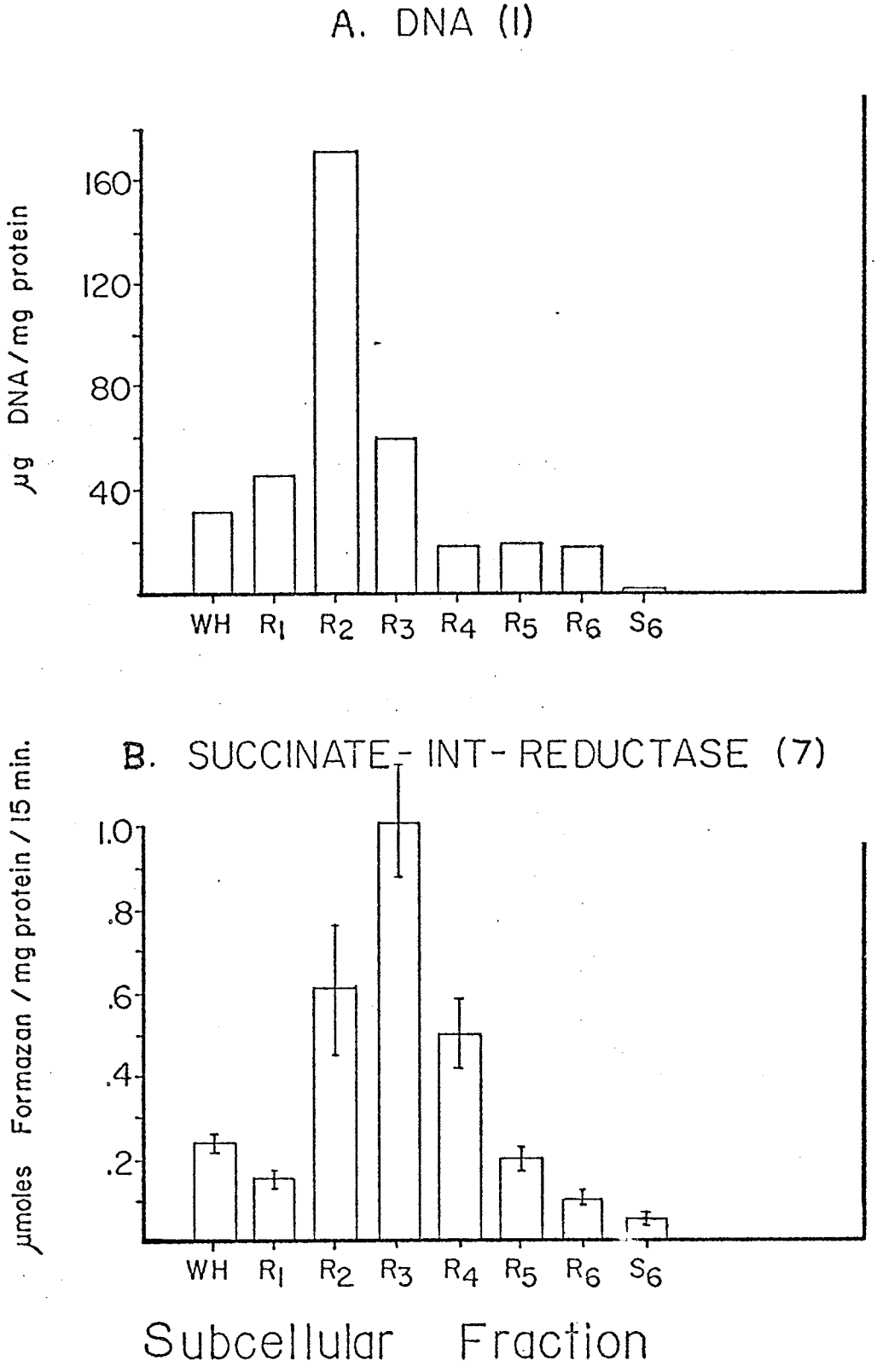
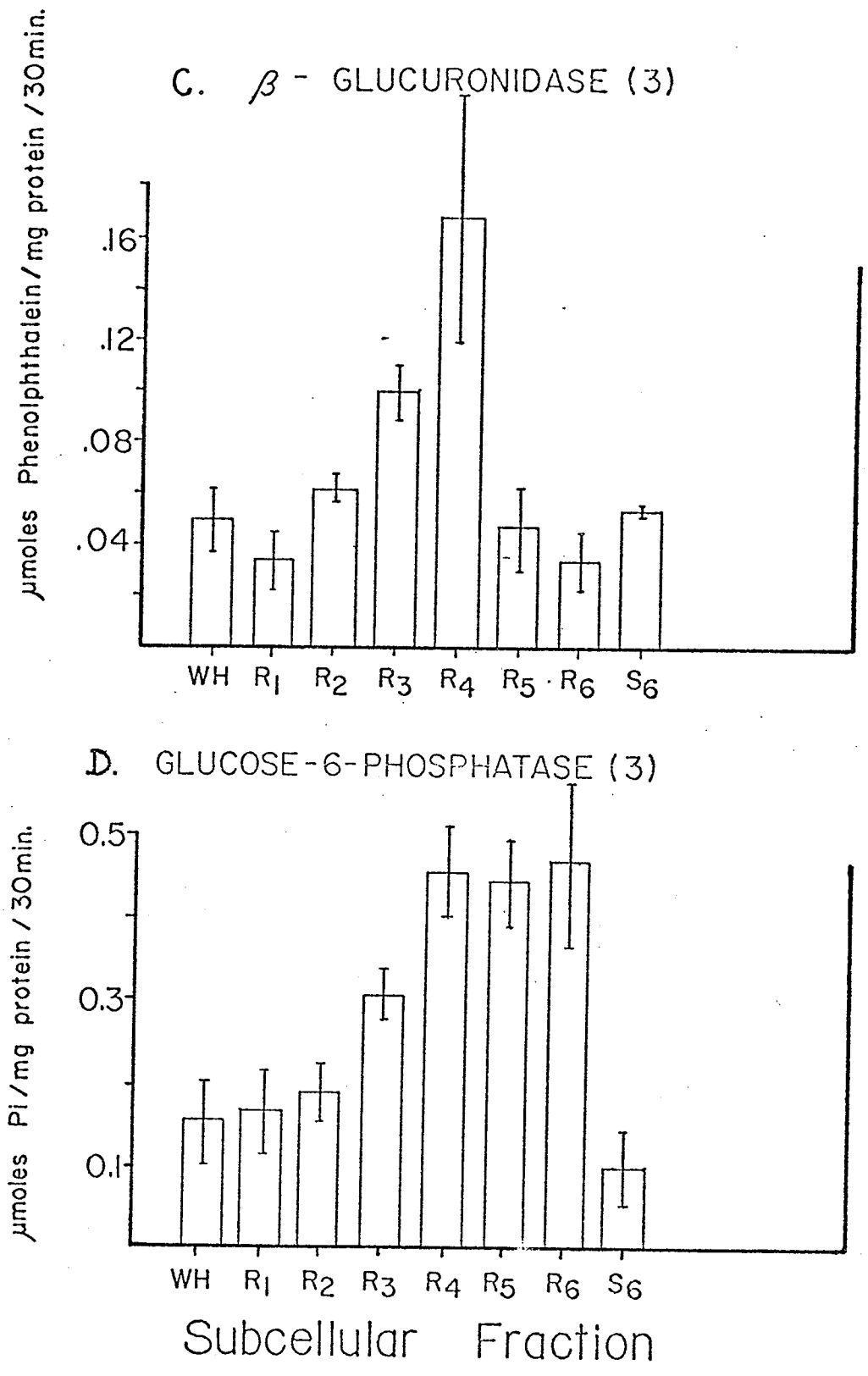


Fig. 6. DNA estimation and enzymic activities in subcellular fractions. Values represent means (number of experiments is given in parenthesis) ± standard error of the mean (I).


C. β - GLUCURONIDASE (3)
µmoles Phenolphthalein / mg protein / 30 min.
.16
.12
.08
.04
WH R1 R2 R3 R4 R5 R6 S6
D. GLUCOSE-6-PHOSPHATASE (3)
µmoles Pi / mg protein / 30 min.
0.5
0.3
0.1
WH R1 R2 R3 R4 R5 R6 S6
Subcellular Fraction

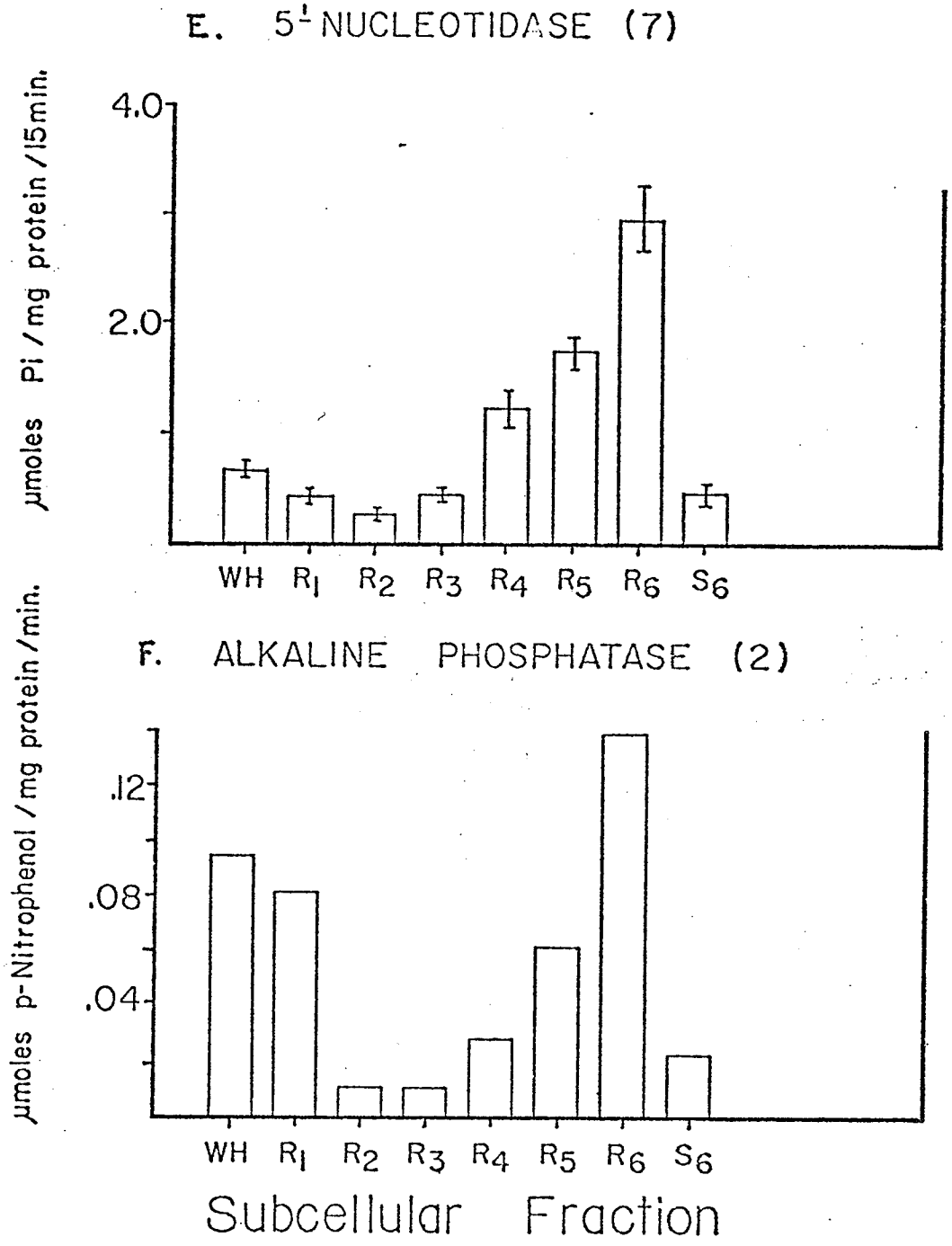

E. 5¹ NUCLEOTIDASE (7)
μmoles Pi / mg protein / 15 min.
4.0
2.0
WH R1 R2 R3 R4 R5 R6 S6
F. ALKALINE PHOSPHATASE (2)
μmoles p-Nitrophenol / mg protein / min.
.12
.08
.04
WH R1 R2 R3 R4 R5 R6 S6
Subcellular Fraction

TABLE 2

AVERAGE PER CENT DISTRIBUTION OF PROTEIN IN SUBCELLULAR FRACTIONS OBTAINED FROM RAT SMSG HOMOGENATE(†)

Fraction	Per Cent Distribution
R_1	44.7 ± 8.9
R_2	3.7 ± 1.9
R_3	3.8 ± 0.4
R_4	2.2 ± 0.5
R_5	2.4 ± 1.0
R_6	2.2 ± 0.7
S_6	40.9 ± 8.4

(†) Values represent the mean of six experiments ± standard error of the mean.

2. Incorporation of palmitic-1-^{14}C acid into lipids

In Fig. 7 a comparison is presented of the ability of isolated subcellular fractions to incorporate free palmitic acid into various lipids in the presence or absence of added carnitine. In all fractions carnitine decreased the incorporation of palmitic-1-^{14}C acid into lecithin (LEC), phosphatidyl ethanolamine (PE), and triglyceride (TG). At the same time (as is shown in Fig. 8), in the presence of carnitine, all subcellular fractions very effectively incorporated palmitic-1-^{14}C acid into palmitoyl-carnitine (PC) and, with the exception of R_6 and S_6, into carnitine lipid (CL). The highest palmitic-1-^{14}C acid incorporation into palmitoyl-carnitine (per mg protein) was associated with the microsomal fractions (R_4 and R_5), while mitochondria were the major site of carnitine lipid synthesis. The formation of these two lipids could be observed only in the presence of carnitine, no detectable palmitoyl-carnitine or carnitine lipid was formed in the absence of carnitine. Therefore, it seems probable that carnitine decreased the incorporation of palmitic-1-^{14}C acid into lipids (LEC, PE, TG) by competing for the available palmitoyl-CoA with other lipid precursors. Furthermore, the presence of carnitine in the incubation mixture resulted in increased uptake of free fatty acid (FFA) by subcellular fractions, as is shown in Table 3 for R_3 fraction (mitochondrial) and R_5 fraction (microsomal). This indicates that under the present experimental conditions, the rate of lipid labelling was limited by the level of

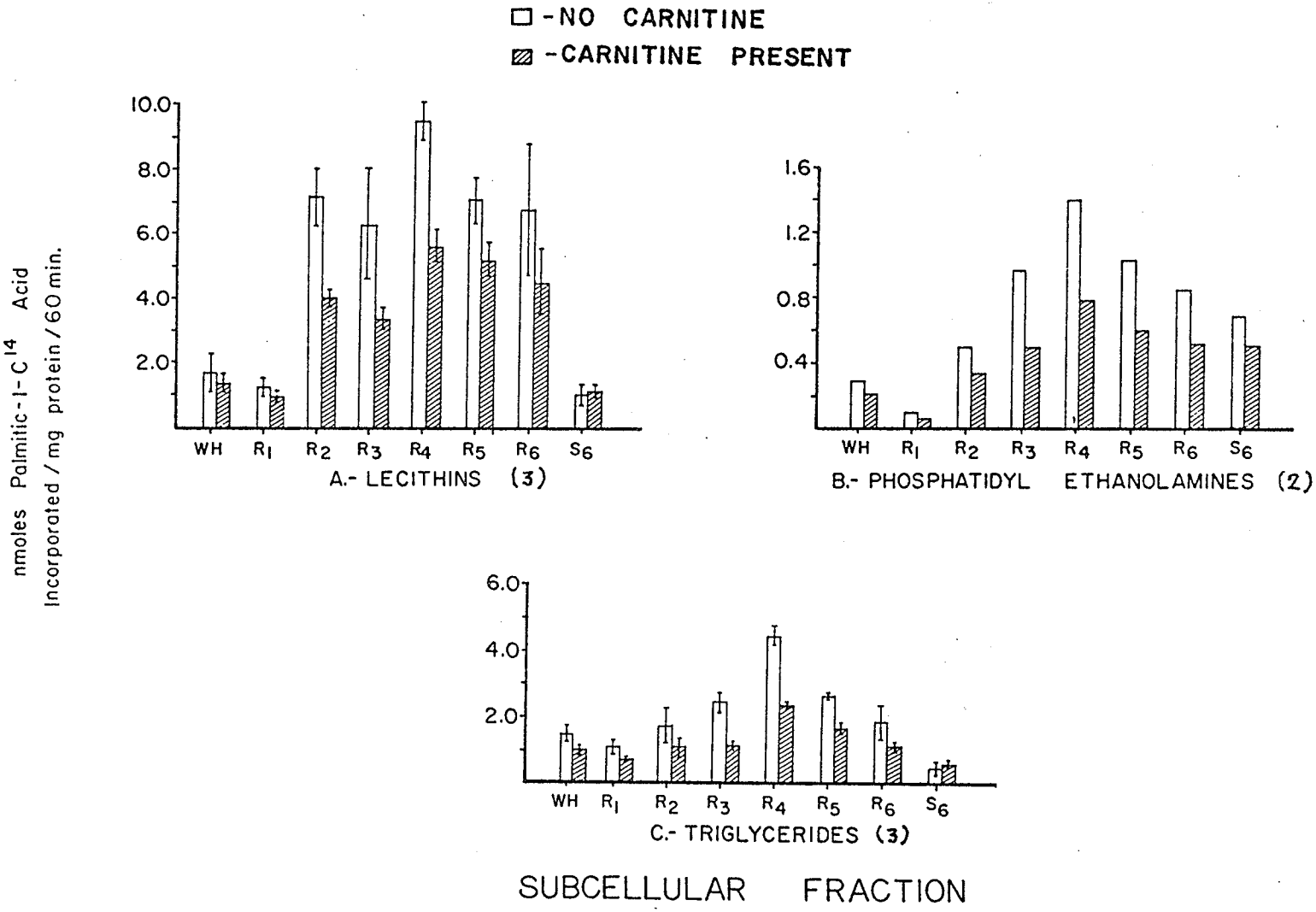


Fig. 7. Incorporation of palmitic-1-^{14}C acid into lipids of isolated subcellular fractions in the presence and absence of carnitine. Incubation conditions are given in Methods, Section E (2). Values represent means (number of experiments is given in parenthesis) ± standard error of the mean (I).

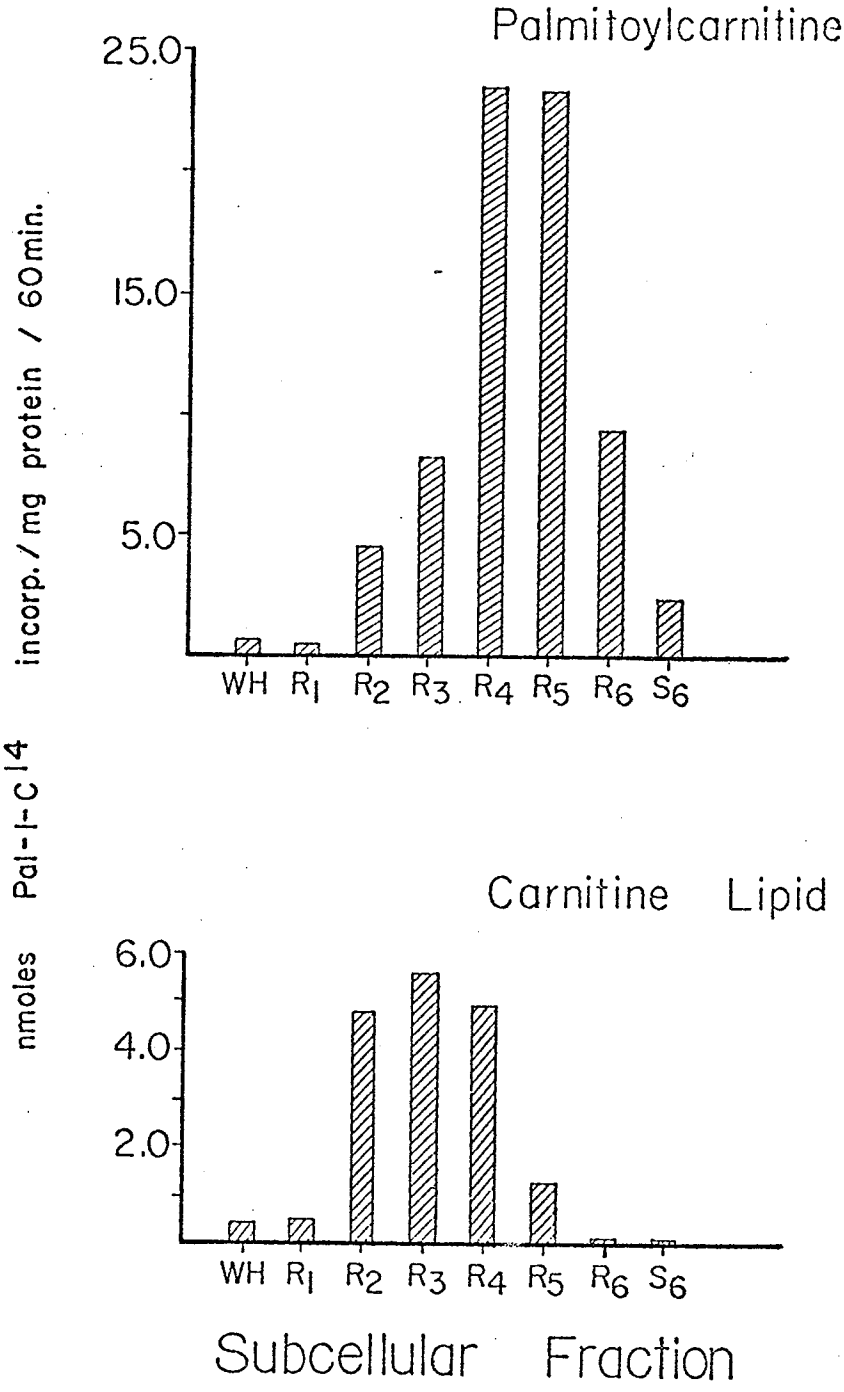


Fig. 8. Incorporation of palmitic-1-^{14}C acid into palmitoyl-carnitine and carnitine lipid. Incubation conditions are given in Methods, Section E (2). Values represent means of three experiments.

TABLE 3

PER CENT DISTRIBUTION OF RADIOACTIVITY AMONG THE LIPIDS OF MITOCHONDRIAL AND MICROSOMAL FRACTIONS IN THE PRESENCE AND ABSENCE OF CARNITINE (†)

		CL	PC	LEC	PE	FFA	TG
R_3 fraction	no carnitine			22.4	1.2	64.2	6.3
(mitochondrial)	+ carnitine	17.3	15.3	13.6	0.7	47.0	3.5
R_5 fraction	no carnitine			22.8	1.2	64.6	7.7
(microsomal)	+ carnitine	2.1	53.0	16.8	0.8	21.9	4.5

(†) Incubation conditions are described in Methods, Section E (2), (palmitic-1-^{14}C acid used as a radioactive precursor). Numbers represent per cent of total radioactivity recovered from TLC plate.

TABLE 4

DISTRIBUTION OF PROTEIN AND SUCCINATE-INT-REDUCTASE AND CYTOCHROME-C-OXIDASE ACTIVITIES AMONG SUBCELLULAR FRACTIONS (†)

	SUCC-INT-RED. (μmoles formazan/mg/15 min.)	CYT-C-OXIDASE (A_{385}/mg/min.)	Per cent Distribution of Protein
WH	0.252	3.67	
R_1	0.194	1.58	56.7
M	1.370	15.64	7.2
S	0.216	1.93	36.1

(†) Isolation of fractions is described in Methods, Section A (2).

carnitine in the presence of only mitochondria and palmitoyl-CoA. No detectable carnitine-^{14}C incorporation into palmitoyl-carnitine occurred in the absence of palmitoyl-CoA. Apart from palmitoyl-carnitine, the mitochondrial fraction from SMSG catalyzed the incorporation of carnitine-^{14}C into carnitine lipid in the presence of palmitoyl-CoA. No detectable carnitine lipid was formed in the absence of palmitoyl-CoA.

1. Effect of palmitoyl-CoA concentration

Fig. 9 shows the dependance of L-carnitine incorporation into palmitoyl-carnitine and carnitine lipid on palmitoyl-CoA concentration in the incubation medium. The maximum incorporation of carnitine into palmitoyl-carnitine was reached at 0.25 mM palmitoyl-CoA, increasing this concentration 2x had no effect on the incorporation. Carnitine lipid formation was completely inhibited at a relatively high concentration of palmitoyl-CoA (0.25 mM), possibly due to the strong surface active properties of this compound (83) (cf. effects of freezing and thawing and Triton X-100 on carnitine lipid formation; Tables 5, 6).

2. Effect of fluoride

The presence of carnitine ester hydrolase, an enzyme that catalyzes the hydrolysis of carnitine ester to free fatty acid and carnitine, was reported in rat liver (94). The activity of this enzyme was inhibited 50% by 1 mM NaF. However, we did not find any effect of

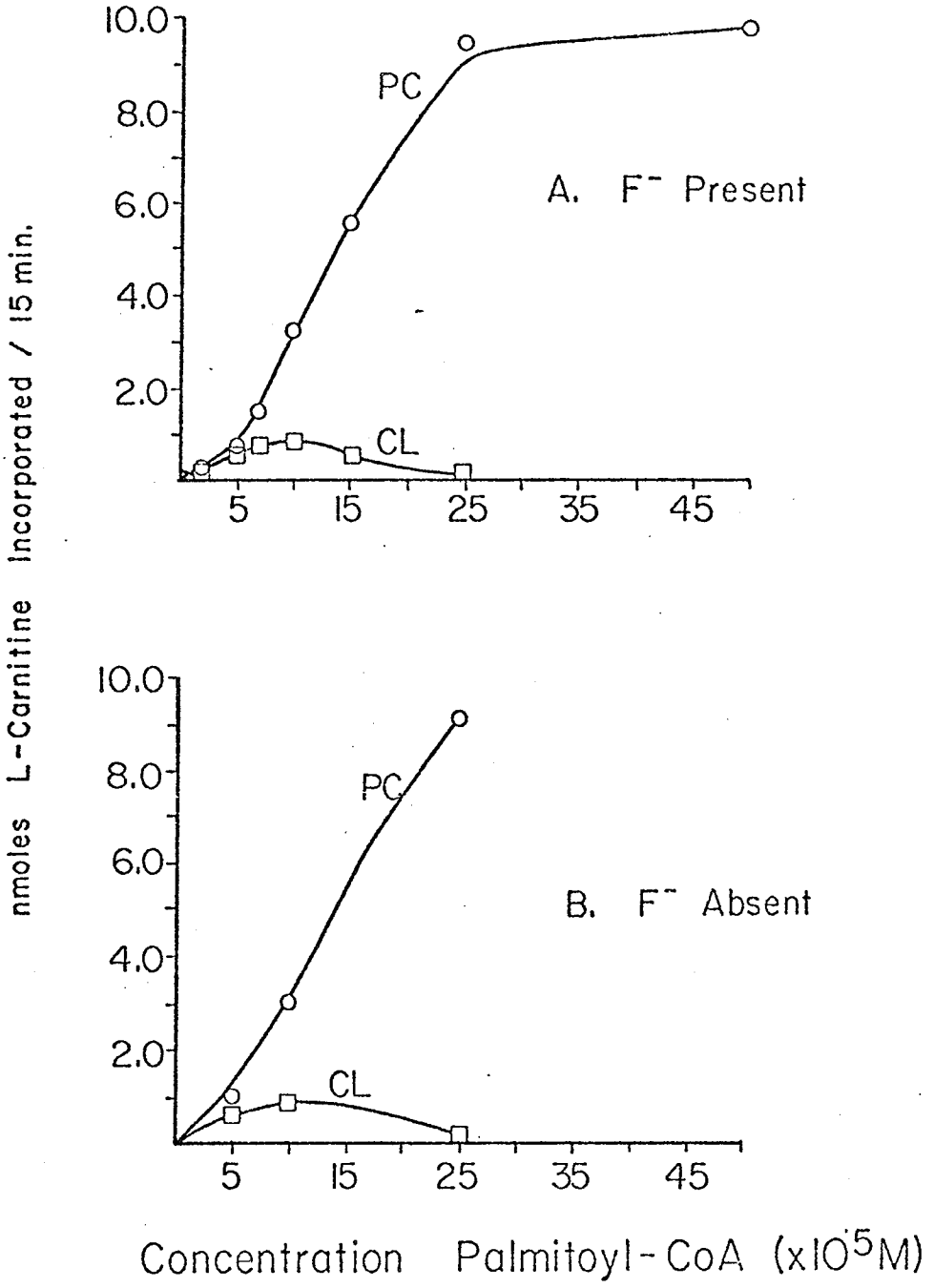


Fig. 9. Dependence of L-carnitine incorporation into palmitoyl-carnitine and carnitine lipid on palmitoyl-CoA concentration. The incubation was performed for 15 min. in a medium (final vol. 0.2 ml) containing 50 mM TES-KOH buffer, pH 7.4; 0.25 mM L-carnitine (^{14}COOH-labelled); 40 mM KF (only in experiment shown in Fig. 9A); 0.272 mg of mitochondrial protein and palmitoyl-CoA as shown in the graph.

F^- on palmitoyl-carnitine formation (compare Fig. 9A and 9B), excluding the possibility of carnitine ester hydrolase interference in our system. Similarly, F^- did not affect the formation of carnitine lipid (Figs. 9A, 9B).

3. Effect of L-carnitine concentration

Fig. 10 shows the dependence of palmitoyl-carnitine formation upon L-carnitine concentration (at the concentration of palmitoyl-CoA used no carnitine lipid was formed). The incorporation of L-carnitine into palmitoyl-carnitine increased on increasing the L-carnitine concentration from 0.02 mM to 0.4 mM. Further increasing the concentration of carnitine was not tried because increased dilution of radioactive carnitine with the non-radioactive compound resulted in an exceedingly small fraction of radioactive carnitine being incorporated into palmitoyl-carnitine. If one wished to keep the specific activity of carnitine constant it would be necessary to use increasingly high concentrations of radioactive carnitine. A carnitine concentration of 0.25 mM was used in subsequent experiments to spare radioactive carnitine. Norum (42) reported that a saturation of purified carnitine-palmitoyl transferase was not obtained even when 3 mM L-carnitine was added to the incubation mixture.

4. Effect of time

When the incorporation of L-carnitine into palmitoyl-carnitine

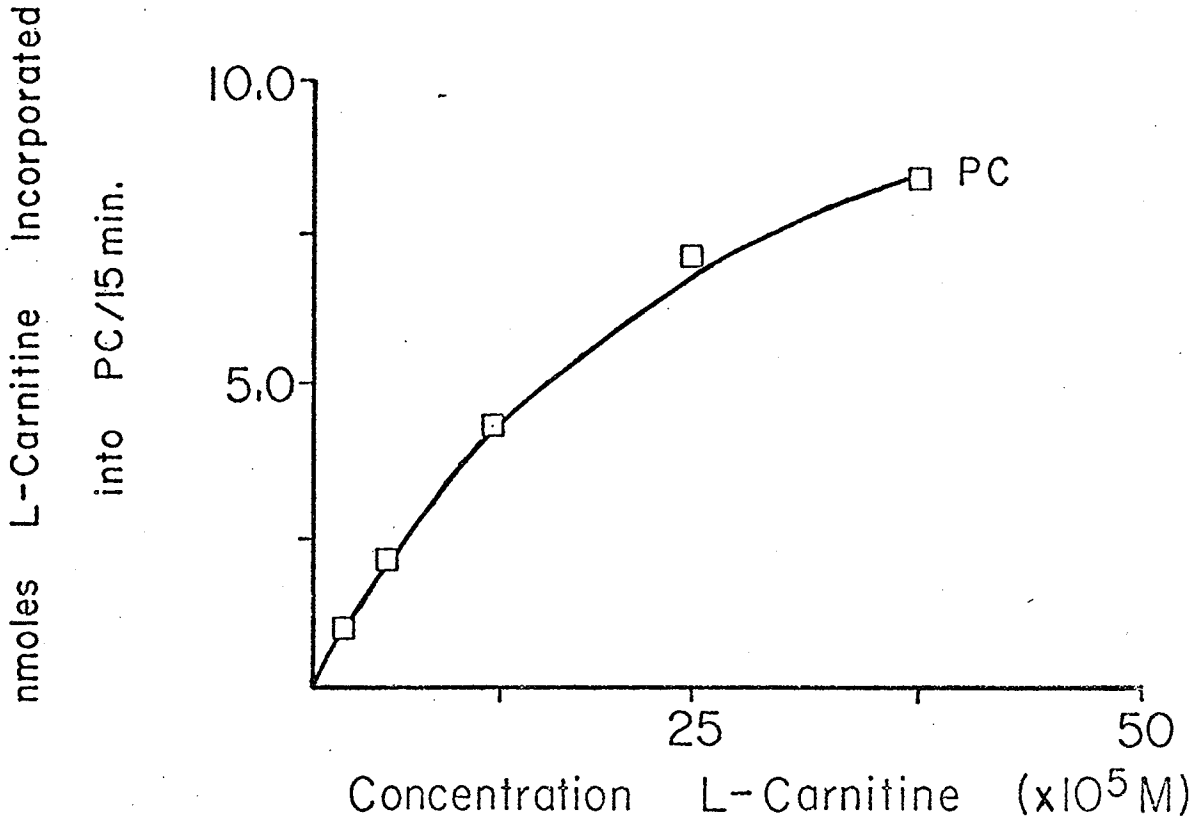


Fig. 10. Dependence of L-carnitine incorporation into palmitoyl-carnitine upon L-carnitine concentration. The incubation was performed 15 min. in a medium (final vol. 0.2 ml) containing 50 mM TES-KOH buffer, pH 7.4; 0.25 mM palmitoyl-CoA; 40 mM KF; 0.210 mg of mitochondrial protein and L-carnitine (^{14}COOH-labelled) as shown in the graph.

and carnitine lipid was followed (in a medium containing palmitoyl-CoA and carnitine) as a function of time (Fig. 11), it was observed that palmitoyl-carnitine formation reached a maximum after 5 min. of incubation, rapidly declining upon prolonged incubation. On the other hand, carnitine lipid gradually increased reaching a maximum after 15 min. of incubation.

Similar observations were made when palmitic-1-^{14}C acid was used as a precursor (Fig. 12). Palmitoyl-carnitine formation reached a maximum after approximately 20 min. of incubation, declining upon prolonged incubation. Carnitine lipid formation gradually increased, reaching the same level as palmitoyl-carnitine after 60 min. of incubation. These results suggested that palmitoyl-carnitine might be a precursor of carnitine lipid. An experiment designed to examine this problem is reported in Section C (4), and the reason for the rapid disappearance of palmitoyl-carnitine upon prolonged incubation is discussed on p. 78 .

5. Effect of pH

As shown in Fig. 13, maximal L-carnitine incorporation into both palmitoyl-carnitine and carnitine lipid occurred between pH 7.0 and pH 8.0. The optimum pH for palmitoyl-carnitine formation is in agreement with results of Norum (42) obtained for purified carnitine palmitoyl transferase. The enzyme exhibited its maximum activity between pH 7.0 and 8.2.

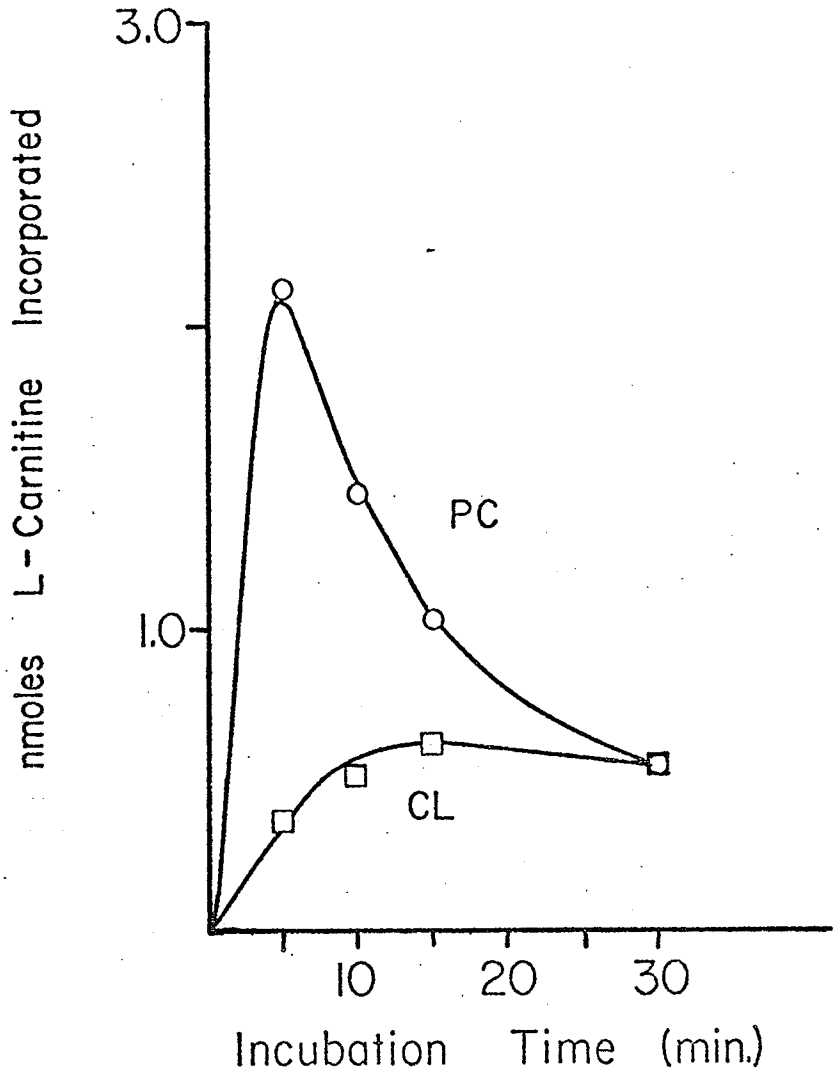


Fig. 11. Dependence of L-carnitine incorporation into palmitoyl-carnitine and carnitine lipid upon time. The incubation was performed in a medium (final vol. 0.2 ml) containing: 50 mM TES-KOH buffer, pH 7.4; 0.05 mM palmitoyl-CoA; 0.25 mM L-carnitine (^{14}COOH-labelled); and 0.272 mg of mitochondrial protein.

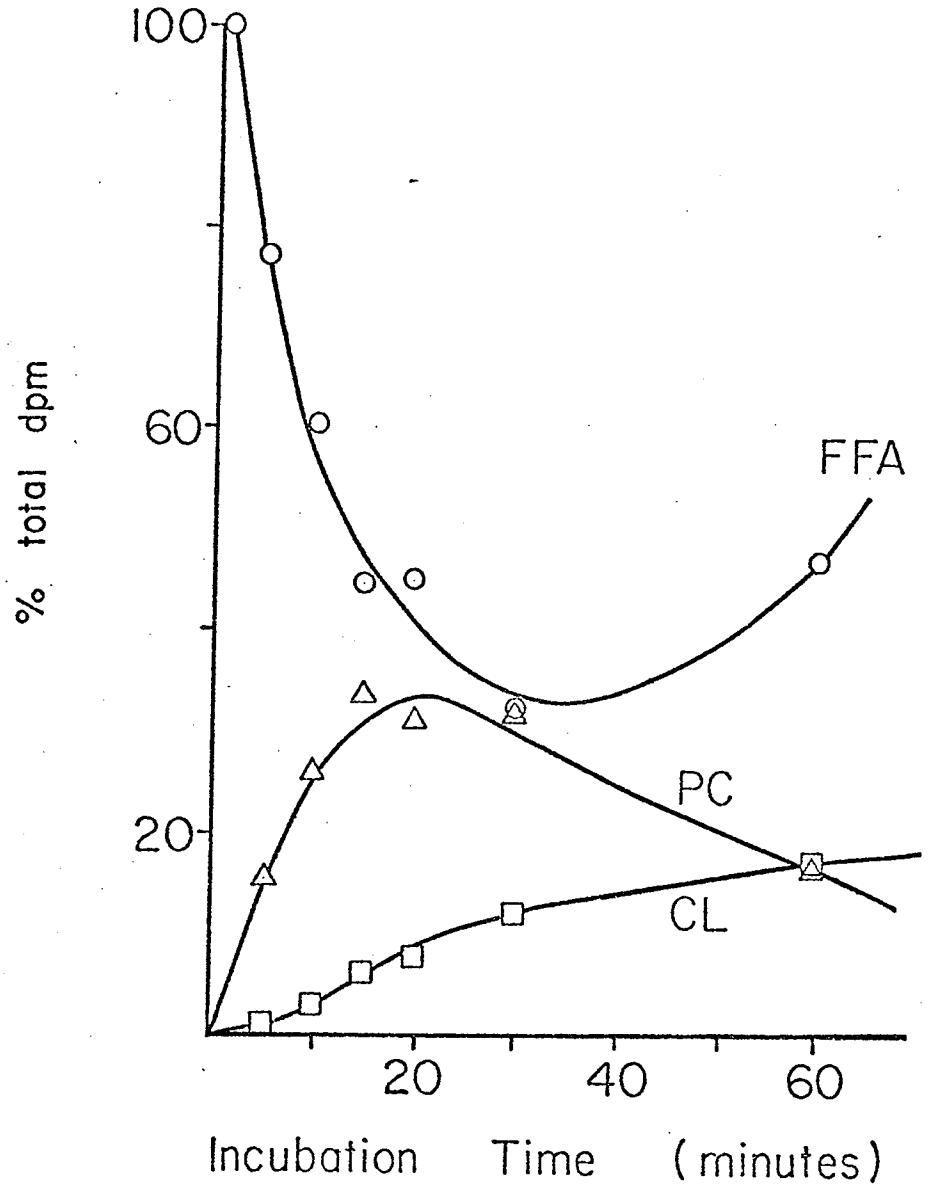


Fig. 12. Dependence of palmitic-1-^{14}C acid incorporation into palmitoylcarnitine and carnitine lipid upon time. Incubation mixture (composition described in Methods, Section E (2)) contained 0.168 mg of R_3 fraction protein (preparation of the fraction given in Methods, Section A (1)). Results are expressed as per cent of total radioactivity recovered from TLC plate.

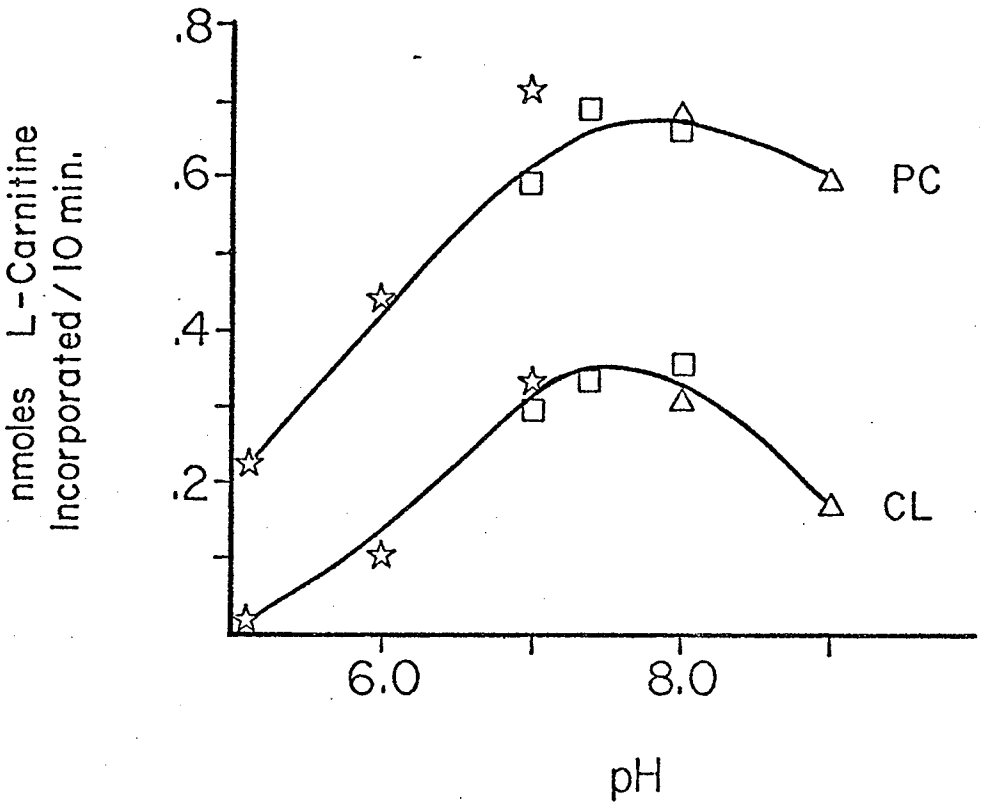


Fig. 13. pH dependence of palmitoyl-carnitine and carnitine lipid formation. The incubation was performed for 10 min. in a medium (final vol. 0.2 ml) containing: 0.25 mM L-carnitine ($^{14}CH_3$-labelled); 0.05 mM palmitoyl-CoA; 0.247 mg of mitochondrial protein and 50 mM MES-KOH buffer from pH 5.0 to 7.0 (☆——☆); 50 mM TES-KOH buffer from pH 7.0 to 8.0 (□——□); 50 mM TRICINE-HCl buffer from pH 8.0 to 9.0 (△——△).

6. Effect of freezing and thawing and Triton X-100 treatment

It was reported that mitochondria contain both non-latent and latent carnitine palmitoyl transferase activities (51). With rat kidney mitochondria, carnitine palmitoyl transferase activity increased 2x and with ox heart mitochondria the activity increased 8x (51) upon ultrasonic treatment of the preparation. Tables 5 and 6 show effects of two other treatments known to result in a disintegration of mitochondrial structure (50) (i.e. freezing and thawing and exposure to Triton X-100 (a non-ionic detergent)) on L-carnitine incorporation into palmitoyl-carnitine and carnitine lipid by SMSG mitochondrial fraction.

As shown in Table 5, at 0.05 mM palmitoyl-CoA concentration, the incorporation of L-carnitine into palmitoyl-carnitine approximately doubled in a freeze-thawed mitochondrial fraction when compared with the freshly prepared mitochondrial fraction. At 0.1 mM palmitoyl-CoA, the incorporation increased 50%, at 0.15 mM palmitoyl-CoA freezing and thawing of mitochondria had no effect on palmitoyl-carnitine formation. Possibly, palmitoyl-CoA which has strong surface active properties (83), caused partial lysis of mitochondria at higher concentrations and thus no effect of freezing and thawing on palmitoyl-carnitine formation could be observed. At all concentrations of palmitoyl-CoA, carnitine lipid formation considerably decreased in a frozen and thawed preparation in comparison to the freshly prepared

TABLE 5

EFFECT OF FREEZING AND THAWING OF THE MITOCHONDRIAL FRACTION ON L-CARNITINE INCORPORATION INTO PALMITOYL-CARNITINE AND CARNITINE LIPID (†)

Palmitoyl-CoA Concentration	n moles L-carn incorp. into PC		n moles L-carn incorp. into CL	
	Fresh Mito.	Frozen + Thawed Mito.	Fresh Mito.	Frozen + Thawed Mito.
0.05 mM	1.00	1.99	0.50	0.30
0.1 mM	2.75	4.07	0.40	0.12
0.15 mM	5.25	5.31	0.20	0.00

(†) Composition of the medium was the same as given in Fig.11, except that 0.210 mg of mitochondrial protein was used, and palmitoyl-CoA concentration was as indicated in the table. Incubations were performed for 15 min.
Mito. = Mitochondria.

TABLE 6

EFFECT OF TRITON X-100 TREATMENT ON L-CARNITINE INCORPORATION INTO PALMITOYL-CARNITINE AND CARNITINE LIPID (†)

n moles L-carn incorp. into PC		n moles L-carn incorp. into CL	
Untreated Mito.	Triton X-100 Treated Mito.	Untreated Mito.	Triton X-100 Treated Mito.
1.88	2.49	0.57	0.13

(†) Composition of the medium was the same as given in Fig. 11, except that palmitoyl-CoA concentration was 0.07 mM, and 0.205 mg mitochondrial protein was present. Mitochondrial fraction was allowed to stand for 20 min. with 0.1% Triton X-100 before use. Incubations were performed for 15 min.
Mito.=Mitochondria.

mitochondrial fraction. Also, treatment of the mitochondrial fraction with Triton X-100 resulted in a decreased L-carnitine incorporation into carnitine lipid (Table 6). Thus, it would seem that the formation of carnitine lipid is dependent on a relatively intact mitochondrial structure.

C. Identification of Carnitine Lipid

1. Thin layer chromatography

R_F values of carnitine lipid and palmitoyl-carnitine as obtained with ascending TLC on Silica Gel G plates using chloroform-methanol-water (14:6:1) as a developing solvent were found to be 0.20 and 0.30, respectively. Carnitine lipid moved more slowly than palmitoyl-carnitine in a non-polar developing solvent, suggesting that the compound is more polar than palmitoyl-carnitine. On the other hand, carnitine lipid was less polar than any other short chain carnitine ester described. It was assumed that acetylcarnitine (75), propionyl-carnitine (95) or acetoacetylcarnitine (96) would not be extracted into the chloroform phase during purification of lipids by Bligh and Dyer procedure (see Methods, Section E (3)).

2. Incorporation of radioactive precursors

Following the incorporation of fatty acid-^{14}C and carnitine-^{14}C labelled in different positions into carnitine lipid it was found that:

(1) both palmitic-1-^{14}C as well as palmitic-16-^{14}C were equally well incorporated into carnitine lipid, suggesting that the lipid contains a C-16 fatty acid or its higher homologue. When other long chain fatty acids (linoleic, linolenic, stearic, oleic) were incubated with carnitine, the resulting fatty acid esters of carnitine had the same R_F in the TLC system used (chloroform-methanol-water, 14:6:1) as palmitoyl-carnitine. Therefore, the lipid cannot represent a simple ester of carnitine and unsubstituted long chain fatty acid. (2) $^{14}CH_3$-labelled carnitine was incorporated into carnitine lipid to the same extent as ^{14}COOH-labelled carnitine (Table 7), suggesting that the lipid did not contain a partially or completely demethylated carnitine molecule. The small decrease (10%) in the incorporation of carnitine-$^{14}CH_3$ as compared to carnitine-^{14}COOH was probably due to experimental error occurring during the dilution of radioactive carnitine with non-radioactive compound as the same decrease in incorporation was also observed in palmitoyl-carnitine.

3. Mild alkaline hydrolysis

From the results presented above it seemed reasonably obvious that carnitine lipid can represent neither an ester of carnitine and short chain fatty acid, nor an ester of carnitine with unsubstituted long-chain fatty acid. Mehlman and Wolf (97) reported the presence of a "lipid bound" carnitine, phosphatidyl carnitine, in chick embryo and liver and rat embryo and liver (however, recent evidence has shown

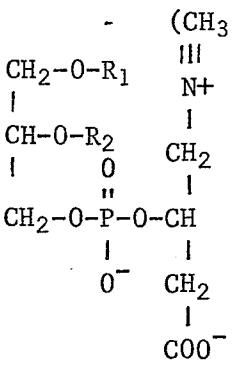
TABLE 7

COMPARISON BETWEEN CARNITINE-^{14}COOH AND CARNITINE-$^{14}CH_3$ INCORPORATION INTO CARNITINE LIPID AND PALMITOYL-CARNITINE (†)

Radioactive precursor	n moles L-carn incorp. into CL	n moles L-carn incorp. into PC
carnitine-^{14}COOH	0.30	1.99
carnitine-$^{14}CH_3$	0.27	1.71

(†) Composition of the medium was the same as given in Fig. 11, except that 0.210 mg of mitochondrial protein was used. Incubations were performed for 15 min.

that these authors did not have a phosphatidyl-carnitine - J. D. Erfle, personal communication). This lipid, judging by its number of polarizable groups - should be more polar than



palmitoyl-carnitine. Therefore, it was assumed that carnitine lipid might be a phospholipid containing carnitine and Dawson's hydrolysis procedure was used to aid in identification. Because of the lack of material and therefore the impossibility of chemical analysis of the hydrolysis products, radioactive carnitine was employed in the procedure. Mild alkaline hydrolysis of fatty acid-1-^{14}C labelled carnitine lipid and subsequent thin layer chromatography of its radioactive hydrolysis products could provide information as to the type of bond by which a fatty acid is bound in carnitine lipid (ester bonds are being selectively hydrolysed, while ether and amide bonds are not hydrolysed under the conditions of mild alkaline hydrolysis (73).

i. Mild alkaline hydrolysis of fatty acid-1-^{14}C labelled lipids

In Fig. 14 a radioautogram of a TLC plate is presented showing the position of radioactive hydrolysis products obtained after mild

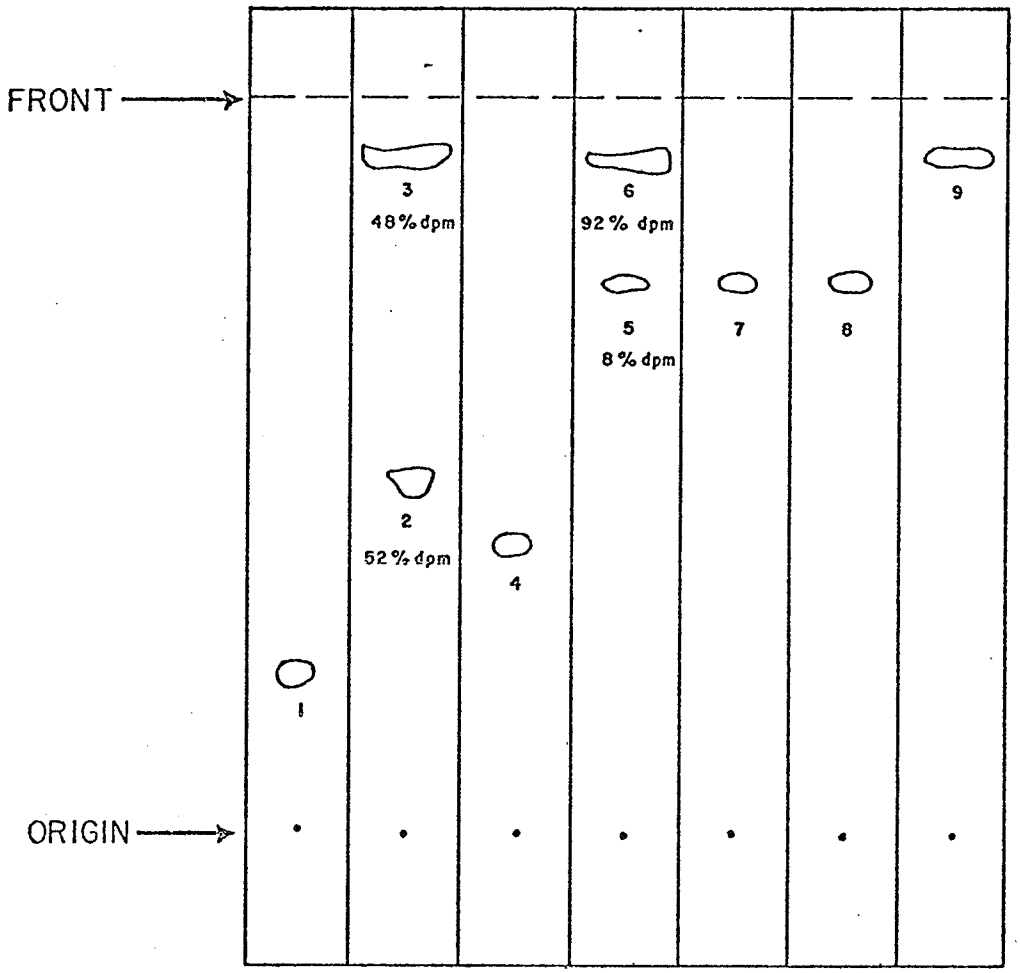


Fig. 14. Radioautogram of a TLC plate showing the radioactive products obtained by mild alkaline hydrolysis of fatty acid-1-^{14}C labelled carnitine lipid and palmitoyl-carnitine. Solvent: chloroform-methanol-water (14:6:1). 1- carnitine lipid unhydrolysed; 2,3-radioactive products of mild alkaline hydrolysis of carnitine lipid (spot 2 containing 52% and spot 3 containing 48% of total dpm recovered from TLC plate); 4- palmitoyl-carnitine unhydrolysed; 5,6- radioactive products of mild alkaline hydrolysis of palmitoyl-carnitine; 7- palmitic-1-^{14}C acid (taken through mild alkaline hydrolysis procedure); 8- standard palmitic-1-^{14}C acid; 9- methyl ester of palmitic-1-^{14}C acid.

alkaline hydrolysis of fatty acid-1-^{14}C labelled carnitine lipid and palmitoyl-carnitine. The chromatogram also shows the positions of unhydrolysed carnitine lipid, palmitic-1-^{14}C acid (that was taken through all the hydrolysis steps), a standard palmitic-1-^{14}C acid and the methylester of palmitic-1-^{14}C acid (obtained by heating palmitic-1-^{14}C acid for 4 hr. at 100^{o}C in 2 N methanolic HCl). The following conclusions can be made on the basis of these results (Fig. 14):

(a) Mild alkaline hydrolysis produced fatty acid methyl esters from both carnitine lipid and palmitoyl-carnitine (spots 3, 6). The reason why palmitic-1-^{14}C acid taken through mild alkaline hydrolysis procedure (spot 7) was not transformed to methylester while fatty acid methyl esters were released from both carnitine lipid and palmitoyl-carnitine can be explained by transesterification occurring in the presence of either of these two lipids. When esters are heated with alcohols (in the present case methanol) double decomposition takes place, the alcohol residue being more or less completely exchanged (98). This transesterification reaction is accelerated by the presence of small amounts of an acid or alkali and it occurs much more easily than direct esterification (98). The release of a fatty acid or, under the present conditions of fatty acid methyl ester, from carnitine lipid suggests that the fatty acid is bound to some moiety by an ester bond.

(b) Transesterification seemed to be preferred over hydrolysis under the present experimental conditions (the incubation

mixture contained an excess of methanol (Methods, Section G (1)), and thus palmitoyl-carnitine predominantly produced methyl palmitate (spot 6: 92% dpm) with only small amounts of free palmitic acid (spot 5: 8% dpm).

(c) Mild alkaline hydrolysis of carnitine lipid gave rise to another radioactive spot (2), containing approximately 50% of the total dpm.

When the distribution of radioactivity between the two radioactive spots obtained by mild alkaline hydrolysis of carnitine lipid (spots 2, 3 - Fig. 14) was followed as a function of hydrolysis time it was observed that the fatty acid methyl ester (spot 3) was formed first, and its concentration decreased upon prolonged incubation, whilst the radioactivity in spot 2 was increasing (Table 8). A similar, but not so marked, relationship was observed between methyl palmitate and free palmitic acid released from lecithin under the conditions of mild alkaline hydrolysis (the distribution of radioactivity between hydrolysis products of palmitoyl-carnitine as a function of time was not investigated). The level of the methylester of palmitic acid decreased while that of free palmitic acid increased upon prolonged incubation with NaOH. On the basis of these results it seems probable that spot 2 (Fig. 14) obtained by mild alkaline hydrolysis of carnitine lipid represents a free fatty acid which was released from its methyl ester (spot 3) upon prolonged hydrolysis. Assuming that spot 2 represents free fatty acid, then this compound must be more polar than

TABLE 8

DISTRIBUTION OF RADIOACTIVITY BETWEEN THE PRODUCTS OF MILD ALKALINE HYDROLYSIS OF CARNITINE LIPID AS A FUNCTION OF HYDROLYSIS TIME (†)

Time of incubation with NaOH (min.)	Per cent dpm (spot 2)	Per cent dpm (spot 3-fatty acid methyl ester)
5	14	86
10	27	73
25	53	47

(†) Hydrolysis conditions were the same as described in Methods, Section G (1), except that time of incubation with NaOH was as shown in the table.

palmitic acid as it moves more slowly in a non-polar solvent system (carnitine lipid was later identified as β-hydroxy-palmitoyl-carnitine and therefore spot 2 in Fig. 14 represents probably free β-OH-palmitic acid).

ii. Mild alkaline hydrolysis of carnitine-^{14}COOH labelled lipids

It was noted in the previous paragraph that a fatty acid was released from carnitine lipid by mild alkaline hydrolysis. Information as to the nature of the carnitine containing hydrolysis product can be obtained by hydrolysis of carnitine-^{14}COOH labelled carnitine lipid and subsequent chromatography of its radioactive hydrolysis products.

R_F values (obtained by paper chromatography) of the hydrolysis products of carnitine-^{14}COOH labelled carnitine lipid and palmitoyl-carnitine are presented in Table 9. This table also shows the R_F values of free carnitine that was taken through all the hydrolysis steps and of a standard sample of carnitine-^{14}C. From the results presented in Table 9 it is obvious that mild alkaline hydrolysis releases free carnitine from both carnitine lipid as well as palmitoyl-carnitine. As would be expected by analogy with other phospholipids (see Methods, Section G), the release of free carnitine could not occur, if carnitine lipid were a glycerophospholipid containing carnitine as the base. The results rather suggest carnitine lipid to be a simple ester.

TABLE 9

R_F VALUES OF THE RADIOACTIVE PRODUCTS OBTAINED AFTER A MILD ALKALINE HYDROLYSIS OF CARNITINE-^{14}COOH LABELLED CARNITINE LIPID AND PALMITOYL-CARNITINE

Solvent	R_F Value			
	CL	PC	Carnitine-^{14}COOH*	Standard Carnitine-^{14}COOH
propanol-ammonia-water (85:5:10)	0.105	0.102	0.101	0.100
ethanol-ammonia-water (90:5:5)	0.129	0.130	0.130	0.131
phenol-dioxane-water-KCl	0.350	0.350	0.360	0.366

(*) Carnitine-^{14}COOH taken through all the hydrolysis steps.

4. Incubations with palmitoyl-carnitine

Time studies using carnitine-^{14}C or palmitic-1-^{14}C acid (Fig. 11, 12) suggested a precursor-product relationship between palmitoyl-carnitine and carnitine lipid. To examine this problem, mitochondrial fraction was incubated with radioactive palmitoyl-carnitine for different time periods and the formation of carnitine lipid as well as other radioactive products was followed (Fig. 15). It is seen that radioactivity appeared in palmitoyl-CoA and free palmitic acid before its appearance in carnitine lipid. These results suggest that palmitoyl-carnitine must be first converted to palmitoyl-CoA, which can be subsequently utilized for carnitine lipid formation.

5. Studies with respiratory inhibitors

In order to determine whether or not carnitine lipid represents an ester of carnitine and a fatty acid obtained from incomplete oxidation of palmitate, the effect of two respiratory inhibitors, cyanide and rotenone, on the formation of carnitine lipid as well as palmitoyl-carnitine was studied (Fig. 16A, B). Cyanide is known to inhibit cytochrome oxidase (99), while rotenone specifically inhibits NADH-cytochrome b-dehydrogenase (99).

Fig. 16A shows that both respiratory inhibitors caused a decrease in the rate of palmitoyl-carnitine disappearance during prolonged incubation periods. Thus, the rate of palmitoyl-carnitine disappearance (cf. Fig. 11 and in Fig. 16A) was due, in part, to the oxidation of

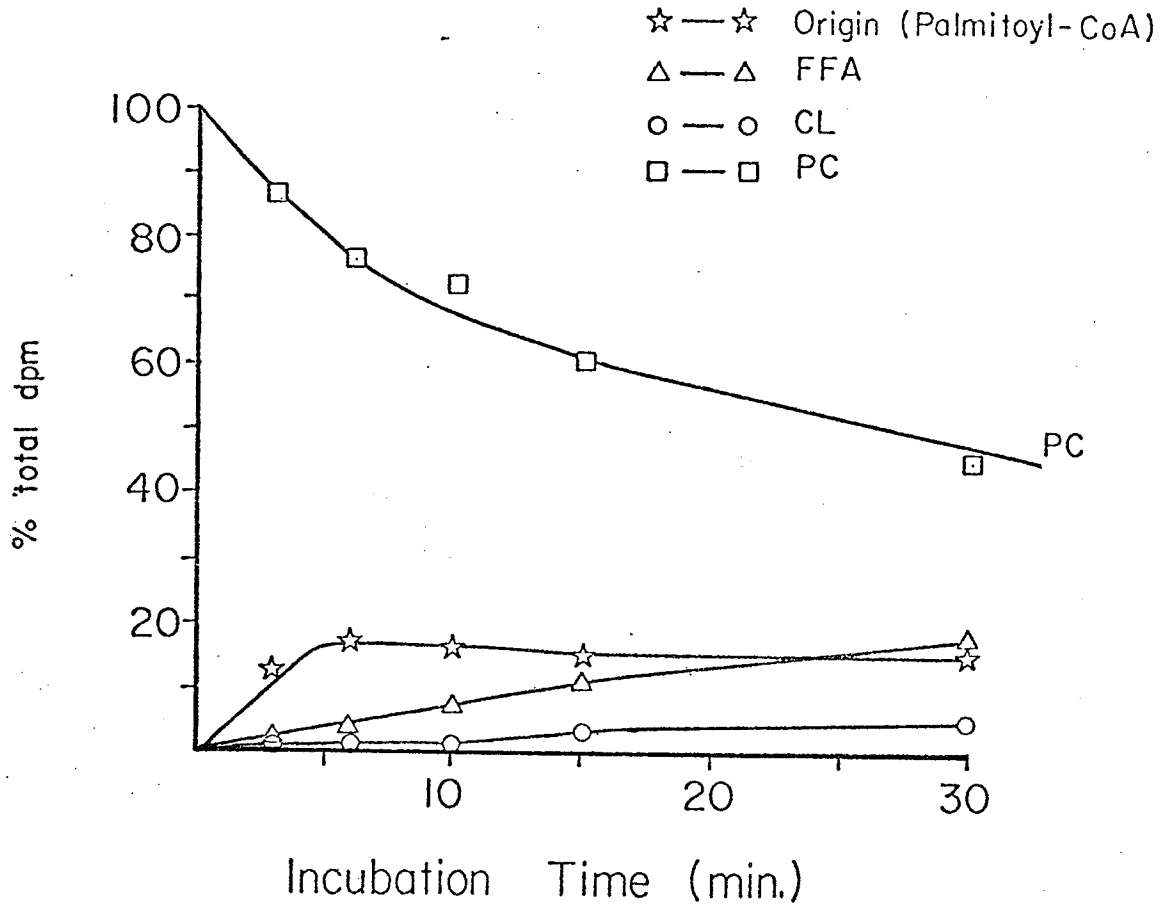


Fig. 15. Incubation of the mitochondrial fraction with fatty acid-1-^{14}C labelled palmitoyl-carnitine. The incubation was performed in a medium (final vol. 0.2 ml) containing: 50 mM TES-KOH buffer, pH 7.4; 2.3 nmoles of palmitoyl-carnitine (approximately 51700 dpm); 0.285 mg mitochondrial protein (preparation of mitochondrial fraction see Methods, Section A (2)). Results are expressed as per cent of total dpm recovered from TLC plate.

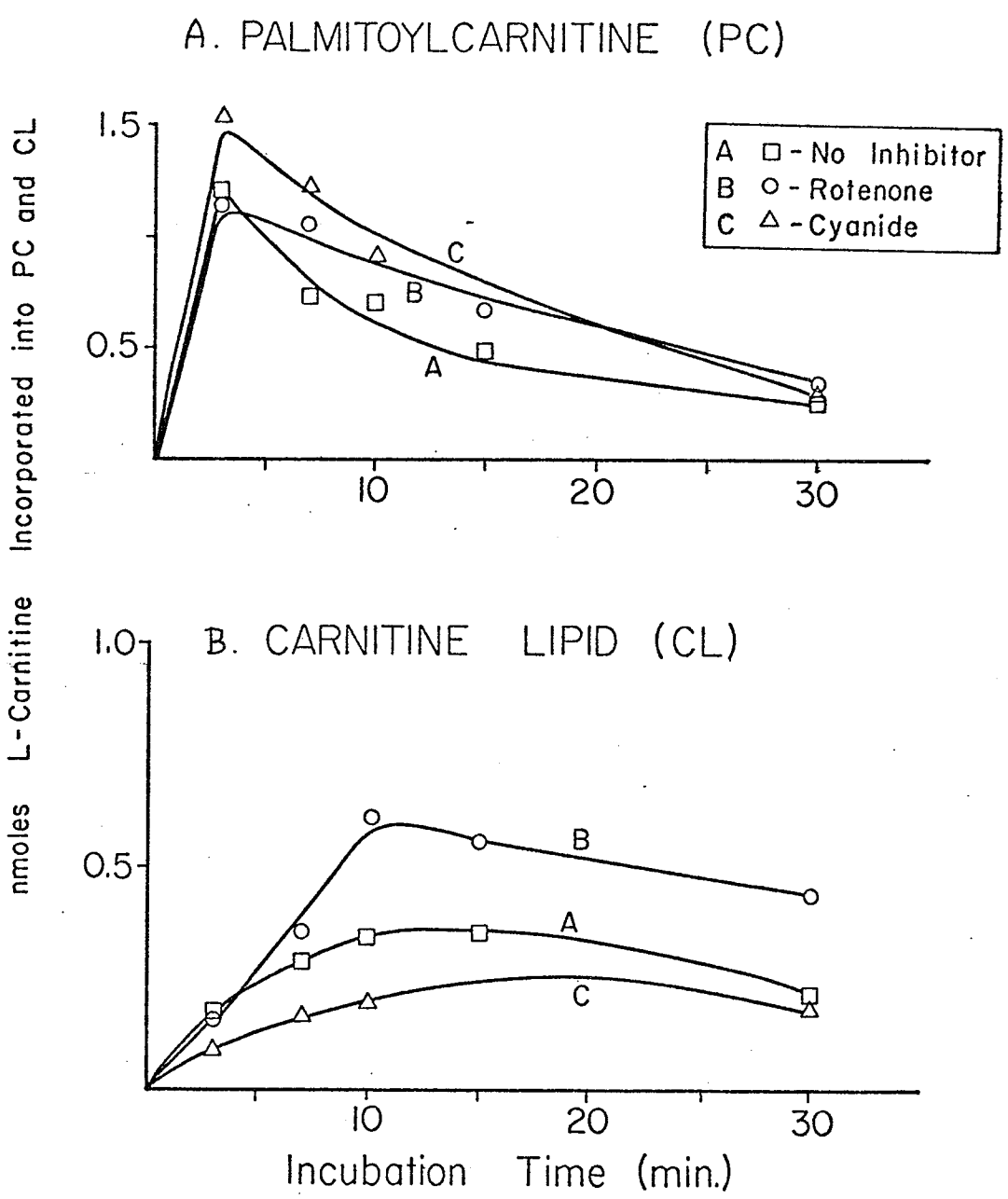


Fig. 16. Effect of cyanide and rotenone on palmitoyl-carnitine and carnitine lipid formation. Composition of the incubation mixture was the same as shown in a legend to Fig. 11, except that 0.193 mg of mitochondrial protein was present. The concentration of NaCN, when present, was 1mM and that of rotenone 0.04 mM. Rotenone was added in ethanolic solution (final concentration of ethanol in the incubation mixture was 1%).

palmitoyl-carnitine. However, oxidation alone could not have been the main reason for the disappearance of palmitoyl-carnitine as the rapid decline in its concentration upon prolonged incubation was observed even in the presence of cyanide or rotenone (Fig. 16A, line B, C). A decline in accumulated palmitoyl-carnitine after prolonged incubations has been also noted in calf liver mitochondria by Norum (42), who suggested that this effect was caused by the rapid deacylation of palmitoyl-CoA by acyl-CoA hydrolase (this enzyme catalyzes the hydrolysis of fatty acyl-CoA esters to free fatty acid and CoA). As palmitoyl-CoA was found to be readily hydrolysed by all subcellular fractions prepared from rat SMSG (66), the same explanation may be applicable here. Therefore, the reversible reaction catalyzed by carnitine palmitoyl transferase (palmitoyl-CoA + carnitine $\rightleftharpoons$ palmitoyl-carnitine + CoA) never attains equilibrium due to the interference by acyl-CoA hydrolase (83), which would shift the reaction back to the left, resulting in a decrease in palmitoyl-carnitine. This suggestion is also supported by results in Fig. 15, where palmitoyl-CoA and subsequently free palmitic acid were released during prolonged incubation of mitochondrial fraction with palmitoyl-carnitine.

Fig. 16B shows that carnitine lipid formation was inhibited by cyanide but stimulated by rotenone. As was mentioned above, rotenone is a specific inhibitor of respiratory chain NADH-cytochrome b-dehydrogenase. In the β-oxidation of palmitate the first substance from which hydrogen atoms are transferred to NAD (which, in turn is

oxidized by NADH-dehydrogenase of the respiratory chain (100)) is β-hydroxy-palmitoyl-CoA. Rotenone, by inhibiting the reoxidation of NADH must have secondarily caused an increased accumulation of β-hydroxy-palmitoyl-CoA. On the basis of the results reported in Fig. 16, coupled with observations reported earlier in this section, it is suggested that carnitine lipid is β-hydroxy-palmitoyl-carnitine, formed enzymatically from β-hydroxy-palmitoyl-CoA and carnitine. Cyanide, by inhibiting cytochrome oxidase, inhibited the reoxidation in the respiratory chain of a reduced form of fatty acyl-CoA dehydrogenase (which converts fatty acyl-CoA to trans, α, β, unsaturated acyl-CoA) and thus decreased the oxidation of palmitoyl-CoA to β-hydroxy-palmitoyl-CoA.

6. Effect of NAD

Table 10 shows the effect of NAD on carnitine lipid and palmitoyl-carnitine formation in mitochondrial preparations of submandibular glands. The presence of NAD in the incubation medium resulted in a 10-fold decrease of carnitine lipid formation, and at the same time palmitoyl-carnitine formation decreased approximately 40% compared to the controls without NAD. As noted above, NAD acts as hydrogen acceptor in a dehydrogenation reaction converting β-hydroxy-palmitoyl-CoA to β-keto-palmitoyl-CoA. These results, therefore, (Table 10) support the suggestion that carnitine lipid is β-hydroxy-palmitoyl-carnitine.

TABLE 10

EFFECT OF NAD ON CARNITINE LIPID AND PALMITOYL-CARNITINE FORMATION (†)

Additions to medium	n moles L-carnitine incorp. into CL	n moles L-carnitine incorp. into PC
	0.371	1.049
0.4 mM NAD	0.038	0.624
1.0 mM NAD	0.032	0.614

(†) Composition of the medium was the same as given in Fig. 11, except that 0.250 mg of mitochondrial protein was present, and NAD, when present, was added in a concentration shown in the table. Incubations were performed for 10 min.

7. Thin layer chromatography of carnitine lipid and β-hydroxy-palmitoyl-carnitine

Fig. 17 shows that radioactive carnitine lipid had the same R_F value as a standard sample of β-hydroxy-palmitoyl-carnitine when run on Silica Gel G coated plates in three different solvent systems. Non-radioactive standard sample of β-hydroxy-palmitoyl-carnitine was visualized by exposure to iodine vapours. Radioactive carnitine lipid did not yield any yellow spot after exposure to iodine vapours, as the quantity of material applied was too small and therefore it was visualized by autoradiography. When the radioactive compound and non-radioactive standard sample were mixed, and applied together on TLC plate, then, after development in each of the three solvents, the radioactivity was always associated with the iodine spot (spot 3, Fig. 17).

8. Gas-liquid chromatography

Fig. 18 shows the position of peaks obtained on gas-liquid chromatography of fatty acid esters prepared (Methods, Section I) from a standard sample of β-hydroxy-palmitoyl-carnitine as well as from carnitine lipid which was obtained enzymatically as described in Methods, Section F. Samples of other fatty acid esters are also shown in Fig. 18. The chromatographic patterns of fatty acid ester prepared from carnitine lipid and β-hydroxy-palmitoyl-carnitine are identical and this, together with results reported in the previous

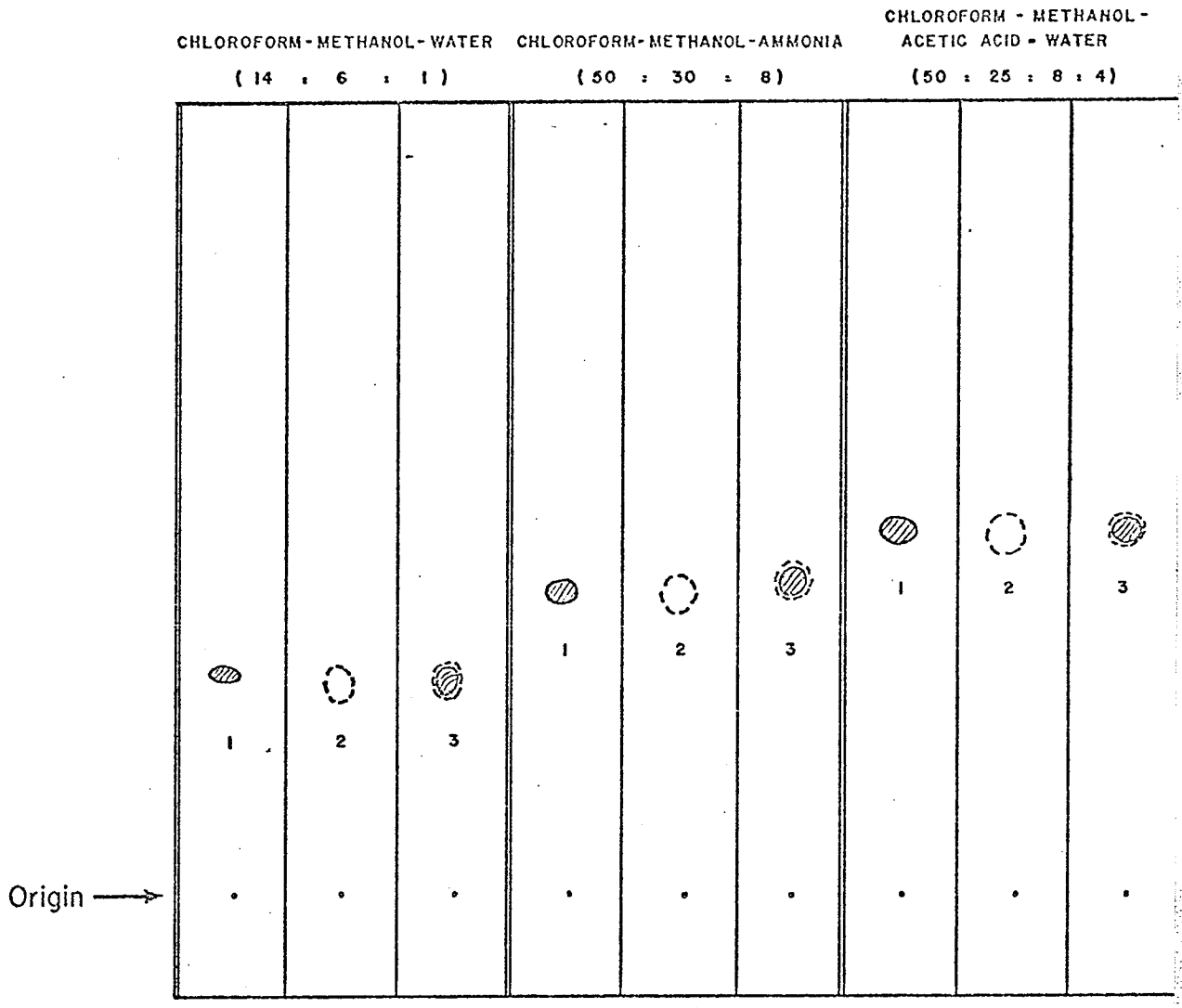


Fig. 17. Thin layer chromatograms of carnitine lipid-^{14}C and standard sample of β-hydroxy-palmitoyl-carnitine. 1- radioactive carnitine lipid; 2- standard sample of β-hydroxy-palmitoyl-carnitine; 3- radioactive carnitine lipid + standard sample of β-hydroxy-palmitoyl-carnitine.

radioactive spots

iodine positive spots

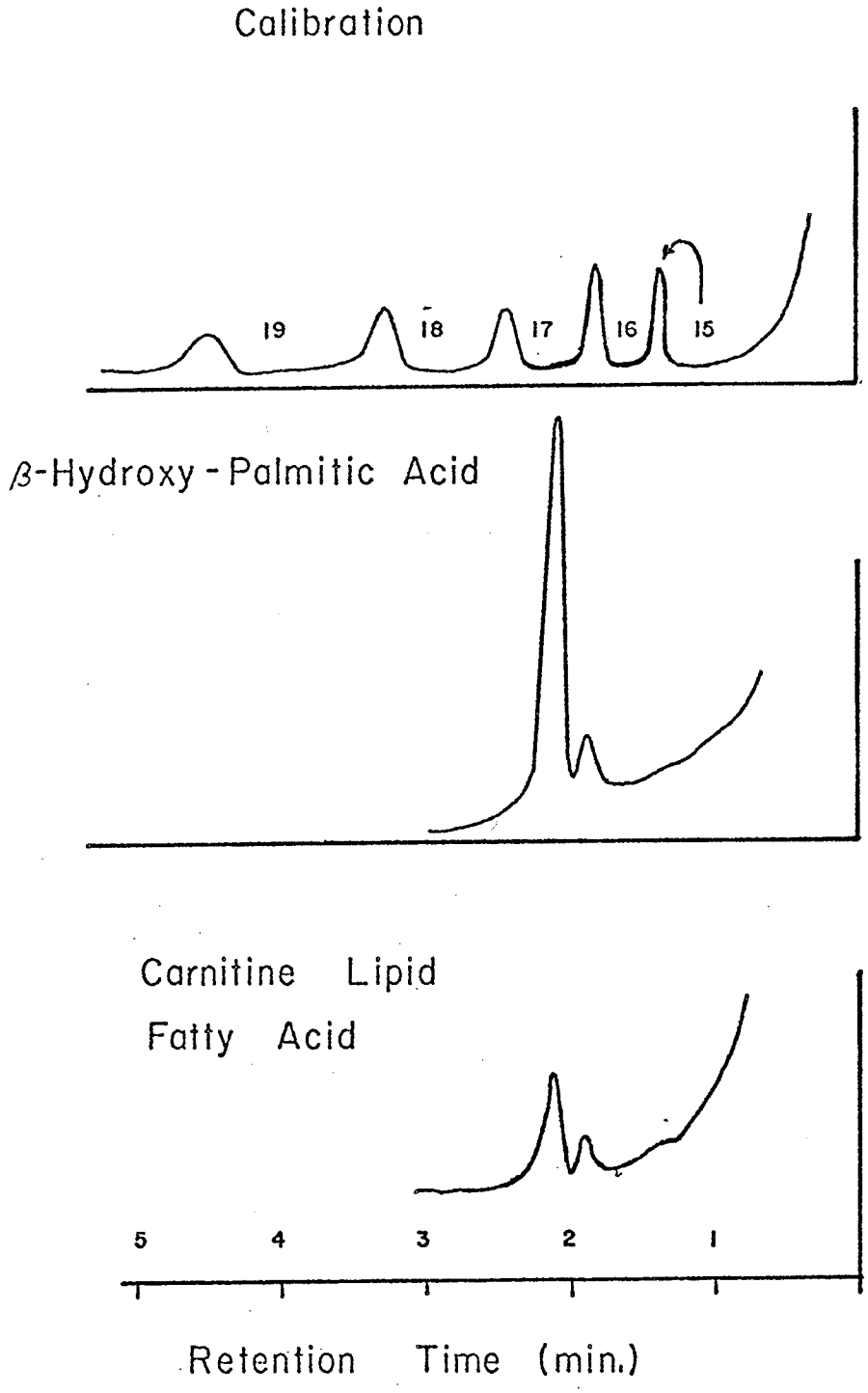


Fig. 18. Gas chromatograms of fatty acid methyl esters from carnitine lipid and β-hydroxy-palmitoyl-carnitine. Calibration with standard fatty acid methyl esters (15 to 19 carbon atoms) is also shown.

paragraph, proves that the compound referred to until now as carnitine lipid is β-hydroxy-palmitoyl-carnitine.

D. Fatty Acid Oxidation in Coupled Mitochondria Isolated from Rat SMSG

In order to study the ability of SMSG mitochondria to oxidize fatty acids and the effect of carnitine thereon, it was necessary to prepare intact mitochondria. As stated in Methods (Section A (2)), a mitochondrial fraction prepared by simple mechanical disintegration of submandibular gland tissue (preparation see Methods, Section A (2)) failed to exhibit a respiratory control ratio, which is considered to be one of the most sensitive criteria for mitochondrial intactness. Therefore, a method combining digestion of the tissue with bacterial proteinase and gentle mechanical homogenization (53) was used (Methods, Section A (3)). The properties of SMSG mitochondria prepared by this procedure are reported below.

1. Respiratory and phosphorylative activities of isolated mitochondria

The ability of rat SMSG mitochondria to oxidize various substrates is shown in Table 11. Succinate was oxidized at the highest rate. O_2-uptake by NAD-linked substrates was 2 to 3 times higher than reported by Feinstein and Schramm (5) for rat parotid gland mitochondria and the present preparation also exhibited higher respiratory control ratios. β-hydroxybutyrate, however, was oxidized at only half the

TABLE 11

OXIDATION OF SUBSTRATES BY SUBMANDIBULAR GLAND MITOCHONDRIA (†)

Substrate	Oxidation Rate (μmoles O_2/min./g protein) State 3	State 4	Respiratory Control Ratio	ADP/O	No. of Experiments
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-hydroxy-butyrate	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-keto-glutarate	61.9	12.4	5.0	2.3	1

(†) 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 (see Fig. 5). The basic medium (pH 7.2) was composed of 0.23 M mannitol, 0.07 M sucrose, 0.02 M Tris-HCl, 5 mM K 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 brackets indicate the range.

rate of other NAD-linked substrates. Balad et al. (3) 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 this material might be the endogenous substrate intimately associated with secretory processes. In more recent studies, however, with isolated parotid gland mitochondria (5) it was demonstrated that β-hydroxybutyrate was oxidized more slowly than other NAD-linked substrates. This suggested that the previous observation (i.e., specific stimulation of secretion in slice preparation) might have been due to preferential penetration of this material into the cells. The fact, that rat SMSG mitochondria oxidized β-hydroxybutyrate at about half the rate obtained with other NAD-linked substrates supports the view that this material is not preferentially favoured by salivary gland mitochondria.

As indicated in Table 12, the use of 0.6% bovine serum albumin in the polarographic medium resulted in higher State 3 respiration and higher respiratory control and ADP/0 ratios. Similar results have been reported for brain (101), pancreas (102) and liver mitochondria (103).

Since rat parotid gland mitochondria have been reported to lose NAD during their isolation (5), 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/0 ratios when pyruvate-malate, glutamate, α-keto-glutarate or β-hydroxybutyrate were employed as substrates.

TABLE 12

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

Substrate	Bovine Serum Albumin (0 or +)	Rate of Oxidation (μmoles O_2/min./g protein)		Respiratory Control Ratio	ADP/O
		State 3	State 4		
5 mM pyruvate +	0	58.9	14.9	3.9	2.4
1 mM L-malate	+	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

(†) Conditions were the same as those noted in Table 11. With succinate 0.645 mg mitochondrial protein was used, with the others, 0.860. Bovine serum albumin concentration, when added, was 0.6%.

2. Electron microscopy

Figures 19 and 20 are electron micrographs of isolated submandibular gland mitochondria. The mitochondrial pellet was processed immediately after isolation, hence they are not in any physiologically uniform state as is evident from the presence of diverse structural forms.

3. Palmitoyl-CoA oxidation

The ability of submandibular gland mitochondria to oxidize long chain fatty acids was measured with palmitoyl-CoA as substrate. De Jong (104) has shown that Nagarse treatment almost completely destroys both rat heart and liver fatty acyl-CoA synthetase, and work in our laboratory has demonstrated that this is also true for rat submandibular gland mitochondria. The active form of palmitic acid, i.e., palmitoyl-CoA, had to be employed as substrate.

i. Effect of carnitine

It can be seen from Tables 13, 14 and 15 that under optimum conditions palmitoyl-CoA oxidation was dependent on the addition of L-carnitine. If carnitine was added to a medium free of palmitoyl-CoA no increase in O_2 utilization was observed (unreported studies). Also tracer experiments (8, 66) have shown that palmitoyl-carnitine is not formed from palmitoyl-CoA by submandibular mitochondria in the absence

Fig. 19. Electron micrograph of isolated SMSG mitochondria.x 6550.

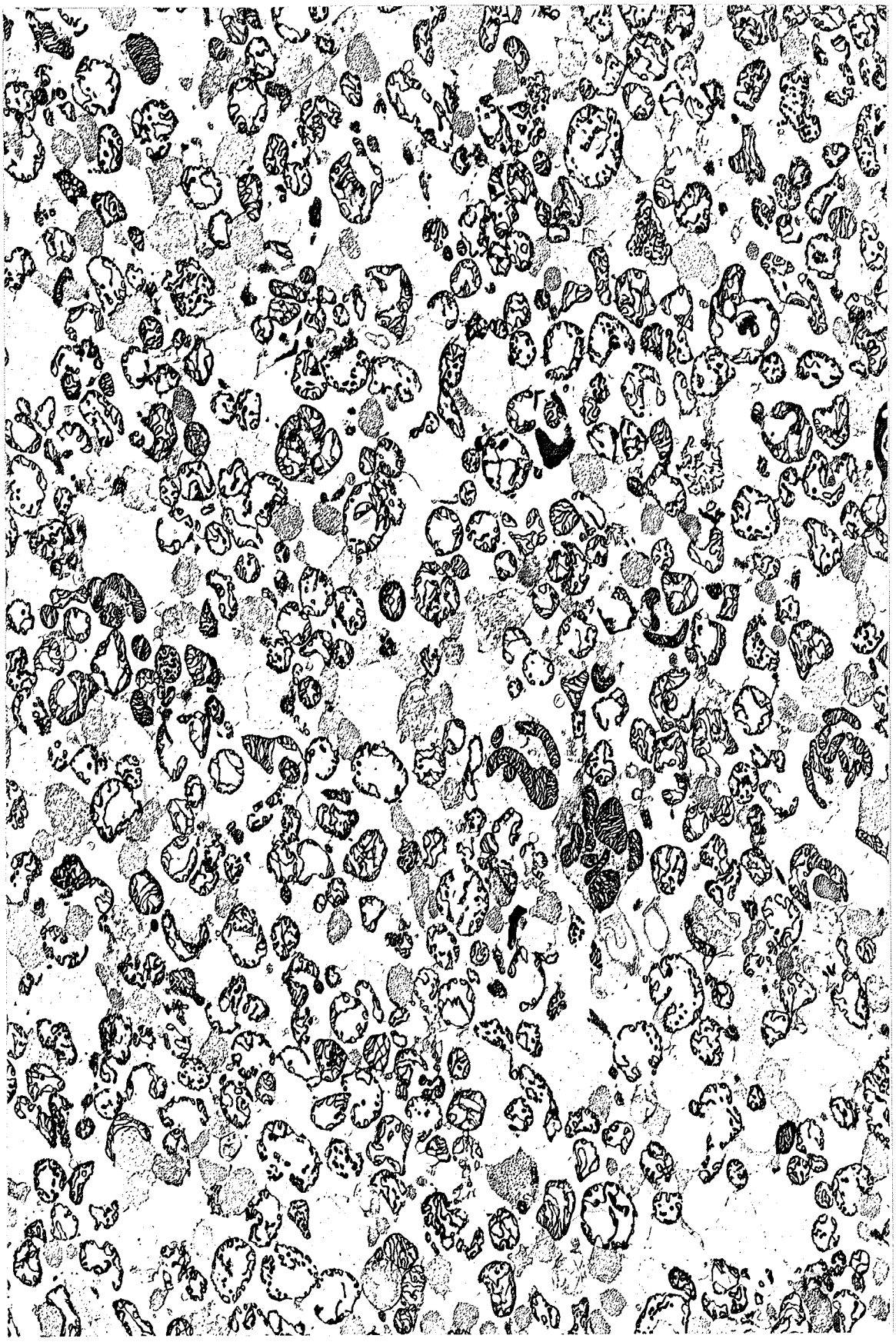
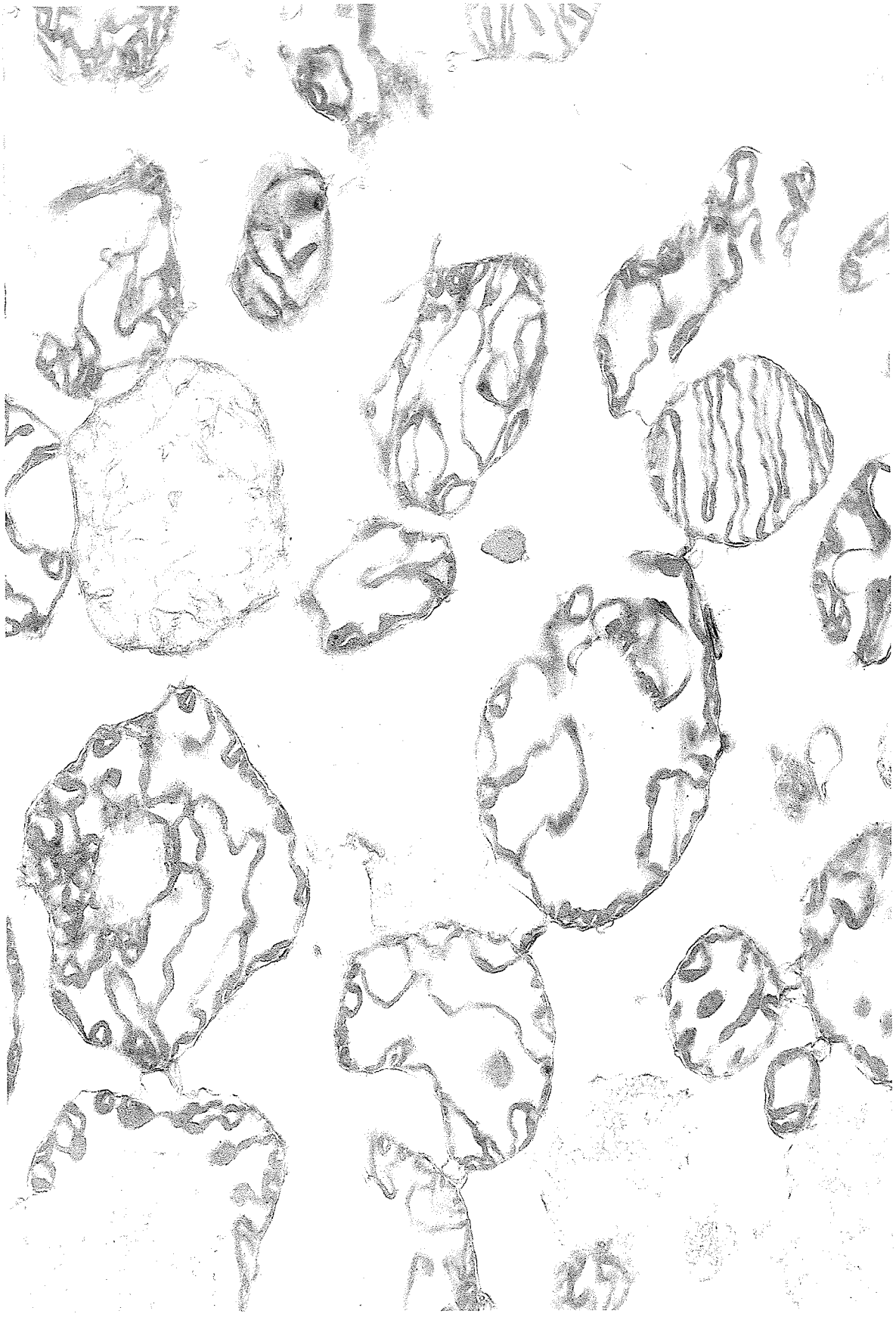


Fig. 20. Electron micrograph of isolated SMSG mitochondria.x 39900.



of L-carnitine, nor from L-carnitine in the absence of palmitoyl-CoA (Results, Section B).

ii. Effect of bovine serum albumin

When palmitoyl-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 13). If 0.6% bovine serum albumin was added to the medium (as used by Koerker and Fritz (103)), a stimulation of palmitoyl-CoA oxidation by carnitine was observed, but the resulting rate of O_2 utilization was still rather low (Table 13). The importance of proper bovine serum albumin concentration for optimum oxidation is evident from the results described in Table 13. A level of 0.15 to 0.20% appears to be best for carnitine-mediated palmitoyl-CoA oxidation. The albumin effect may be a result of its protection of mitochondrial integrity from surface active compounds such as palmitoyl-CoA and palmitoyl-carnitine (11, 9). The inhibition of oxidation by high bovine serum albumin concentrations could be caused by its binding palmitoyl-CoA, thereby decreasing the availability of oxidizable substrate (39). Goodman (105) 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.

TABLE 13

DEPENDENCE OF PALMITOYL-CoA OXIDATION ON BOVINE SERUM ALBUMIN (†)

Bovine Serum Albumin (w/v %)	Oxidation Rate (μmoles O_2/min./g protein)				
	Additions to Medium				
	1. None	2. Palmitoyl-CoA	Difference (2 - 1)	3. L-carnitine	Difference (3 - 1)
0.00	3.6	8.7	5.1	8.9	5.3
0.05	3.8	6.5	2.7	8.1	4.3
0.10	6.0	13.6	7.6	22.2	16.2
0.15	7.2	9.1	1.9	29.0	21.8
0.20	7.5	8.3	0.8	25.3	17.8
0.60	12.2	13.4	1.2	19.5	7.3

(†) Besides the basic medium (Table 11) the vessels contained 1 mM ADP, 0.015 mM L-malate and 0.765 mg mitochondrial protein. The numbers indicate sequence of addition of palmitoyl-CoA (0.05 mM) and L-carnitine (0.3 mM).

iii. Effect of ADP

The importance of added ADP to the carnitine stimulated oxidation is shown by the data of Table 14. Palmitoyl-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 addition of ADP to this system caused a very rapid increase in O_2 utilization (18.2 μmoles/min.). When ADP was present in the medium from the start, the addition of carnitine caused an 11 fold increase in palmitoyl-CoA oxidation (1.9 to 21.8 μmoles/min.).

iv. Effect of malate

It was found that for optimum oxidation of palmitoyl-CoA in the presence of carnitine, a low concentration of L-malate was needed (Table 15). In the absence of malate, carnitine stimulated palmitoyl-CoA oxidation but this oxidation rate (12.7 μmoles/min.) was about half of that given in the presence of malate (24.1 μmoles/min.). The addition of malate to a 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 15). This latter observation suggested that a metabolite accumulates during palmitoyl-CoA oxidation in the absence of L-malate, then, after malate supplementation, this material becomes readily available for oxidation.

TABLE 14

EFFECT OF ADP ON PALMITOYL-CoA OXIDATION (†)

	Oxidation Rate (μmoles O_2/min./g protein)							
	Additions to Medium							
	1. None	2. Palmitoyl-CoA	Difference (2 - 1)	3. L-Carnitine	Difference (3 - 1)	4. ADP	Difference (4 - 1)	
Absence ADP	2.8	5.0	2.2	5.0	2.2	21.0	18.2	
Presence ADP	7.2	9.1	1.9	29.0	21.8	--	--	

(†) Besides the basic medium (Table 11) 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 palmitoyl-CoA (0.05 mM), L-carnitine (0.3 mM) and ADP.

TABLE 15

EFFECT OF L-MALATE ON PALMITOYL-CoA OXIDATION (†)

	Oxidation Rate (μmoles O_2/min./g protein)								
	Additions to Medium								
	1	2		3		4		5	
	None	Palmitoyl-CoA	Difference (2 - 1)	L-carnitine	Difference (3 - 1)	L-malate	Difference (4 - 1)	4 min. After Malate Addition	Difference (5 - 1)
No malate	5.6	8.2	2.6	18.3	12.7	41.4	35.8	28.7	23.1
+ Malate	5.3	9.4	4.1	29.4	24.1	--	--	--	--

(†) Incubation vessels contained the basic medium (Table 11) 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 palmitoyl-CoA (0.05 mM), L-carnitine (0.3 mM), malate.

The experimental results depicted in Figs. 21 and 22 illustrate the correlation between palmitoyl-carnitine formation and the rate of O_2 uptake. When L-malate was present in the medium prior to the addition of palmitoyl-CoA and carnitine, the level of palmitoyl-carnitine increased rapidly after the addition of carnitine and then gradually decreased (Fig. 21). In the absence of L-malate, the amount of palmitoyl-carnitine formed was greater, and when L-malate was added, the level decreased rapidly (Fig. 22). These results suggest that the metabolite responsible for the increased O_2 uptake after L-malate addition (cf. Table 15) is palmitoyl-carnitine and that the rate of palmitoyl-CoA oxidation in the presence of carnitine is dependent upon the effective concentration of palmitoyl-carnitine, as has been suggested for other tissues (83). One possible explanation of the effect of malate on palmitoyl-CoA oxidation is the increased availability of oxaloacetate for citrate formation after the oxidation of added malate by malate dehydrogenase. Williamson et al (106) 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.They provided experimental evidence (107) that addition of malate to liver mitochondria oxidizing palmitoyl-carnitine resulted in a switch of metabolism from acetoacetate to citrate formation. Thus the presence of malate favours complete palmitoyl-carnitine oxidation to CO_2 through the tricarboxylic acid cycle.

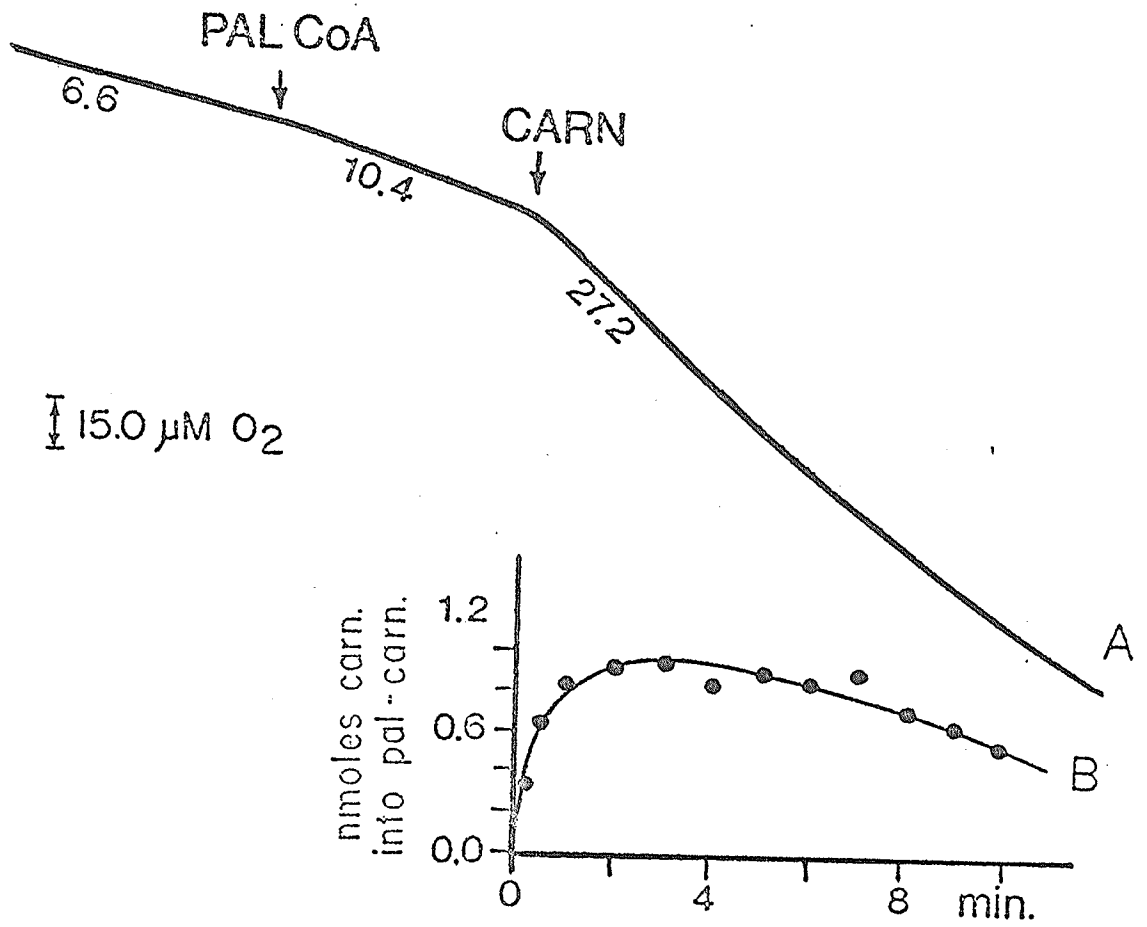


Fig. 21. Palmitoyl-CoA oxidation and palmitoyl-carnitine formation by SMSG mitochondria in the presence of L-malate. A. Polarographic trace showing rates of O_2 consumption. B. Palmitoyl-carnitine levels after adding carnitine (^{14}C-methyl) to the medium . No incorporation of labelled carnitine occurred in the absence of palmitoyl-CoA. Composition of medium and additions (indicated by arrows) were as described in legend to Table 15 (malate present). Mitochondrial protein content was 0.880 mg. Abbrev.: pal-CoA - palmitoyl-CoA; pal-carn - palmitoyl-carnitine; carn - L-carnitine.

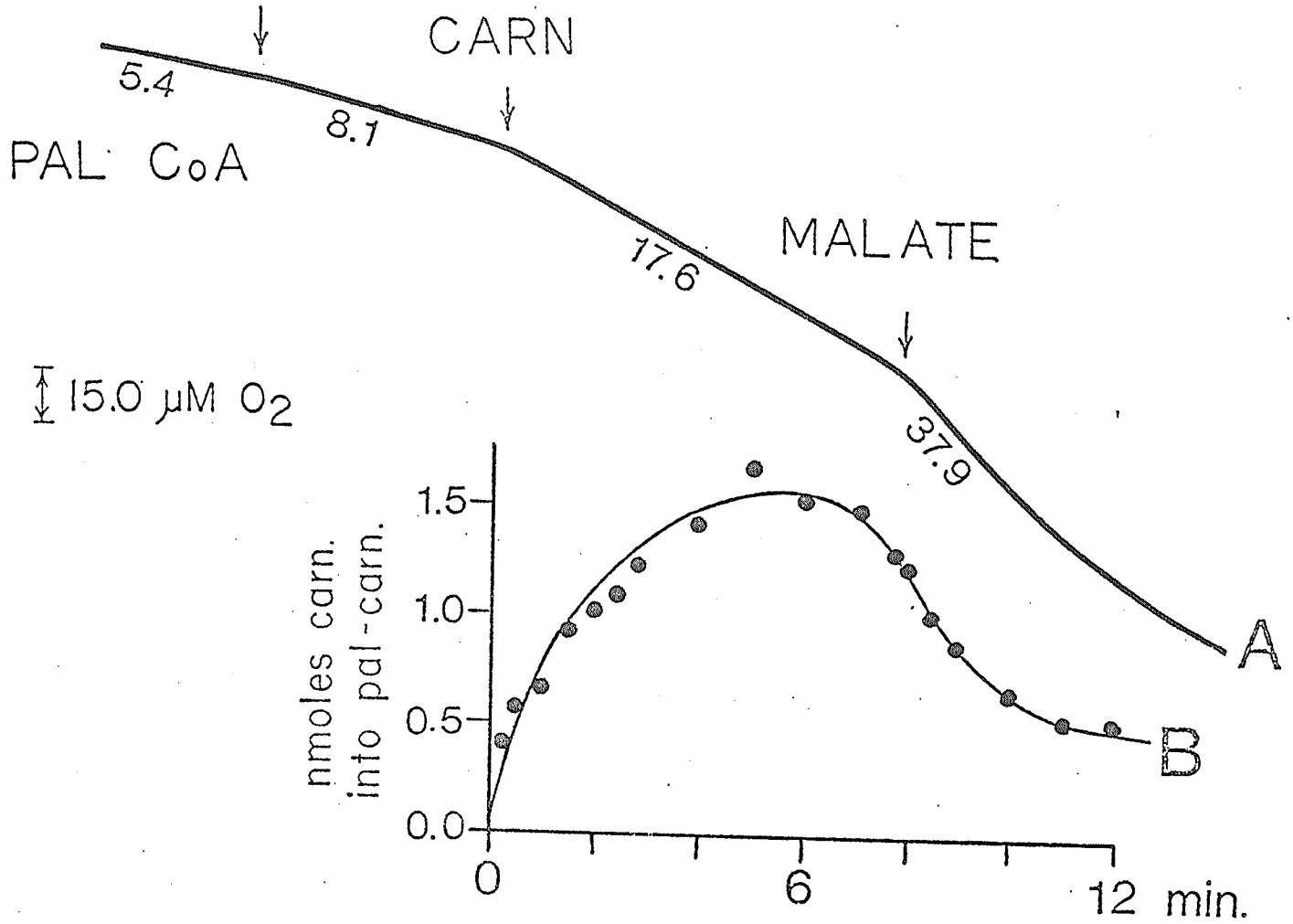


Fig. 22. Effect of L-malate on palmitoyl-CoA oxidation and palmitoyl-carnitine formation. All conditions as noted in Fig. 21, except 0.015 mM L-malate was added as shown instead of being present from the start of incubation.

GENERAL DISCUSSION AND CONCLUSIONS

As most of the results presented in individual figures and tables have been discussed in the previous chapter, only the most important observations presented in this thesis and their physiological significance will be dealt with here. In particular, the subcellular localization of carnitine palmitoyl transferase, the significance of β-hydroxy-palmitoyl-carnitine formation and the preparation of coupled mitochondria and their ability to oxidize fatty acids will be discussed.

A. Subcellular Localization of Carnitine Palmitoyl Transferase

Results in Fig. 8 provide evidence that apart from mitochondrial carnitine palmitoyl transferase, rat SMSG contain highly active extra-mitochondrial carnitine palmitoyl transferase. All subcellular fractions isolated from rat SMSG had an ability to form palmitoyl-carnitine, particularly high formation being associated with microsomal fractions. Carnitine palmitoyl transferase has been reported to be present in both mitochondrial and microsomal fractions prepared from rat liver by Bremer (10) and van Tol and Hülsmann (46). Norum (12) found the enzyme in mitochondria, microsomes as well as the particle-free supernatant of several human organs. However, all these authors found the activity of the enzyme to be highest in mitochondria. One of the possible reasons for relatively low palmitoyl-carnitine formation in SMSG mitochondrial fraction (Fig. 8) was partial oxidation of activated palmitic acid to β-hydroxy-palmitoyl-CoA and subsequent formation of β-hydroxy-palmitoyl-carnitine (cf. Discussion, Section B).

The function of nitochondrial carnitine palmitoyl transferase has been discussed in detail in the Literature Review and will be also dealt with in the Discussion,-Section C. The physiological function of extramitochondrial carnitine palmitoyl transferase is unclear. Rat liver microsomes contain an enzyme which is capable of forming lecithin by condensation of lysolecithin and acyl-CoA (108). Several workers investigated a possibility of carnitine (or acylcarnitine) being involved in the acylation reaction. Bressler and Friedberg (109) reported that addition of carnitine to guinea pig heart homogenate resulted in increased palmitate-^{14}C incorporation into lecithin. On the basis of their results they suggested that carnitine may facilitate the translocation of acyl-CoA between lecithin and lysolecithin. However, McLeod and Bressler (110) reported that addition of carnitine to a red blood cell membrane preparation resulted in increased incorporation of palmitate-^{14}C into palmitoyl-carnitine while decreasing incorporation of palmitate-^{14}C into lecithin, an identical result to the observations with SMSG (Fig. 7). Therefore suggestion that carnitine may facilitate direct translocation of acyl-CoA between lysophospholipids and phospholipids, does not seem to be generally applicable.

Fritz and Hsu (111) reported that palmitoyl-carnitine as well as free carnitine, stimulated fatty acid synthesis in a rat liver supernatant fraction. The stimulation of fatty acid synthesis by long chain acylcarnitine and by free carnitine appeared to be at the level of malonyl-CoA formation because these compounds increased acetate

incorporation into fatty acids, but had only minimal effects on the incorporation of malonyl-CoA (111). The acetyl-CoA carboxylase enzyme was found to have an increased activity in the presence of carnitine or palmitoyl-carnitine, and the inhibition of this enzyme by palmitoyl-CoA could be reversed by carnitine (111). It appeared, therefore, that carnitine increased the activity of acetyl-CoA carboxylase by removing its inhibitor, palmitoyl-CoA, via palmitoyl-carnitine formation, which itself acts as an activator of acetyl-CoA carboxylase. Palmitoyl-CoA inhibits a variety of key enzymes involved in the regulation of lipogenesis and glycolysis (112) and, as suggested by Marquis et al (112), it is conceivable that the levels of carnitine and carnitine palmitoyl transferase may play important regulatory roles by altering the levels of acyl-CoA esters in various physiological states.

B. β-hydroxy-palmitoyl-carnitine Formation in Mitochondrial Fraction

It has been shown that SMSG mitochondrial preparations synthesize β-hydroxy-palmitoyl-carnitine from palmitoyl-CoA and carnitine. This means that palmitoyl-CoA (or more precisely palmitoyl-carnitine after reconversion to palmitoyl-CoA and carnitine) must have undergone partial oxidation to β-hydroxy-palmitoyl-CoA, which in turn reacted with carnitine to yield β-hydroxy-palmitoyl-carnitine. One reason for β-hydroxy-palmitoyl-CoA accumulation was probably because of an insufficiency in endogenous NAD in the mitochondrial fraction prepared by simple mechanical homogenization in a medium either lacking EDTA

(Methods, Section A (1)) or containing a low EDTA concentration (Methods, Section A (2)). Although the NAD concentration in these mitochondrial fractions was not measured, the addition of exogenous NAD (Table 10) resulted in a decreased β-hydroxy-palmitoyl-carnitine formation probably due to the further oxidation of β-hydroxy-palmitoyl-CoA. While the present studies were in progress, Feinstein and Schramm (5) reported that rat parotid glands contained a high concentration of Ca^{++} (10 times higher than liver) which, unless complexed with EDTA or EGTA (1 mM), caused extensive damage to the mitochondria, including the depletion of NAD. Concentrations of Ca^{++} comparable to those measured in parotid gland were also found in submaxillary gland by Driesbach (113) and by Kraintz (114).

Formation of β-hydroxy-palmitoyl-carnitine from palmitoyl-CoA and carnitine was decreased by treatment of the mitochondrial fraction with a detergent (Triton X-100, Table 6) or freezing and thawing of the preparation (Table 5). Possibly these treatments prevented oxidation of palmitoyl-CoA by damaging electron transport chain assembly (115) or enzymes associated with β-oxidation.

The finding that the SMSG mitochondrial fraction can form β-hydroxy-palmitoyl-carnitine from palmitoyl-CoA and carnitine demonstrates: 1) the presence of a system responsible for β-hydroxy-palmitoyl-carnitine formation; 2) the accumulation of an intermediate of palmitate oxidation, β-hydroxy-palmitoyl-CoA, under certain conditions.

Hollis and Blecher (43) reported that the oxidation of not only

palmitate but also of β-oxidation derivatives of palmitate by liver and heart muscle mitochondria was stimulated by carnitine. These results were later confirmed by Mahadevan et al (44). The latter workers also demonstrated that rat liver mitochondria readily oxidized carnitine derivatives of β-keto-palmitate, β-hydroxy-palmitate and α, β-unsaturated derivatives of palmitate. On the basis of these findings, coupled with the present observation of β-hydroxy-palmitoyl-carnitine formation, it is possible to suggest that carnitine functions as a carrier across the mitochondrial membrane of not only saturated fatty acids but also of the β-oxidation derivatives of fatty acids, with appropriate acylcarnitines as intermediates in this process. However, as mentioned in the Literature Review, it is not possible to say at this time, whether there exists only one long chain acylcarnitine transferase having a broad substrate specificity, or whether there are specific transferases for each β-oxidation derivative of a long chain fatty acid.

The answer as to the physiological function of carnitine stimulated translocation of intermediates of long chain fatty acid oxidation can be only speculative. A clue to a possible function, has been provided by the observations of Nugteren (116). He described a rat liver microsomal fatty acid elongation mechanism involving malonyl-CoA and NADPH, in which non-protein bound β-keto-acyl-CoA, β-hydroxy-acyl-CoA and α, β-trans unsaturated acyl-CoA occurred as intermediates. Hollis and Blecher (43) postulated, therefore, that, whenever required, these

intermediates produced by either oxidative or reductive processes can be transported across the mitochondrial or microsomal membrane by a carnitine mediated mechanism, for oxidative or reductive purposes. At the same time, this postulation provides another possible physiological role for extramitochondrial carnitine palmitoyl transferase.

In the oxidation of palmitoyl-CoA to acetyl-CoA, 27 possible acyl-CoA compounds are involved (117). The failure to detect these intermediates led to a suggestion that they do not exist, but rather that the intermediates of β-oxidation of fatty acids are bound to thiol groups of the enzyme proteins (118). However, the results of Mahadevan et al (44), i.e., the rapid oxidation of β-substituted palmitoyl-carnitine derivatives by rat liver mitochondria, suggest the direct integration of CoA esters of these intermediates (produced from their carnitine derivatives) into the β-oxidation scheme. Furthermore, Davidoff and Korn (119, 120) found that mitochondria from the slime mold, Dictyostelium discoideum, and guinea pig liver mitochondria on incubation with palmitoyl-CoA can accumulate measurable quantities of trans unsaturated derivatives of palmitoyl-CoA as well as β-hydroxy-palmitoyl-CoA. This is in agreement with our finding, i.e., the accumulation of β-hydroxy-palmitoyl-carnitine (formed presumably from β-hydroxy-palmitoyl-CoA and carnitine) on incubation of rat SMSG mitochondrial fractions with palmitoyl-CoA and carnitine. Therefore several lines of evidence, including the present observation of β-hydroxy-palmitoyl-carnitine formation, argue against the occurrence of enzyme

linked β-oxidation intermediates and rather indicate the involvement of their CoA esters in the process.

C. Preparation of Coupled Mitochondria and Their Ability to Oxidize Fatty Acids

The study of salivary gland mitochondrial metabolism has received little attention, although the importance of energy-yielding processes to secretory function of these glands is well-established. There are no reports in the literature on the isolation of phosphorylating submandibular gland mitochondria, possibly due in part, to the difficulty encountered in the preparation of such mitochondria. The presence of proteases and nucleases which are released by homogenization (121), the toughness of the glands, the high amount of connective tissue present, and the innate high content of Ca^{++}, which reportedly disrupts salivary mitochondria (5), makes the isolation of phosphorylating mitochondria from this tissue rather difficult. Recently, Feinstein and Schramm (5) reported the preparation of coupled parotid gland mitochondria. They used a very loose-fitting Teflon pestle (0.8 mm clearance), to minimize pressure during the homogenization and avoid rupture of zymogen granules. They also included 1 mM EDTA (or EDTA/EGTA) in their medium complex endogenous Ca^{++}. However, we have found the direct application of their procedure to submandibular gland tissue unsuccessful. Using a loose-fitting Teflon pestle, a high percentage of tissue was not disintegrated, and the prolonged homogenization period resulted in

mitochondria which did not exhibit respiratory control ratio. The modification of Chance and Hagihara's method (53), wherein a brief digestion of the tissue with bacterial protease precedes the homogenization, yielded the best results. At the same time, 0.5 mM EDTA and 0.5 mM EGTA, as used by Feinstein and Schramm (5), were included in the homogenization medium to complex endogenous Ca^{++}.

The mitochondria prepared by this procedure (Methods, Section A (3)) showed respiratory rates for glutamate, α-keto-glutarate and β-hydroxybutyrate (Table 11) comparable to those reported for liver mitochondria, but for pyruvate-malate and succinate the rates were 2 to 3 times greater than those of liver mitochondria (122, 123). The isolated mitochondria exhibited respiratory control ratios of 5.0 to 5.5 for glutamate, α-keto-glutarate and pyruvate-malate; 4.5 for succinate and 2.5 for β-hydroxybutyrate (Table 11).

Palmitoyl-CoA was oxidized by salivary mitochondria at rates comparable to those reported for liver mitochondria (83, 124) and this oxidation was found to be dependent on the presence of ADP, bovine serum albumin and L-carnitine in the medium (Tables 13-15). It was further demonstrated that palmitoyl-carnitine was formed during the oxidation and its level determined the O_2 utilization rate (Figs. 21, 22). From these observations it can be concluded that carnitine functions in SMSG in a similar manner to that reported for other tissues, i.e., as a carrier of fatty acids across the mitochondrial membrane.

The present work showed the capacity of SMSG to oxidize fatty

acids under the conditions when no secretagogue was used to stimulate secretory mechanisms of the gland. Preliminary work reported by Pritchard (8) has shown that epinephrine can increase the conversion of endogenous fatty acid to CO_2 by rat SMSG slices, suggesting that fatty acid may serve as one of the substrates supplying energy during secretory processes. It is hoped that further work will provide a definitive answer as to the relationship of fatty acid oxidation to secretion.

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