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Studies of the Incorporation of Palmitate-1-C¹⁴

into Tissue Lipides in Vitro*

by

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At present, relatively little is known of the mechanisms by which fatty acids are incorporated into or released from cellular lipides. It might be assumed that the closing as well as the opening of ester linkages is catalysed by intracellular counterparts of the digestive lipases and lecithinases, inasmuch as the reversibility of their action has been repeatedly demonstrated (1,2). There are, however, several reasons why such a conclusion might be questioned.

Lipases of the pancreatic type are not generally found in tissues. Though esterases are present, these enzymes do not attack the long chain glycerides or phosphatides, but act only on short chain glycerides or esters of unphysiological nature (1). The formation of an ester bond, like the peptide or glycoside bond, is an endergonic process. It is now evident that such bonds are not formed in cells by reversal of the action of hydrolitic enzymes, but in part at least by transeptidation (3) or transglycosidation (4,5). It is conceivable that the formation of ester bonds in glycerides and phosphatides may occur analogously by transesterification.

In the present report experiments are described, using C^{14} -labeled

palmitate with isolated tissues in vitro, in which it has been found that incorporation of long chain fatty acids into glycerides and phosphatides is a general property of rat tissues. The incorporation into phosphatides was found to be associated with respiratory activity and to depend on the presence of coenzyme A. These findings are regarded as indicative of the probable occurrence of transesterification of fatty acids between coenzyme A esters and phosphatides.

Methods

As a first approach to this problem it was considered desirable to learn to what extent and under what conditions a typical long chain fatty acid, such as palmitic acid, could be incorporated into lipides of surviving tissues in vitro. No difficulties were anticipated in using customary Warburg techniques with a long-chain fatty acid, since previous studies had demonstrated that palmitic acid was accessible to oxidative enzymes of tissue slices and homogenates in vitro (6). However, because of the relatively large amounts of tissue lipides with which any newly formed radioactive lipides would be diluted, a wide disparity was expected between the specific activity of the substrate and that of the

isolated lipides. For this reason it was essential to make certain that lipide fractions could be isolated without contamination by the palmitate used as substrate.

In the procedure to be described, phospholipides were completely separated from the substrate and other labeled lipides by repeated precipitation with acetone in the presence of olive oil to "trap" labeled glycerides. Subsequently the substrate was removed from the acetone-soluble lipides by "washing out" with unlabeled palmitic acid. This was achieved by repeated additions of palmitic acid and precipitations as the lead salt.

Preparation of Labeled Palmitic Acid-1-C¹⁴. The labeled acid was prepared by Murray E. Volk as previously described (6) from radioactive barium carbonate allocated by the Atomic Energy Commission. It had an activity of 20,000 cpm. when combusted to CO₂ (7) and counted as barium carbonate in an "infinitely thick" layer 7.5 sq. cm. in area.

Preparation and Incubation of Tissues. All experiments were conducted with stock colony rats fed a diet of Allied Mills rat checkers. The fasted animals were given no food for 24 hours prior to an experiment;

the fed animals were fasted for 24 hours, then refed for 24 hours on a high carbohydrate diet consisting of: dried milk, 30 per cent; sucrose, 63 per cent; salt mixture, 3 per cent; and roughage, 4 per cent. The animals were decapitated, the tissues were dissected, and either sliced with the Stadie-Riggs slicer (8) or homogenized by the Potter-Elvehjem technique (9). About 7 gms. of slices were suspended in 40 ml. of a saline solution 0.13 M in NaCl, 0.005 M in KCl, and 0.01 M in pH 7.4 phosphate buffer. Homogenates, prepared in 5 volumes of a 0.013 M phosphate-buffered 0.13 M KCl solution, were fortified by the addition of 0.002 M final concentration of ATP (adenosine triphosphate) for activation of fatty acids (10). The large amounts of tissue were necessary to provide sufficient lipide for the separation procedure. The palmitic acid was dissolved in sufficient hot NaOH solution to give a pH of 7, and was brought to a final concentration of 0.005 M. The soap, which separated as a gel on cooling, was dissolved by warming just before use. Unless otherwise indicated, it was added directly to the main compartment of large Warburg vessels in a final concentration of 0.0005 M. Details of incubation and recovery of respiratory CO₂ will be found elsewhere (6).

Unless otherwise indicated, experiments were conducted for 3 hours at 38° with oxygen or nitrogen as the gas phase. Further details are given in the tables.

Extraction and Separation of Lipides. Slices were washed with saline solution and extracted successively in a Soxhlet apparatus with ethanol for 8 hours and ethyl ether for 2 hours. In experiments with homogenates lipides were precipitated together with protein by addition of alcohol to a final concentration of 20 per cent. The residue was removed by centrifugation at 8550 g. in a Sorvall centrifuge, and was extracted successively with alcohol and ether. This procedure resulted in complete removal of radioactivity from the tissue. The extracts were evaporated almost to dryness, the residue was taken up in petroleum ether, and the lipid layer washed with water. It was then transferred to a centrifuge tube, evaporated to about 2 ml. and the phospholipides were precipitated by addition of 40 ml. of ice-cold acetone. The phospholipides were centrifuged off and the supernatant solution removed and set aside for removal of palmitate. The residue was redissolved in 2 ml. of petroleum ether, 0.5 ml. of olive oil was added, and phospholipides were

reprecipitated by addition of 40 ml. of acetone. Three precipitations in this manner gave a phospholipide fraction with constant specific activity, uncontaminated by palmitic acid or radioactive triglycerides. When the whole procedure was carried out with tissue and labeled palmitate (zero time control) no activity was found in the isolated phospholipide.

The non-phospholipide fraction is a heterogeneous mixture of lipides, containing principally glycerides, together with small amounts of free and esterified cholesterol, phospholipides escaping precipitation, uncharacterized lipides, and labeled palmitate used as substrate. No attempts were made to isolate individual lipides from this fraction; it was assumed that the fatty acids not present as such were preponderantly in combination as glycerides. The labeled palmitate was removed from this acetone-soluble fraction as follows: The acetone solution was evaporated to 10 ml. Non-isotopic palmitic acid in an amount of 150 mg. was added in 1 ml. of ethanol, followed by 150 mg. of lead acetate dissolved in 1 ml. of hot ethanol. The precipitate was chilled in an ice-bath for one hour, and filtered off with suction. Exploratory experiments showed that three such precipitations completely removed all labeled palmitate, but to make

certain of its removal five precipitations were routinely conducted. Zero time controls gave values of about 6 counts per minute in the bound fatty acids of the non-phospholipide fraction. This was regarded as probably due to the occurrence of exchange esterification during the isolation procedure, but since this was generally very low in comparison with enzymatic incorporation it was neglected.

Isolation of Fatty Acids by Saponification. The phospholipide (PL) and non-phospholipide (NPL) fractions were saponified by refluxing for four hours in 50 ml. of 2.0 M alcoholic KOH solution. After three volumes of water were added, non-saponifiable matter was removed by three extractions with 20 ml. portions of petroleum ether. The aqueous solution was then acidified with nitric acid, and the free fatty acids were taken up in petroleum ether. A portion was combusted for radioactivity assay (?), and the remainder was weighed and assayed as such.

Results

The data of Table I represent results of an exploratory survey of the capacity of a variety of rat tissues to oxidize and to incorporate palmitate into lipides. All of the tissues studied were able to oxidize

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palmitate, the most active in this respect being kidney, heart, spleen and liver. Incorporation of palmitate was generally greater in non-phospholipides than in phospholipides, and occurred in all tissues except adipose tissue. The data clearly demonstrate that the ability to incorporate fatty acids into lipides is quite general among rat tissues. From the relative specific activities we can estimate minimum values for incorporation of the exogenous, labeled palmitate ranging from about 0.1 to 2 micromoles per 7 gm. of tissue. Actual rates of formation of ester bonds cannot be calculated from these data, since it is uncertain to what extent the exogenous palmitate was diluted by tissue fatty acids, and to what extent the various fatty acids differ in their rates of incorporation. It seems probable, however, that the actual rates are at least several times greater than these minimal values.

The remainder of this study was devoted to a more detailed investigation of factors affecting palmitate incorporation into lipides. Because of the special function of the liver in fatty acid metabolism, attention was focussed principally on slices and homogenates of this organ. Although the rate of palmitate incorporation decreased with time, paralleling its rate of oxidation, it was approximately linear during the

first hour and was still appreciable after three hours. For this reason, all experiments were conducted for three hours.

Effect of Palmitate Concentration. As shown in Table II, increases in palmitate concentration in slices resulted in increased oxidation of palmitate; a four-fold increase in the former resulted in a doubling of the specific activity of the respiratory CO_2 . This was accompanied by comparable increases in incorporation of palmitate into both fatty acid fractions. The effect of palmitate concentration in liver homogenate deviated considerably from that of slices however. As observed previously (6), increased palmitate concentration provoked a marked inhibition of its oxidation. Although incorporation into phospholipide fatty acids was inhibited to about the same extent as was oxidation, incorporation into non-phospholipides was not only not inhibited, but increased in proportion to the level of palmitate concentration. These findings, in conjunction with others to be discussed later, suggested that fundamentally different mechanisms were operative for the incorporation of palmitate into the two lipid species and suggested further that the incorporation of fatty acids into phospholipides may be associated with respiratory activity, whereas incorporation into non-phospholipides may be independent thereof.

Effect of Dietary Condition. Recent studies of fatty acid synthesis in vitro have demonstrated a marked dietary effect on lipogenesis in liver slices, the lipogenic activity of liver of a fasted animal being only a small fraction of that of the well-fed animal (11,12). The site of this block in fasting is not yet established, and the present experiments were conducted to ascertain whether it lay in an impairment in the capacity to incorporate fatty acids into tissue lipides. In these experiments further information was also sought concerning the effects of respiratory activity on incorporation of palmitate into phospholipide and non-phospholipide fatty acids. In each experiment palmitate incorporation was measured in "fed" and "fasted" livers with either nitrogen or oxygen in the gas phase. The results of five such experiments, two with slices and three with homogenates, are displayed in Table III. As previously noted (13), respiratory activity as measured by the radioactivity of the CO_2 was generally somewhat higher in the "fed" than in the "fasted" liver. In every experiment, and regardless of the gas phase, incorporation of palmitate into phospholipide was higher in the "fed" than in the "fasted" liver. The differences observed in each experiment were highly variable, ranging from 1.2 fold in number 4 to almost 4-fold in number 1. Essentially

the same results were obtained for incorporation into non-phospholipides; in only one instance was the incorporation lower in the fed animal. Some overlapping of values was observed, emphasizing the individual variability among animals, but on the whole the findings clearly indicate that the liver of the fasting animal has a lower capacity for incorporating fatty acid into tissue lipides. The differences would be even more marked if corrections were made for the glycogen content of the "fed" livers. It seems likely, however, that esterification of the fatty acid is not the major factor in the impairment of lipogenesis in fasting, since the effects of fasting on palmitate incorporation were small compared with previously observed effects of fasting on lipogenesis from acetate (11,12).

Effects of Respiration. The same data reveal that incorporation of palmitate in phospholipide is markedly dependant on respiration. In every experiment shown in Table III incorporation ranged from 1.3-fold to 6-fold higher in the presence of oxygen than in nitrogen, and the same behavior was exhibited by tissues from both fed and fasted animals. In contrast, no regular effects of respiratory activity were observed on incorporation into non-phospholipides. Wide variability in incorporation was observed, but increases were found as often as decreases. These findings again

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suggested that formation of the ester bond in phospholipides is coupled with respiratory activity.

Further corroboration of this view was obtained in studying the effects of respiratory inhibitors. The data given in Table IV show that an inhibitor of respiration such as cyanide, or inhibitors of coupled phosphorylation such as azide and dinitrophenol, greatly impair incorporation of palmitate into phospholipides, but have little or no effect on its incorporation into non-phospholipides. It is interesting to note that malonate, which inhibits respiration by preventing the oxidation of succinate, but probably has no direct action on electron transport or coupled phosphorylation, had little effect on the incorporation of palmitate into tissue lipides. Since the incorporation of fatty acids into phosphatides is dependant on respiratory energy, it was expected that ATP would stimulate incorporation under nitrogen, but the effects of this nucleotide were small; they were found on occasion with oxygen as well as with nitrogen, and with non-phospholipide as well as phospholipide fatty acids.

Incorporation of Palmitate into Phospholipide Promoted by Acetone

Powder Extracts. It is now apparent that fatty acids undergo oxidation in the form of S acyl esters of coenzyme A, and that the priming effects of ATP or citric acid cycle components on fatty acid oxidation are due to their involvement in CoA esterification mechanisms (14-16). On these grounds it seemed likely that the dependence on respiration for the incorporation of fatty acids into phosphatides may also have its explanation in the intermediate participation of CoA. To test this possibility a coenzyme A effect on this reaction was sought, using acetone powder extracts of liver in the manner that these preparations were successfully employed by Kaplan and Lipmann (17) and others in demonstrating CoA effects in various acylation reactions.

In exploring the feasibility of such preparations for this purpose, it was found that suspensions of acetone powders of rat liver in saline media incorporated palmitate into their phosphatides. Though aqueous extracts of the acetone powders contained no endogenous phospholipides, such extracts were quite active in incorporating palmitate into rat liver phospholipides when these were added as an aqueous emulsion. Subsequent experiments were conducted with extracts made with phosphate or

bicarbonate solutions, to which rat liver phosphatide was added in emulsion form.

Using the method of Kaplan and Lipmann (17) fresh rat or pigeon liver was homogenised in a Waring blender with 20 volumes of cold acetone, and the solid material was filtered with suction, washed with cold acetone, and dried in air. This preparation was stored in vacuo in the cold room. In some experiments a commercial pigeon liver acetone powder obtained from the Armour Laboratories was used. In preparing the extracts, 7.5 gm. of the powder was mixed with approximately 5 volumes of 0.1 M phosphate buffer at pH 7.4 or 0.02 M sodium bicarbonate in a Potter-Elvehjem homogeniser, and the mixture was allowed to stand at near 0° for forty-five minutes. The residue was centrifuged off and the supernatant solution, together with 25 ml. of washings, were made up to 50 ml. Each test flask contained 10 ml. of the extract, plus other additions shown in the tables, plus 0.2 gm. rat liver phospholipide emulsified in 2 ml. of water. The phospholipide was prepared from an alcoholic extract of rat liver by repeated precipitation with acetone and re-solution in petroleum ether. The tubes were incubated for two hours at 38° with occasional shaking. In the early experiments the phospholipides were

recovered directly from the incubation mixture by repeated extraction with 20 ml. portions of ethyl ether; however only about half of the total phosphatide was recovered in this manner, and in the experiments with pigeon liver the procedure was modified as follows to allow complete extraction of all of the phosphatide. The suspension was acidified to pH 2 with hydrochloric acid and centrifuged; after removal of the supernatant, which contained no phospholipide, the residue was extracted with three successive portions of boiling alcohol. The phospholipide fraction was purified as before by repeated precipitation with acetone from solutions to which olive oil was added to "wash out" any contaminating fatty acids and glycerides.

ATP invariably stimulated incorporation, and the effect was usually, though not always enhanced by the addition of fluoride. However, a disappointing feature of the rat liver preparation was the inability to demonstrate a consistent coenzyme A effect. This was probably due to the fact that these preparations were not inactivated by "ageing", such as was observed by Kaplan and Lipmann (17) for acetylation in pigeon liver preparations. When we resorted to pigeon liver acetone powder extracts

no difficulty was experienced in consistently demonstrating a coenzyme A effect. Two coenzyme A preparations were used in this study. One was a grossly impure preparation obtained from Armour and Company which, when assayed by the sulfanilamide acetylation method of Kaplan and Lipmann (17), had an activity of 5 Lipmann units per mg; the other, obtained through the kindness of David E. Green, was about 75 per cent pure, having an activity of 300 units per mg. Effects, unit for unit, were about the same for each preparation. As shown in the experiments in Table V, preparations "aged" for four hours exhibited a reduced capacity to incorporate palmitate into added phospholipide, and this was partially restored by adding coenzyme A. Though the magnitude of the effects varied with different enzyme preparations the results leave no doubt that CoA is involved in the formation of the phospholipide structure.

Discussion

The present results confirm and extend previous investigations indicating that the formation of the phospholipide molecule is coupled with energy-yielding reactions.

Fishler et al (18) observed incorporation of P^{32} -labeled inorganic phosphate ions into liver slice phosphatides in vitro, and Taurog et al. (19)

found this process to be markedly respiration-dependent. Incorporation of choline into rat liver slice phosphatides was also observed by Aron et al. to be oxygen-dependent under certain circumstances (20), and more recently Kennedy (21) has demonstrated the incorporation of C^{14} -labeled choline into the lecithin of rat liver mitochondria when these were incubated in air with adenylic acid, magnesium ions, and succinate. The dependence on energy-coupling was further shown by inhibition of incorporation by 2,4-dinitrophenol. While our work was in progress Kornberg and Pricer (22,23) reported the isolation from rat liver of an enzyme system carrying out the synthesis of two chromatographically separable substances tentatively identified as mono- and diacyl-phosphatidic acids, from P^{32} -labeled α -glycerophosphoric acid and C^{14} -labeled stearic acid. This reaction was found to have absolute requirements for ATP and CoA. These investigators have since resolved this system into two components, one of which carries out the ATP-promoted esterification of fatty acid with CoA, the other catalyzes the transesterification of the CoA ester with α -glycerophosphoric acid (24). The present studies supplement these findings by demonstrating an

oxygen-dependent incorporation of palmitate into intact phospholipides, occurring generally in rat tissues, and by demonstrating a CoA dependency for this process.

Though the incorporation of all three of the phosphatide components, vis., fatty acids, phosphate, and choline are respiration dependent, it does not necessarily follow that the three separate reactions require oxidative energy. It is more likely that a single, common step is respiration-dependant, and the studies of Kornberg and Pricer (22-24) together with the present results indicate that this step is the formation of the acyl CoA ester. Oxidative energy would provide the ATP necessary for this reaction (equation 1). Since the free energy of hydrolysis of acetyl CoA is of the order of -8 to -13 KCal (25,26), it can be expected that the S acyl CoA band will have sufficient energy for transesterification with glycerol or glycerophosphoric esters. Though the exact sequence of steps involved in the synthesis of lecithin still is uncertain, the studies of Kornberg and Pricer (22-24) and particularly those of Kennedy (21) leave hardly any doubt that the first step is the formation of α -glycerophosphate (equation 2). Whether, in the intact cell,

the incorporation of acyl groups occurs with glycerophosphorylcholine (equation 3) or with glycerophosphoric acid (equation 4) remains uncertain. Though the enzymatic studies already cited (22-24) are in favor of mechanism 4, the presence of free α -glycerophosphorylethanolamine in liver and brain (27) suggests that reactions of the type shown in equation 3 may also be involved in the synthesis and breakdown of phosphatides.

The lack of any effect of respiration on the incorporation of palmitate into glycerides does not eliminate the possibility of the occurrence of CoA-involved transesterifications in the formation of the glyceride ester linkage, but it does point to the existence of other preponderant mechanisms.

Though a reversal of lipase action would explain the present results, such an explanation leaves undisclosed the nature of the enzymes concerned, in view of the absence of lipases in tissues (1).

Summary

Palmitate-1-C¹⁴ was found to be incorporated into lipides of rat tissues in vitro. In experiments with liver slices and homogenates incorporation into phospholipides and non-phospholipides was significantly higher in tissue from fed than from fasted animals. A dependency on respiratory activity for incorporation into phospholipides was indicated by a higher incorporation with oxygen^O than with nitrogen^N in the gas phase, and by inhibiting effects of respiration inhibitors. Similar effects were not observed for incorporation of palmitate into non-phospholipides.

Coenzyme A was found to stimulate incorporation of palmitate into phospholipides added as an aqueous emulsion to extracts of pigeon liver acetone powder. These results are regarded as indicating that the fatty acid ester bonds of phosphatides are formed by stepwise transesterification between acyl coenzyme A and either glycerophorylcholine or glycerophosphate. A similar mechanism is probably not involved in the formation of the glyceride ester bond. (auth)

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Table I

Survey of Rat Organs with Respect to Oxidation of
Palmitate-1-C¹⁴ and Its Incorporation into Lipides

Each experiment was carried out with oxygen in the gas phase at 38° for three hours. Values are in counts per minute per 7.5 sq. cm. dish as barium carbonate. PL and NPL represent phospholipide and non-phospholipide, respectively.

	S l i c e s			H o m o g e n a t e s		
	Respiratory CO ₂	Fatty Acids PL	NPL	Respiratory CO ₂	Fatty Acids PL	NPL
Liver	248	50	364	1493	60	62
Heart	588	12	85	1010	15	45
Kidney	1410	65	227	1110	80	124
Brain	147	20	41	127	10	27
Spleen	630	152	74	60	3	14
Large intestine	201	16	17			
Small intestine	59	15	8			
Adipose tissue				21	1	7

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Table II

Effect of Concentration of Palmitate-1-C¹⁴ on its
Incorporation into Lipides of Rat Liver

Slices or homogenates from 7 gm. of liver were incubated for 3 hours at 37° with oxygen as the gas phase. Radioactivity data are given in c/m per dish as barium carbonate.

Liver Tissue	Palmitate Conc'n	Oxygen uptake	Respiratory CO ₂		Fatty Acids			
					PL		NPL	
			amt.	Sp. Act.	amt.	Sp. Act.	amt.	Sp. Act.
	Molar	mM	mg.	c/m	mg.	c/m	mg.	c/m
Slices	0.00025	1.29	0.67	355	143	29	45	162
Slices	0.0005	1.11	0.61	473	96	37	41	263
Slices	0.001	1.24	0.72	610	132	53	39	388
Homogenate	0.00025	0.72	0.41	790	63	87	37	64
Homogenate	0.005	0.25	0.19	570	66	49	50	214
Homogenate	0.001	0.23	0.19	580	76	58	32	511

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Table III

Effect of Dietary Condition on Incorporation of
Palmitate into Lipides of Liver Slices and Homogenates

Flasks were incubated for three hours at 37° with either oxygen or nitrogen as the gas phase. Radioactivity values are given in c/m per dish as barium carbonate. Each experiment was conducted with aliquots of the same pooled tissue removed from several rats.

Exp. No.	Liver Tissue	Gas Phase	Respiratory CO ₂		Fatty Acids			
					PL		NPL	
			Fasted	Fed	Fasted	Fed	Fasted	Fed
1	Slices	O ₂	583	1075	38	65	209	205
	"	N ₂	-	-	13	48	146	245
2	"	O ₂	252	-*	21	49	156	261
	"	N ₂	-	-	0	20	116	77
3	Homogenate	O ₂	925	1100	40	110	70	323
	"	N ₂	-	-	14	38	161	277
4	"	O ₂	1240	1260	72	122	160	164
	"	N ₂	-	-	17	20	163	243
5	"	O ₂	1000	1493	45	60	24	62
	"	N ₂	-	-	17	24	56	88

* not determined

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Table IV

Effect of Respiratory Inhibitors on the Incorporation of
Palmitate into Lipides of Liver Homogenates

Experiments were conducted for 3 hours at 38° with oxygen in the gas phase. Palmitate concentration was 0.00025 M. Radioactivities are given in c/m per dish of purified lipides as such before saponification.

Inhibitor	Oxygen uptake	Respiratory CO ₂		Phospholipides	Non-Phospholipides
		Amt.	Sp. Act.	Sp. Act.	Sp. Act.
	mM	mM	c/m	c/m	c/m
None	0.76	0.56	1520	621	722
Aside, 0.005 M	0.27	0.21	334	169	512
DNP, 1×10^{-4} M	0.22	0.21	468	150	590
Cyanide, 0.005 M	0.13	0.13	28	43	498
Malonate, 0.01 M	0.26	0.15	525	544	880

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Table V

Effects of Coenzyme A on Incorporation of Palmitate into
Added Phosphatides Promoted by Extracts of Acetone Powder
of Pigeon Liver

The "aged" extract was allowed to stand at room temperature for 4 hours prior to the experiment. The mixture in each tube contained 10 ml. of the extract, 0.2 gm. of phospholipide, and was 0.001 M in cysteine 0.005 M in ATP, and 0.0003 M in palmitate. Incubation was conducted for 2 hours at 37°. Values are given in c/m per dish of phospholipide as such.

Exp. No.	Type of Extract	Additions	Specific Activity of Phospholipides	
			"Fresh" Extract	"Aged" Extract
1	Phosphate	None	525	19
		Fluoride, 0.015 M	560	34
		" + CoA, 65 μ	606	76
2	Bicarbonate	None	237	81
		Impure CoA, 65 μ	-	226
		" " 500 μ	481	298
3	Bicarbonate	None		157
		Purified CoA, 65 μ		393
		" " 260 μ		596
		Impure CoA, 600 μ		660

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