creased incorporation of labeled linoleic acid into phospholipids was found in EFA deficient animals(7).

In normal, growing rats similar half lives of palmitic and linoleic acids in the adipose tissue were found(1). The estimation of half lives of fatty acids in the selectively labeled adipose tissue of EFA deficient rats was difficult owing to irregular loss of body weight, accompanied by rapid mobilization of the labeled fatty acids from the pads. However, using the 2 isotopic labels the rate of disappearance of linoleic acid in relation to that of palmitic acid in the same pad could be determined and neither conservation of the essential fatty acid nor an increased rate of its removal from the depot was encountered. This is in accord with the findings of oxidation of linoleic acid in EFA deficient mice in which no conservation of the essential fatty acid was reported(8).

Summary. *In vivo* incubated epididymal fat pads of EFA deficient rats were labeled selectively with radioactive palmitic and linoleic acid. The comparative uptake of both fatty acids by the same pad and their dissimilation from the contralateral pad during 47 days after labeling were determined. No preferential uptake or disappearance of linoleic acid in the EFA deficient adipose tissue was found.

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Post-Heparin Lipolytic Activity Following Intravenous Heparin-S^{35}. (29926)

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The relation between circulating heparin and the lipolytic factors which appear in plasma after its injection has been of considerable interest since the original observation of post-heparin clearing(1). Recent studies in rats using relatively high doses of S^{35}-labelled heparin (200 units/kg or about 2 mg/kg) have suggested that the appearance and decay of post-heparin lipolytic activity is parallel to radioactive heparin disappearance (2), raising the possibility of a physicochemical relationship between circulating heparin and the lipolytic enzyme. Other studies in rabbits and man using much lower doses of nonradioactive heparin (0.1 mg/kg)(3) have suggested that heparin rapidly leaves the circulation and does not circulate in significant quantities during the decline in plasma levels of the lipolytic enzyme. This dissociation of heparin and lipolytic activity would make a combination of heparin with the lipolytic enzyme highly unlikely. On the other hand rapid heparin disappearance would allow a quantitative determination of the relationship between the heparin injected and the lipolytic activity appearing in plasma(3). The present study was designed to test the validity of this latter observation by using

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TABLE I. T 1/2 of S^{35} Removal and Lipolytic Activity After Intravenous Heparin.

Rabbit No.	Heparin dose (mg/kg)	Diet	Initial (0-10 min) T 1/2 of S^{35} removal (min)	Peak lipolytic activity (μeq/ml/min)	Lipolytic activity T 1/2 of decay (min)	Basal plasma triglyceride (μmole/ml)
			S^{35} heparin			
1	.1	Fed	9.5	.050	8.0	.63
2	.1	"	8.0	.076	8.0	.93
3	.2	"	7.5	.110	10.0	1.52
4	.6	"	9.0	.154	17.5	.58
5	.6	"	12.0	.152	19.5	1.26
6	.6	Fasted 24 hr	7.0	.119	33.0	.42
7	.6	"	9.0	.171	31.0	.39
		Mean $\pm$ S.D.	8.85 $\pm$ 1.7			
			Non-radioactive heparin			
8	.6	Fed		.147	21.0	.61
9	.6	"		.176	19.0	.43

a high specific activity S^{35}-labelled heparin in low doses, and comparing the observed disappearance of radioactive heparin with the disappearance curve for plasma lipolytic activity.

Methods. Male rabbits weighing between 2.5-4.0 kg were fed a stock rabbit chow diet either *ad lib* up to the time of experiment or until 24 hours prior to experiment. General anesthesia was induced with intramuscular Sernyl® or i.v. pentobarbital. The femoral vein was isolated and catheterized with a polyethylene catheter. Non-radioactive or S^{35}-labelled Na heparin (0.1-0.6 mg/kg), dissolved in 0.85% NaCl, was rapidly injected and blood samples removed through the same catheter at 2, 4, 6, 8, 10, 15, 25, 35, 45, 60, 90, 120 and 180 minutes. Sodium chloride (.85%) was infused in amounts equal to the volume of whole blood removed. The S^{35}-labelled heparin used was prepared from commercial Na heparin with a clotting activity > 120 units/mg as described by Levy and Petracek.† Thrombin-time clotting activities of a solution with 0.5 μg heparin-S^{35} per ml and a similar solution of non-labelled heparin were comparable.‡ Blood samples were prevented from clotting with commercial Na heparin, centrifuged at 4°C, and frozen at —19°C until analyzed.

Lipolytic activity was measured by the Fredrickson assay(4). Plasma triglyceride was determined by the method of Bierman (5) except for the use of isopropyl ether instead of ethyl ether to elute the neutral lipids from silicic acid. Plasma-S^{35} radioactivity was measured in a liquid scintillation spectrometer after preparation for counting by the method of Jeffay *et al*(6). In addition selected plasma samples were dialyzed for 48 hours against 5 changes of 0.85% NaCl prior to preparation for S^{35} analysis. Other selected plasma samples were extracted with phenol and precipitated with alcohol(7,8). The precipitates were washed with ether and counted directly as described above.

Results. Plasma lipolytic activity following injection of 0.6 mg/kg S^{35}-labelled Na heparin was comparable to that seen following the same dose of non-labelled heparin from which it was made (Fig. 1)($p>0.9$ for both slope and intercept). Peak activity occurred at 2 or 4 minutes and was proportionately elevated as the dose of heparin was raised. The peak was followed by a logarithmic disappearance rate which varied both with the dose of heparin and previous dietary state. The T ½ for lipolytic activity was shorter with the lowest dose of heparin and longer after a 24-hour fast (Fig. 2, 3, 4 and Table I). Although there was a lower mean triglyceride level after a 24-hour fast, there was no correlation between basal plasma triglyceride and lipoprotein lipase (LPL) turnover (Table I). The heparin-S^{35} activity *vs* time curve was more complex. The first 8-10 minutes appeared to be a period of logarith-

† S^{35}-labelled heparin was kindly supplied by Dr. Leon Freeman, Riker Laboratories, Northridge, Calif.

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mic decrease with a mean half time of 8.9 minutes. This early uptake rate did not vary with the dose of injected heparin-S^{35} or the nutritional state. After 10 minutes the latter part of the curve could not be resolved into any straight line component but became flatter with time clearly paralleling the lipolytic activity curve from 10 to 90 minutes (Fig. 2, 3, 4 and Table I). Beyond 120 minutes a definite plateau appeared. Part of the expla-

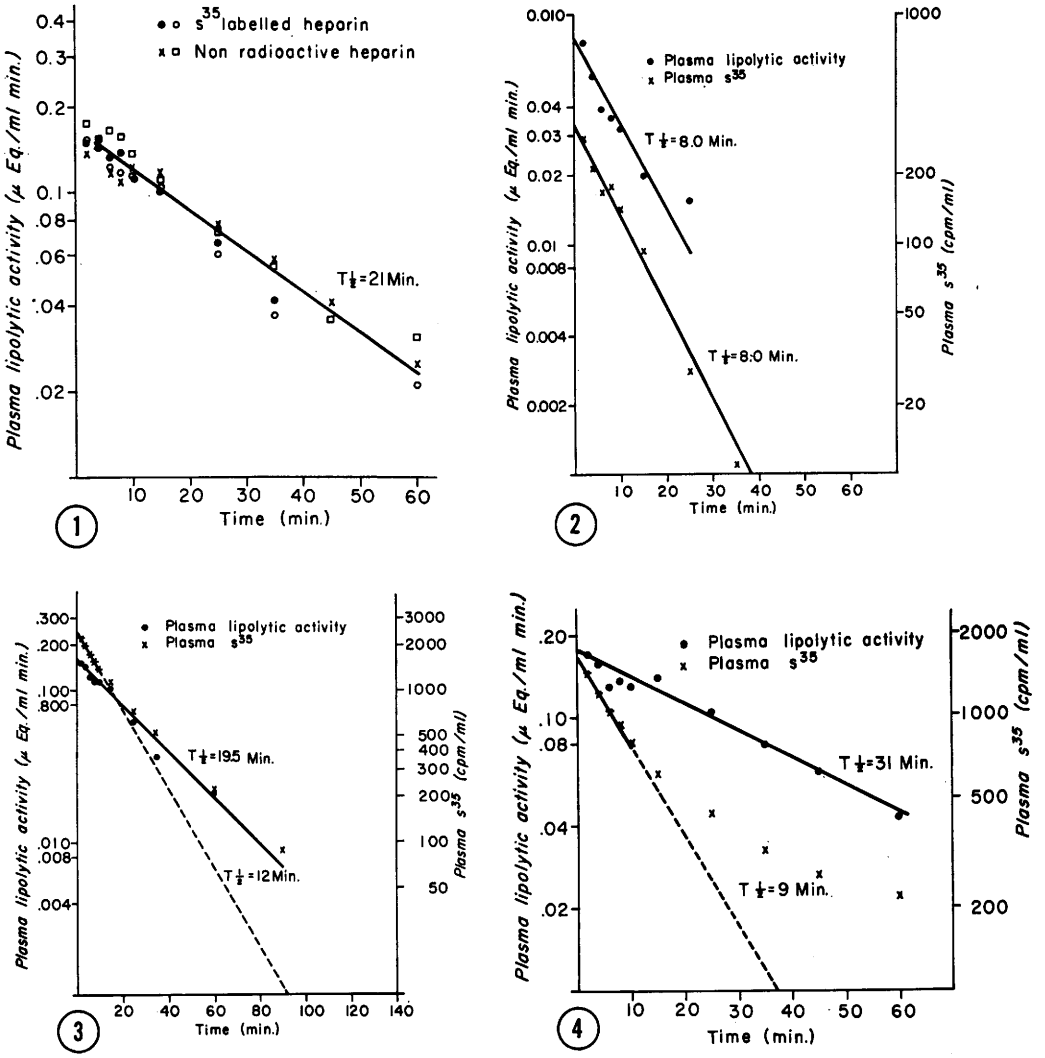

FIG. 1. Time course of lipolytic activity after heparin. Four fed rabbits (#4, 5, 8, 9) were injected with 0.6 mg/kg of either S^{35}-labelled or non-radioactive heparin at 0 time. The line is drawn by least squares for all data as there was no significant difference between the slope or intercept for lipolytic activity curve for the 2 heparin preparations ($p > 0.9$).

FIG. 2. Time course of lipolytic activity and plasma-S^{35} radioactivity after heparin-S^{35}. Fed rabbit, 0.1 mg/kg (#2).

FIG. 3. Time course of lipolytic activity and plasma-S^{35} radioactivity after heparin-S^{35}. Fed rabbit, 0.6 mg/kg (#5). The line for heparin-S^{35} disappearance is drawn using only the 2-10 min points. The line for lipolytic activity is drawn using all of the lipolytic activity data from 2-90 min.

FIG. 4. Time course of lipolytic activity and plasma-S^{35} radioactivity after heparin-S^{35}. Fasted rabbit, 0.6 mg/kg (#7). The line for heparin-S^{35} disappearance is drawn using only the 2-10 min points. The line for lipolytic activity is drawn using all of the lipolytic activity data from 2-60 min.

nation for this late plateau is revealed by analysis of the nature of the S^{35}-labelled plasma. Comparison of plasma-S^{35} radioactivity after dialysis in selected samples taken from 0 to 60 minutes after injection showed 95 $\pm$ 2.4% recovery inside the dialysis membrane (9 samples from rabbits no. 5, 6 and 7). Three samples taken between 90 and 120 minutes showed 92, 75, and 60% recovery while 3 samples taken beyond 170 minutes showed complete loss of plasma radioactivity after dialysis. To identify further the nature of the radioactive material, 11 samples from a fed (no. 5) and fasted (no. 6) rabbit injected with 0.6 mg/kg were assayed for S^{35} after extraction and precipitation of the contained heparin. The precipitate contained 75.5 $\pm$ 6.3% of the original plasma-S^{35} activity with comparable recovery from 2-120 minutes after injection. A similar extraction and precipitation of heparin-S^{35} added to unlabelled plasma *in vitro* recovered 75% of the S^{35} in the precipitate.

Discussion. The data presented suggest that most of the S^{35} circulating during the initial 90-minute observation period was present as intact heparin-S^{35}. Day *et al*(9) showed in rats that after intraperitoneal injection of heparin-S^{35}, dialysis could separate heparin-S^{35} from its radioactive metabolites since all non-dialyzable-S^{35} could be isolated by procedures which extract native heparin. Their observation is supported by the present experiments with phenol extraction and ethanol precipitation in which the bulk of the non-dialyzable radioactivity was recovered as a polysaccharide precipitate. Although recovery was somewhat low (75%), this method has usually been found to give recoveries of only 80%(8), and it was similar when the procedure was applied to a sample of heparin-S^{35} added to unlabelled plasma *in vitro*. The same recovery was found on samples drawn between 2 and 120 minutes after injection; this means that the S^{35} disappearance curve would not be altered by plotting either dialyzable or precipitable S^{35} activity *vs* time curves. On the other hand, a significant percentage of S^{35} in plasma after 120 minutes, and virtually all after 170 minutes, appears to be in dialyzable heparin metabolites, probably $S^{35}O_4$. Comparable experiments by Eiber and Danishefsky(10), in the dog, also using intravenous heparin, showed that all of the radioactivity was present as heparin-S^{35} 15 and 30 minutes after injection. Significant quantities of dialyzable S^{35} were noted by Day *et al*(9) at all times after intraperitoneal administration of heparin, with a gradual decrease in plasma sulphated muco-polysaccharide-S^{35} during the experiment. It appears likely that the different route of administration accounts for the quantitative differences in percentage of S^{35} in plasma as heparin-S^{35} soon after injection.

The parallel disappearance from plasma of lipolytic activity and heparin-S^{35} is striking. Since these disappearance rates are similar, even though they vary widely with the dietary state and heparin dose, it seems likely that this similarity is not fortuitous but related to some physicochemical combination of the circulating substances and/or a common removal site or mechanism. *In vivo* studies using hepatectomized rabbits(3) and *in vitro* experiments using isolated liver perfusion(11,12) suggest that the liver either removes or inactivates the enzyme. The present data show marked prolongation of the lipolytic activity decay curve by a 24-hour fast and by higher doses of heparin, indicating that factors other than liver function are probably involved, since it is unlikely that liver function in the rabbits of the present study was altered. The observation that significant quantities of heparin circulate during the decay portion of the lipolytic curve necessitates a reevaluation of the model proposed by Yoshitoshi *et al*(3), for quantitating lipolytic response to heparin. Either the heparin disappearance rate which is implied by their data is related to the relative binding rate of heparin to tissue or enzyme and is not a measure of the presence of the material in circulating plasma, or the "trivial" solution of the equation for LPL turnover which provides for identical rates of turnover for heparin and lipolytic activity should be applied. This latter approach unfortunately reduces the calculations to meaningless values. In either event it is apparent that further investigation is required before the Yoshitoshi model can be used for quantitative

evaluation of post-heparin plasma lipolytic activity in intact animals.

Summary. Following intravenous injection of S^{35}-labelled heparin in doses from 0.1-0.6 mg/kg, in rabbits, the time course of heparin radioactivity and lipolytic activity was measured. After 10 minutes, both S^{35} and lipolytic activity decay curves follow an essentially similar course whether prolonged by larger doses of heparin or a previous 24-hour fast. These data suggest a circulating combination between these two substances. The data are not compatible with previous studies which suggested a much faster rate of turnover for heparin.

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Inhibition of Enzymic (*Thrombokinase*) Activation of Blood Clotting by Organic Phosphates (ATP, ADP, AMP), Involving a Calcium Complex.* (29927)

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Pilgeram and Loegering(1) recently reported inhibition of certain blood-clotting test systems by additions of adenosine triphosphate (ATP) and other organic phosphates. Questions as to how much this might be related to pH, to calcium ions, and to other possible variables, were raised but were not easily answered by these test systems.

In our weakly-buffered 2-stage test system for activating prothrombin to thrombin enzymatically(2), *Sigma's*† ATP, ADP, or AMP, in 1-8 mM stable acidic solutions with plain physiological saline, reduced the pH and proved markedly inhibitory to the enzymic thrombin formation. We have attempted to quantitate this in terms of thrombin yields at controlled concentrations of prothrombin substrate, pH, and calcium ions. Even more interesting than the specific results with these organic phosphates, are the broad generalizations uncovered with respect to the roles of these 3 variables in connection with the enzymatic activation of prothrombin to thrombin.

Materials and methods. ATP, $Na_2 \cdot 3H_2O$ (610), ADP, $Na_{1.5} \cdot 3H_2O$ (524), AMP, $Na \cdot 2H_2O$ (406, apparent molec wt), and ATP-ase, of high quality, were commercially available (Sigma). Reagents and methodology for the 2-stage thrombin-generating system have been described(2). Briefly, (a) 5 ml incubation mixture containing canine eluate (prothrombin, with factor X), factor V (bovine serum AcG), prothromboplastic phospholipid (human brain cephalin), thromboplastic enzyme (2 μg/ml Milstone's(3) thrombokinase), any additive, and finally calcium, in imidazole-buffered 0.85% NaCl (at stated pH), is sampled at minute inter-

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† Sigma Chemical Co., St. Louis, Mo.