Uptake of C^{14}-1-Palmitic Acid-P^{32}-Phospholipid Labeled Chylomicrons By Perfused Liver and Liver Slices.* (30100)

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Tissue incubation *in vitro* has been used in the study of lipid metabolism for many years. Several studies were concerned with chylomicrons or particulate fat. Hamosh and Shapiro(1) studied the uptake and release of triglycerides by liver slices and Bezman, Felts and Havel(2) have demonstrated the assimilation of $d<1.006$ lipoproteins by the same tissue *in vitro*. We have tested the *in vitro* incubation technique as a tool for the study of chylomicron uptake by liver slices. We were particularly interested in learning whether chylomicron phospholipid is taken up together with the triglyceride. Since it is known that chylomicron phospholipids exchange rapidly with serum lipoproteins(3), it is very likely that studies with chylomicrons labeled in the phospholipid moiety will give misleading results when lipoproteins are present. In the present study on liver slices, this difficulty was avoided by incubating liver slices with doubly labeled chylomicrons in a protein-free medium. In addition, we compared the uptake of doubly labeled chylomicrons by rat liver slices with that of the perfused rat liver.

Methods. Chylomicron collection. Thoracic ducts were cannulated in overnight fasted female dogs anesthetized with sodium pentobarbital. C^{14}-1-palmitic acid (100 μC)§ and 0.5-1.5 mC of inorganic P^{32} in 150-250 ml of milk were fed by stomach tube after completion of the cannulation. In most instances 100-150 ml of milk was given an hour before the cannulation to initiate fat absorption. After removal of the clot the radioactive lymph (100-200 ml) was dialyzed twice against 3 liter portions of 0.9% saline for a total of 18 hours which caused a 70% decrease in the P^{32} content of the lymph. Further dialysis did not cause any decrease in P^{32} content. The lymph chylomicrons were isolated in a Spinco Model L ultracentrifuge for 30 minutes at 80,000 $\times$ *g*. They were washed twice with 0.9% saline after dispersion with a 23 gauge needle.

Female Sprague-Dawley rats weighing over 250 g were fed 2 ml of corn oil. About 2 hours later they were anesthetized with ether and the thoracic duct was cannulated according to Bollman *et al*(4). When the lymph was clear the next day the rat was fed 3-5 μC of C^{14}-1-palmitic acid and 30 μC inorganic P^{32} either together with 1.5 ml whipping cream (to produce butterfat chylomicrons) or with 1 ml corn oil (for corn oil chylomicrons). Lymph was collected overnight on ice. During lymph collection the rats were allowed to drink a solution containing 0.64% NaCl and 0.04% KCl. After removal of the lymph clot the lymph was dialyzed and chylomicrons were isolated as described for the dog experiments.

Incubation technique. Dogs of both sexes fasted overnight were killed by inhalation of CO_2. The livers were taken out and about 0.5 mm thick liver slices were cut with a Martin slicer(5). The slices were transferred as quickly as possible to ice-cold Krebs-Ringer phosphate buffer(6) and washed twice with buffer to remove blood. Incubation of tissues was carried out in a Dubnoff metabolic shaker at 38°C. About 200 mg of liver was incubated in 4 ml Krebs-Ringer phosphate buffer pH 7.4 plus 0.1-1.0 ml of chylomicrons in stoppered 25 ml Erlenmeyer flasks filled with 95% O_2 and 5% CO_2. Incubations were performed from 0 to 3 hours after which the tissues were taken out, rinsed 3 times in buffer, drained against the side of

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§ Palmitic acid, obtained from Isotope Specialties, Burbank, Calif., was dissolved in ethanol before addition to the milk.

the glass, blotted on filter paper and weighed on a torsion balance.

Liver slice experiments. Female rats fed Purina chow *ad libitum* were killed by decapitation and 0.5 mm thick liver slices prepared with a Stadie-Riggs tissue slicer. The edges were trimmed with scissors while the slices were kept in ice-cold buffer. 250-350 mg of tissue was incubated with radioactive butterfat chylomicrons as described for dogs. Each incubation vessel contained about 7 mg of chylomicrons containing 20,060 cpm C^{14} and 10,030 cpm P^{32}. Liver slices from three rats were used for separate experiments. The slices were incubated with chylomicrons for 3 hours. Before lipid extraction the slices were rinsed 3 times in buffer.

Liver perfusion technique. Female Sprague-Dawley rats weighing 250-350 g were maintained on Purina laboratory chow and water *ad libitum.* The perfusion technique of Heimberg(7) was followed except that the livers were perfused *in situ* instead of being removed from the animal carcass. The livers were perfused up to 45 minutes with 60 ml Krebs-Ringer bicarbonate buffer, pH 7.4, and gassed with 95% O_2 and 5% CO_2. To remove blood constituents from the livers as completely as possible they were perfused with 30 ml of buffer which was discarded and replaced with fresh buffer. After 10 minutes of perfusion with buffer only, the doubly labeled chylomicrons were added. Perfusion with the labeled chylomicrons was continued for 15 or 30 minutes, and the liver was then perfused with 50 ml saline to remove radioactive substrate from the liver blood vessels. The perfused livers were removed from the animal. Liver weights ranged from 7.7 to 14.1 g.

Lipid extraction and separation. The tissues were minced with scissors in 20 ml methanol before 40 ml of chloroform was added. After overnight extraction the extracts were washed according to Folch *et al* (8), taken to dryness in a rotary vacuum evaporator, redissolved in chloroform and separated on 1 g columns of silicic acid-Supercel 1:1 (w/w)(9). The nonphospholipids were eluted with 50 ml chloroform and the phospholipids with 50 ml methanol. Aliquots were evaporated *in vacuo* before counting or further separation. Glyceride glycerol was determined as previously described(10).

Chylomicron nonphospholipid fractions with high specific activities were picked up in chloroform and separated into triglycerides, diglycerides, free fatty acids, and monoglycerides on thin layer silica gel G plates with the developer described by Hofmann(11). Prior to preparing the plates the silica gel was mixed with Ultraphor‖(12) and the lipid spots were localized under ultraviolet light. The silica gel was scraped off and counted in a liquid scintillation counter with the mixture of Gordon and Wolfe(13). Phospholipid fractions were also counted in this mixture. Channel 1 counted both C^{14} and P^{32}, whereas Channel 2 counted only P^{32}. The per cent overlap of P^{32} into Channel 1 was known from P^{32} standards and thus the C^{14} counts in Channel 1 could be calculated. Quenching of P^{32} and C^{14} was determined in each sample with internal standards.

Interpretation of P^{32}/C^{14} ratios. In each experiment the ratio P^{32}/C^{14} of the lipids taken up by the tissues was compared with the same ratio of the chylomicrons. This ratio represents essentially the ratio of phospholipid P^{32} to nonphospholipid lipid C^{14} since in dog chylomicrons the amount of C^{14} in the phospholipid fraction constituted only 3.3 to 6.1% of the total C^{14} and since there was no detectable nonphospholipid P^{32}. The chylomicron nonphospholipid C^{14} in one representative sample was found to consist of 4.2% cholesterol ester, 67.3% triglyceride, 8.1% free fatty acid, 7.1% diglyceride and 3.4% monoglyceride. Thin layer separation of chylomicrons from rats fed butterfat showed that 91.1% of the C^{14} label was in the glycerides, 2.9% in the free fatty acids, 1.4% in the cholesterol esters and 4.6% in the phospholipid fraction.

Results. Uptake by dog liver slices. In 5 experiments, the uptake of doubly labeled chylomicrons by liver slices was measured. The incubation time varied from 15 minutes to 3 hours. In Fig. 1, the relative uptakes

‖ Badische Anilin- & Soda-Fabrik AG, Ludwigshafen am Rhein, Germany.

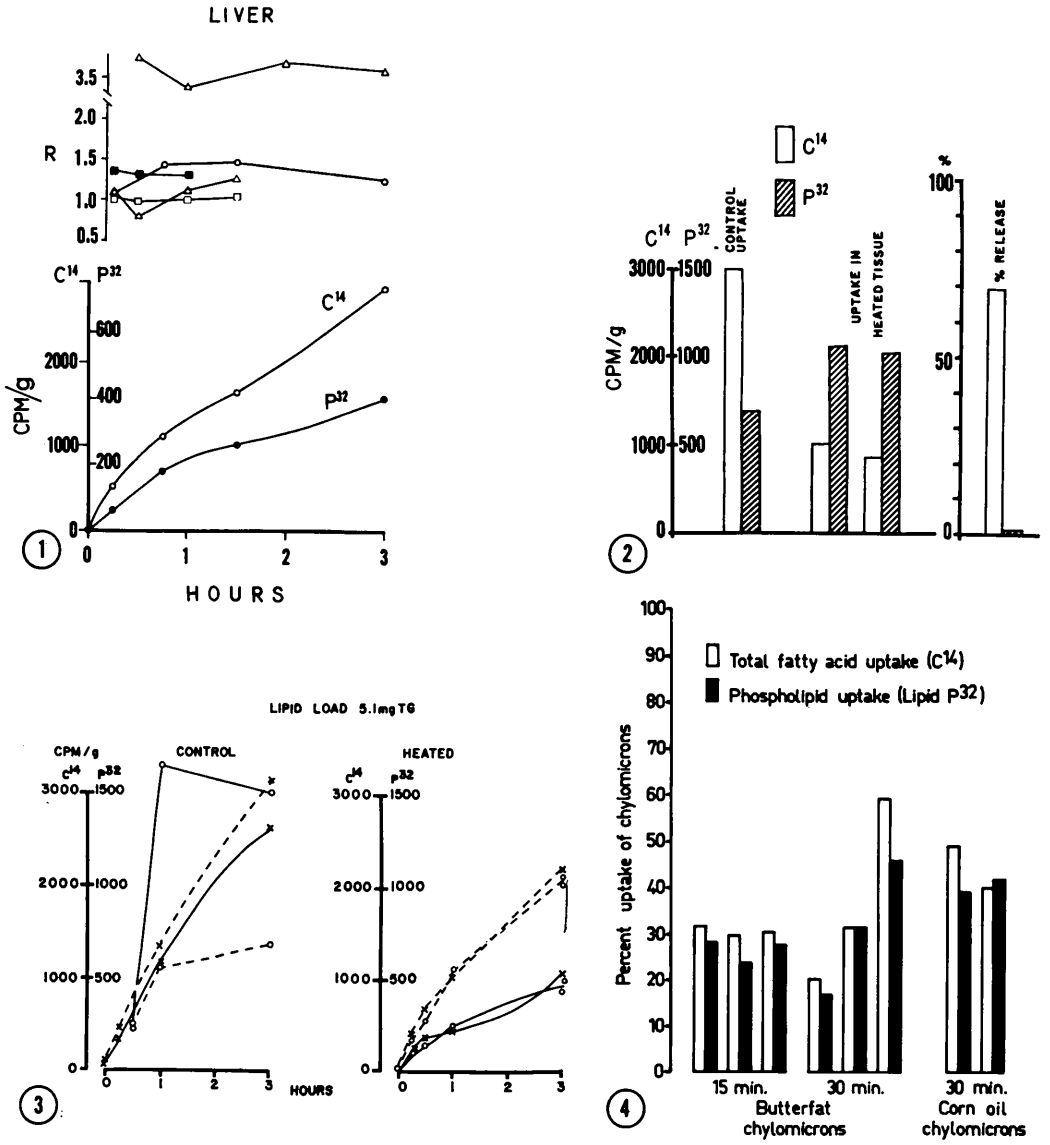

FIG. 1. Uptake of chylomicrons by liver slices *in vitro*. Bottom: uptake of C^{14} and P^{32}-labeled chylomicron lipid by liver slices obtained from a single animal *vs* time of incubation. Top: R values for liver slices from 5 dogs: △ 3.7, ■ 7.7, ○ 19.2, ▲ 1.8, □ 1.8 mg TG/flask.

FIG. 2. Uptake and release of chylomicron lipid by heated liver slices: Slices were incubated for 3 hr with doubly labeled chylomicrons. Uptake in heated slices was measured in duplicate, *i.e.*, in slices from 2 incubation flasks. Percent release of C^{14} and P^{32} from heated slices during 1 hr reincubation with nonradioactive chylomicrons represents the average of duplicates.

FIG. 3. Time course of lipid uptake by heated and control slices: ——— C^{14}, - - - - P^{32}. Two experiments performed on slices from different animals and with different chylomicron preparations are indicated by ○ and ×. The 3 hr values of one experiment (○) are the same as those used in the left-hand portion of Fig. 2.

FIG. 4. Uptake of doubly labeled chylomicrons by perfused rat liver: Percent uptake per liver is given on the ordinate. Liver weights (from left to right) were: 10.0, 14.0, 7.7, 9.2, 9.0, 7.9, 14.1, and 13.3 g.

of P^{32} and C^{14} are expressed as the ratio:

$$R = \frac{P^{32}/C^{14} \text{ in liver}}{P^{32}/C^{14} \text{ in chylomicrons}}$$

The absolute P^{32} and C^{14} uptake for one representative experiment is also shown. In 4 of the experiments, the R varied between 0.8 and 1.5, but in one case it fluctuated between 3.3 and 3.6. In 2 experiments shown in Fig. 1, the same amounts of different chylomicron preparations were incubated with slices from different dogs. The relative uptakes of P^{32} and C^{14} (R) differed by a factor of 3. With the chylomicron preparation that had given the high R values in Fig. 1, the relation between R, lipid uptake and chylomicron loads varying from 0.9 to 9.0 mg per flask was investigated. In Table I no relation between chylomicron load and relative uptake is evident.

Lipid release from tissues. The finding of high uptakes of P^{32} compared to C^{14} by liver slices was puzzling and several experiments were designed to elucidate this finding. Dog liver slices were incubated for 3 hours with buffer containing 5.2 mg doubly labeled chylomicrons. One portion of the incubated slices was dipped into buffer 3 times, blotted on filter paper and extracted (Controls, Table II). Two portions were washed in buffer and transferred for subsequent one-hour incubations in either buffer or buffer containing 5 mg nonradioactive chylomicrons. The control slices showed a relative uptake of nearly twice as much P^{32} as C^{14}. Blotting with filter paper removed about 20% of the C^{14} and P^{32}. In the one-hour reincubation experiments the loss of lipid to buffer was small; about 10% of the C^{14} and less of the P^{32} was released into the medium. When nonradioactive chylomicrons were present in the buffer the release of neither C^{14} nor P^{32} was significantly greater than that in buffer only. Three additional release experiments were performed. Although in some experiments the % of radioactivity released into the medium was 3 times as great as that reported in Table II, the relative amount of P^{32} released was always less than the C^{14}.

Viability of slices during reincubation. Since reincubation of slices was carried out between the third and fourth hour after slicing, it was important to ascertain the via-

TABLE I. Relation Between Chylomicron Load and Uptake by Liver Slices.

Triglyceride per flask	C^{14} uptake	R
(mg)	(% per g tissue)*	
.9	5.8	3.0
1.8	7.4	3.5
3.6	3.6	2.8
9.0	3.4	2.8

* 3 hr incubation.

TABLE II. Release of C^{14}- and P^{32}-Lipid from Liver Slices.

			C^{14}	P^{32}	R
			(cpm/g)	(cpm/g)	
Controls incub 3 hr	Total uptake*	A	2930	493	2.05
		B	3480	528	1.85
	Loss to filter paper	A	549	98	2.18
		B	792	103	1.59
Reincubated in buffer	Total uptake*	A	3780	437	1.41
		B	3090	459	1.82
	Release to medium	A	380	24	.77
		B	385	28	.89
	Loss to filter paper	A	430	73	2.05
		B	449	89	2.43
Idem plus nonradioactive chylomicron	Total uptake*	A	2940	459	1.91
		B	3880	582	1.83
	Release to medium	A	318	29	1.10
		B	500	56	1.37
	Loss to filter paper	A	491	103	2.56
		B	551	107	2.37

* A and B represent duplicate incubations. Total uptake includes radioactivity found in slices and filter paper; in the reincubation experiments also the amount released into the medium was included.

TABLE III. Incorporation of Inorganic P^{32} into Liver Phospholipids.

Hr of incubation	% incorporation/g tissue/hr
0	.00
1	.17
2	.19
3	.35
	.27
4	.40
	.42

bility of the tissue at that time. Dog liver slices were, therefore, incubated with inorganic P^{32} and at hourly intervals the degree of incorporation of the label into phospholipid was determined (Table III). The slices incorporated P^{32} into phospholipid somewhat faster at the later intervals than in the earlier period. Thus, the liver slices were, at least with regard to phospholipid synthesis, metabolically active during 4 hours.

Effect of heating, cooling and KCN. To stop metabolic activity, dog liver slices were heated above 95°C for about 5 minutes. The slices became grey and leathery and incorporated no inorganic P^{32} into phospholipids. The ability of these slices to take up chylomicrons was compared to that of unheated controls from the same animal. One such experiment is shown in Fig. 2. C^{14} uptake after 3 hours of incubation was reduced to almost 30% of the control value, but uptake of P^{32} had increased. When the heated radioactive slices were subsequently incubated with nonradioactive chylomicrons about 70% of the C^{14} was released into the medium but practically none of the P^{32} was lost from the slice.

It was assumed that the uptake of chylomicrons by the heated slice represented a type of adsorption phenomenon that might take place during the first few minutes of incubation. The time course of C^{14} and P^{32} uptake from chylomicrons by normal and heated liver slices from the same dog was, therefore, investigated. Fig. 3 shows that in 2 experiments, C^{14} uptake was again depressed by heating and that uptake of P^{32} by the heated liver slices continued to increase throughout the 3-hour period in a manner qualitatively similar to that observed in the unheated control slices. Thus, the continuous uptake of chylomicron lipid does not prove that the tissue under investigation is functioning normally.

The effect of cooling was studied by incubating dog liver slices at 0°C and at 37°C for one hour with doubly labeled chylomicrons. Cooling reduced uptake of C^{14} to 16% of the control at 37°C. R values were reduced from 1.37 in the control to 0.97 in the cooled slices. Addition of 0.001 M KCN did not inhibit uptake of C^{14} or P^{32} (Table IV).

Relation between chylomicron size and P^{32}/C^{14} ratio. Chylomicrons from dogs were fractionated in sucrose gradients as previously described(14). Whole chyle and washed chylomicrons were centrifuged for one hour at 13,000 $\times$ g (maximal value, at origin). Table V shows that the average particle size of washed chylomicrons is somewhat larger than that of whole lymph. Whereas in whole lymph only 21% of the C^{14} apeared in the top fraction of the centrifuge tube, the corresponding fraction of the washed chylomicrons contained 38% of the C^{14}.

The ratio of P^{32} to C^{14} shows a definite inverse relationship with particle size. In the washed chylomicrons this ratio was 0.044 for the large particles and 0.10 for the small ones—a difference of nearly 2.5-fold. The range for the native chyle was even greater. This finding raised the question whether liver slices might preferentially take up particles smaller than the average which might explain the high R values. Dog liver slices were incubated with washed chylomicrons or with whole lymph. The slices took up 12.5% of the C^{14} and 18.5% of the P^{32} from the washed chylomicrons. The R value was, therefore, 1.5. From whole chyle the liver

TABLE IV. Effect of 0.001 M KCN on Uptake of Chylomicron Lipid by Liver Slices.

	C^{14}		R	
	Control	Cyanide	Control	Cyanide
	(cpm/g tissue)			
Liver				
A Control	877	866	3.41	3.03
B "	751	722	3.53	2.45

Lipid load = 1.8 mg TG/flask containing 11,100 cpm C^{14} and 1850 cpm P^{32}.
Incubation time—3 hours.

TABLE V. Sucrose Gradients* of Native Chyle and Washed Chylomicrons Isolated from the Same Chyle.

	Particle diameter (μ)	C^{14} cpm $\times 10^{-3}$	C^{14} %	P^{32}/C^{14}
Native chyle				
Before fractionation		19.3		.12
After "				
Top (~9.1 ml)	>.17	4.46	21	.054
Middle (~9.3 ")	.15	4.96	24	.073
Bottom (~9.2 ")	<.13	10.7	52	.15
Below origin (~4.2 ")		.58	3	.31
Washed chylomicrons				
Before fractionation		50.0		.070
After "				
Top (~9.0 ml)	>.17	19.9	38	.044
Middle (~8.8 ")	.15	21.7	41	.068
Bottom (~7.7 ")	<.12	11.1	21	.10

* Spinco L ultracentrifuge, swinging bucket (SW 25.1) 10,000 rpm (max g = 13,000) for 60 min.

took up 9.3% of the C^{14} and 13.9% of the P^{32} giving the same R value as before.

Rat liver slice experiments. Rat liver slices took up both P^{32}-phospholipid and C^{14}-palmitate activity when incubated for 3 hours with doubly labeled chylomicrons (Table VI). The P^{32} uptake was proportionally much greater than the uptake of C^{14}; in fact P^{32} uptake was approximately twice as high as it would have been if the slices took up intact chylomicrons.

Perfused rat liver. The results of the liver perfusions with doubly labeled butterfat or corn oil chylomicrons are summarized in Fig. 4. Each liver was perfused with 13 mg of chylomicron glyceride. Three perfusions with butterfat chylomicrons were carried out for 15 minutes and 3 for 30 minutes. Two livers were perfused with corn oil chylomicrons for 30 minutes. All the perfused livers produced bile at a rate of approximately 0.1 ml per 15 minutes.

The uptake of chylomicron C^{14} and P^{32} was significant. Only in one experiment was less than 30% of the circulating C^{14} removed by the liver. The ratio of P^{32}/C^{14} in liver and chylomicrons varied from 0.76 to 1.04 with an average of 0.88. Corn oil and butterfat chylomicrons did not differ in this respect. Bile collected during the perfusion showed no detectable radioactivity.

TABLE VI. Uptake by Rat Liver Slices of P^{32}-Phospholipid-C^{14}-1-Palmitate Labeled Chylomicrons.

Rat	C^{14} (cpm/g)	P^{32} (cpm/g)	R
1	867	914	2.1
2	613	662	2.2
3	1,360	1,110	1.6

Discussion. Although other workers have demonstrated that triglycerides are taken up by liver *in vivo* without prior hydrolysis(15, 16), this does not imply that chylomicrons are taken up as intact particles. The uptake of chylomicrons in which both triglyceride and phospholipid are labeled would give evidence to what extent uptake of intact particles by the tissue might take place. However, in the intact animal such experiments are interpreted with difficulty since chylomicron phospholipids exchange rapidly with serum phospholipids(3). In the present study this difficulty was avoided by incubation of liver slices and perfusion with protein-free media.

Several interesting facts emerged. Dog liver slices took up both triglyceride (C^{14}) and phospholipid (P^{32}) continuously for 3 hours. However, the observation that both dog and rat liver slices took up more phospholipid P^{32} than glyceride C^{14} is not consistent with any simple hypothesis of chylomicron uptake. In principle this might be due to selective secretion or oxidation of a C^{14}-rich lipid by the slice or exchange of phospholipids between chylomicrons in the

medium and the surface of the slice. In confirmation of experiments by Hamosh and Shapiro(1) we found that dog liver slices lost little lipid to the protein-free incubation medium, however. Oxidation of C^{14} lipids without simultaneous removal of P^{32} would have given rise to gradually increasing P^{32}/C^{14} in the slices as the incubations proceeded, but this was not observed. Since small chylomicrons were shown to have higher P^{32}/C^{14} than the larger ones, one might speculate that the selective uptake of small fat droplets might give a high tissue P^{32}/C^{14} as compared to the average P^{32}/C^{14} of all chylomicrons. However, incubation of dog liver slices with native chyle which contains a larger proportion of small fat particles than the washed chylomicron preparation failed to show a difference in P^{32}/C^{14} ratio of the liver slice. Uncomplicated exchange of phospholipid between the surface of chylomicrons and tissue slices is not likely since little P^{32} was released from the dog liver slice upon reincubation with nonradioactive chylomicrons. This does not eliminate, of course, the possibility of an irreversible transfer of chylomicron phospholipid to the liver slice. Such transfer was observed in dog liver slices inactivated by heat.

It is also possible that liver slices do not take up chylomicrons in a physiological manner. For this reason we compared the uptake of doubly labeled chylomicrons in rat liver slices and the perfused rat liver. Although rat liver slices took up proportionally more P^{32} than C^{14}, it is interesting that the perfused livers took up chylomicron C^{14} and P^{32} in nearly the same proportion in which they were present in the medium. This indicates that the liver takes up whole chylomicrons, *i.e.*, the triglyceride droplets along with their phospholipid envelopes. It may be concluded that perfused livers take up chylomicrons by a different and possibly more physiological mechanism than liver slices.

Summary. Liver slices from post absorptive dogs and fed rats were incubated for up to 3 hours with chylomicrons containing C^{14}-palmitate and P^{32}-phospholipid suspended in phosphate buffer. The ratio of P^{32}/C^{14} in the liver slices was in most instances higher than that of the substrate chylomicrons. Reincubation of the dog liver slices with buffer or nonlabeled chylomicrons showed some release of C^{14} and P^{32}. Cyanide failed to inhibit lipid uptake by dog liver tissue, but lowered temperatures reduced the uptake. Dog liver slices deactivated by heating took up chylomicron P^{32} but the C^{14} uptake was greatly diminished from normal. Separation of chylomicrons into different size classes by centrifugation in sucrose gradients showed that the smaller particles are relatively richer in P^{32} than the larger chylomicrons. Evidence indicates, however, that the high P^{32}/C^{14} ratios in the liver slice are probably not caused by preferential uptake of small chylomicron particles. The perfused rat liver took up the two labels in nearly the same proportion as present in the circulating chylomicrons. Apparently chylomicrons were taken up as intact particles including their phospholipid envelope. It is suggested that adsorption of chylomicron lipid on the liver slice may account for much of the "uptake" and throws some doubt on the validity of experiments with tissue slices for the study of chylomicron transport into the liver.

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Esterification of Cholesterol by Canine Adrenal Cell Fractions.* (30101)

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The origin and function of the adrenal cholesterol esters has not been adequately elucidated. The adrenal cholesterol esters may be an important reservoir for synthesis of the corticoids. A recent report(1) has shown that cholesterol esters can serve as precursors for synthesis of steroids by canine adrenal homogenates either directly or after the liberation of free cholesterol by hydrolysis of the esters. The mechanisms involved in the formation and hydrolysis of the adrenal cholesterol esters are particularly relevant to utilization of the esters in steroid synthesis. The adrenal cholesterol esters appear to be synthesized primarily *in situ*. It has been shown(2) that the adrenal cholesterol esters possess a characteristic fatty acid spectrum which is different than that of the serum esters. Also, *in vivo* studies(3) with injected C^{14}-cholesterol suggest that the adrenal gland removes cholesterol esters from the blood, hydrolyzes them, and then re-esterifies the liberated free cholesterol to form the characteristic adrenal cholesterol esters.

More direct evidence for the synthesis of adrenal cholesterol esters has been provided by recent reports(4-7) which have shown that adrenal homogenates from a number of species (rat, dog, bovine, guinea pig, rabbit) can esterify cholesterol. The reaction requires ATP and CoA and is inhibited by free fatty acid. A hydrolytic cholesterol ester system has also been shown to be present in canine adrenal(1).

The purpose of the present report was to provide additional information on the canine adrenal cholesterol esterifying system. Data are presented on the occurrence of cholesterol esterifying systems in adrenal cell fractions and on the nature of the cholesterol esters synthesized.

Methods and materials. Adrenal glands were removed from dogs immediately after sacrifice, demedullated, chilled on ice, and homogenized in a medium (100 mg/ml) containing 0.1 M phosphate buffer, pH 7.2, 0.03 M nicotinamide, 0.004 M magnesium chloride, and 0.01 M glutathione as described by Frantz and Bucher(8). The homogenate was centrifuged at 600 $\times$ *g* for 10 minutes to remove cells and debris. The supernatant solution was centrifuged at 10,000 $\times$ *g* for 30 minutes to obtain mitochondria and then at 104,000 $\times$ *g* for 60 minutes to obtain microsomes. The final supernatant solution is referred to in Table I as soluble fraction. Both mitochondria and microsomes were washed with buffer-medium and the solutions centrifuged at 10,000 $\times$ *g* and 104,000 $\times$ *g* for 10 minutes respectively; both fractions were made to a volume equal to that of the 600 $\times$ *g* supernatant. One ml aliquots of the cell fractions or homogenate were incumated with cholesterol-4-C^{14} (added in 0.1 ml acetone) and co-factors as indicated in

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