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A Study of Lipoprotein Lipase Produced by Perfusion of Isolated Rabbit Heart with Heparin.* (26776)

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Intravenous administration of heparin increases a clearing factor in human and animal plasma. This clearance has been shown to be a result of the appearance in the serum of lipoprotein lipase (LPL), which catalyzes the hydrolysis of triglyceride moiety of chylomicron and low density lipoprotein(1). Using an isolated rat heart, Crass demonstrated the appearance of LPL in the perfusing solution, which consisted of 5% serum and 3 to 10 mg % heparin in Ringer buffer (2). In this paper we are interested in the metabolism of transport of lipides relating to the problem of atherosclerosis. The mechanism of appearance of LPL in circulating solution in presence of heparin and serum was studied.

Method. The rabbits used were 8-month-old males weighing 1.5 to 2.0 kg with hearts of 3.5 to 5.0 g. Sodium heparin contains 1,000 U of heparin per ml with a concentration of about 10 mg/ml. A rabbit was anesthetized with ether and exsanguinated. Its heart was immediately isolated and washed well with bicarbonate buffer of pH 7.4 (Krebs-Ringer). Care was taken to avoid introduction of air into the circulating system. The aorta was connected to a glass tube and then by rubber tube to a pump (Sigma motor T-6). Rubber tubing was used to connect the pump to the polyethylene tube which ran to the accepting flask. Rubber contamination appearing during contact with the pump was filtered off through a glass tube filled with gauze. O_2 gas was bubbled through the liquid in the flask at a rate of 2 to 3 bubbles per second. Except in the pump, temperature of the circulating liquid was maintained at 30 to 31.5°C by keeping the entire perfusion system in a water bath of 33°C. With the pump set to deliver 10 to 12 ml/min, the isolated heart contracted well for 2 to 3 hours. The circulating liquid consisted of varying concentrations of heparin and serum (described in Table I) in bicarbonate buffer of Krebs-Ringer (pH 7.4). At the start of the experiment total volume of circulating solution was 120 ml. Eight ml of perfusing solution was taken from the flask at intervals of 20 min and immediately stored at 0°C until determination of LPL activity.

Determination of enzyme activity. Three ml of perfusion solution were incubated with 0.5 ml of 3% oil emulsion (coconut oil : glucose : Tween 60 = 15 : 5 : 0.5), 0.5 ml of

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TABLE I. Effect of Heparin and Serum on LPL Production.

Serum (%)→	5	10	50	—	5	5	—
Heparin (mg %)→	5	5	5	5	—	10	—
Time (min.)							
20	.025	.060	.065	—	.023	.023	—
40	.033	.090	.073	.015	.026	.043	.005
60	.130	.143	.105	.040	.040	.043	.010
80	.135	.150	.155	.050	.043	.047	.008
100	.128	.107	.166	.028	.033	.042	—
120	.030	.090	.083	.018	.020	.035	—

Time: Perfused time.
Values were increased optical density at 570 mμ after incubation for 1 hr in 5 ml reaction mixture with 1 ml of enzyme solution produced per 1 g of heart.
Mean variation was ± 15% with 5 times experiments at maximum enzyme activity.

10% albumin and 1.0 ml of M/10 ammonium chloride buffer containing M/25 $CaCl_2$ at 37°C(3). At 0, 1 and 2 hours of incubation 1 ml of the reaction mixture was put into a glass tube containing 0.5 ml of 10% trichloroacetic acid solution. Glycerol was determined in the deproteinized filtrate according to Korn's method(4), using chromotropic acid reagent.

Results. Effect of serum and heparin on LPL production. LPL activity in each perfusing solution was determined in the presence of various amounts of serum and heparin (Table I). Appreciably high enzyme activity was demonstrated in solutions of 5, 10 or 50% serum with 5 mg % heparin, whereas only slight activity was demonstrated with buffer alone. Addition of heparin or serum alone produced considerable LPL activity; however, higher activity was produced in the presence of both. A concentration of heparin as high as 10 mg % in addition to 5% serum inhibited the appearance of LPL, perhaps because of the inhibiting effect of heparin on LPL activity as reported *in vitro* experiment (I). The pH of the perfusion solution rose to 8.5 soon after the introduction of O_2 into the solution, due to CO_2 liberation. The experiment was, however, carried out with this pH, since the enzyme was not inactivated.

TABLE II. Effect of Heat on Serum to Liberate LPL Activity.

Time (min.)	Control	Serum heated at: 50°C for 1 hr	70°C for 2 min.
20	.027	.030	.035
40	.042	.033	.068
60	.107	.115	.073
80	.133	.142	.099
100	.116	.073	.022
120	.038	.030	.010

Time: Perfused time.
Values are same as Table I.

Effect of heat and dialysis of serum. Serum, 5% in bicarbonate buffer, was heated at 50°C for 60 min and at 70°C for 2 min. Perfusion was carried out with each solution, after addition of 5 mg % heparin (Table II). The serum in a visking cellulose tube was dialyzed by ultrafiltration technic(5) under decreased pressure (60 to 65 mm Hg) at room temperature of 10 to 15°C. The dialyzable serum fraction was computed to be 5% of the original serum. After the addition of 5 mg % heparin, this fraction was perfused; however, LPL did not appear (Table III). The non-dialyzable fraction of serum was prepared by dialyzing serum against distilled water or bicarbonate buffer of pH 7.4 at 5°C overnight. The activity produced by this fraction, though appreciably decreased in quantity, was similar to that produced with untreated serum. (Table III).

Effect of phosphate buffer. Heparin and serum were added in phosphate Ringer buffer (pH 7.4 or 8.5); other conditions for perfusion were the same as with bicarbonate buffer. There was no change in pH during perfusion. LPL activity was not demonstrated in circulating solution of either buffer. Since phosphate ion inhibits LPL activity *in vitro* (1), the results obtained might be due to the inhibiting effect of phosphate on the LPL produced; however, it is possible that LPL is not produced in presence of phosphate.

Effect of inhibitors on LPL. A perfusion

TABLE III. Effect of Dialysis of Serum on LPL Liberating Activity.

Time (min.)	I Control*	II Heparin†	III Heparin 5% serum dialyzed	IV Heparin 5% dialyzable serum fraction
20	.027	—	.030	.012
40	.042	.015	.050	.020
60	.107	.040	.085	.042
80	.133	.050	.118	.043
100	.116	.028	.080	.020
120	.038	.018	.050	.020

* Control solution contained 5% serum and 5 mg % heparin.
† Heparin: 5 mg % used.
Values are same as Table I.

solution obtained from 80 min perfusion in the presence of 10% serum and 5 mg % heparin under conditions producing the highest activity was used as the LPL solution (Table I). The enzyme had the same properties as LPL of adipose tissue(4) and plasma(3) in its behavior in presence of its inhibitors. There was evidence, however, of other esterases which were not inhibited by protamine sulfate or by higher concentrations of NaCl or $CaCl_2$ (Table IV).

Optimum pH of LPL. The enzyme solution used in the above experiment was adjusted to pHs of 7.2, 7.6, 8.0 and 8.4, with addition of M/40 aqueous ammonium solution of M/40 HCl. Buffer, substrate and albumin were adjusted to the corresponding pH values. The optimum pH of enzyme activity was shown to be 8.2, which is similar to the LPL of adipose tissue (Fig. 1).

Thermostability of LPL. Each enzyme solution described above was heated to the temperatures noted in Table V and its enzymatic activity studied (Table V).

TABLE IV. Effect of Inhibitors on LPL.

Inhibitors	Conc.	$O.D._{570\ m\mu}$	Activity, %
NaCl	1 M	.022	35
"	.5 M	.040	63
$CaCl_2$	.2 M	.022	35
"	.1 M	.022	35
Na-protamine sulfate	200 γ/ml	.032	57
Idem	100 "	.042	67
Control	—	.063	100

$O.D._{570\ m\mu}$: Increased optical density in 0.2 ml of reaction mixture, after 1 hr incubation.

LPL activity of intact and perfused rabbit heart. Fresh heart tissue was glass homogenized with 3 volumes of M/10 ammonium chloride buffer (pH 8.5) at 0°C. The homogenate was centrifuged at 0°C after

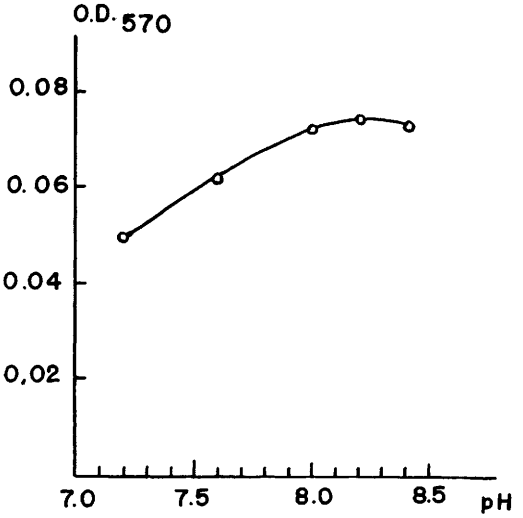

FIG. 1. Optimum pH. $O.D._{570}$: Increased optical density at 570 mμ in 0.2 ml of reaction mixture, after 1 hr incubation.

standing for 30 min at 0°C. After addition of 2 ml of distilled water to make a final volume of 5 ml, the supernatant of 1.0 ml was used for activity assay, under the same conditions described elsewhere.

TABLE V. Thermostability of LPL.

Temperature	Period	$O.D._{570\ m\mu}$	%
37°	30 min.	.068	87
"	60 "	.053	68
50°	30 sec.	.062	79
"	1 min.	.040	51
"	2 "	.011	24
70°	30 sec.	.016	21
Control		.078	100

$O.D._{570\ m\mu}$: Increased optical density in 0.2 ml of reaction mixture, after 1 hr incubation.

Ammonium buffer extractable LPL activities of the intact and perfused hearts were compared. With intact hearts, the increase in optical density at 570 mμ due to glycerol in 0.2 ml of reaction mixture was shown to be 0.090 as a mean value (± 0.015), whereas after the perfusion experiment, LPL activity was completely lost (after 100 min of perfu-

sion). Total activity appearing in the perfusion solution was calculated from Table I: the LPL activity obtained from the perfusion solution was about 1.5 times that obtained from the ammonium extractable LPL of heart.

Summary. With a perfused, isolated rabbit heart LPL activity appeared in the perfusion solution of bicarbonate buffer in the presence of 5 mg % heparin and 5 to 10% serum. However, under the same conditions, LPL did not appear with phosphate buffer. The LPL produced was inhibited by protamine sulfate, $CaCl_2$ and NaCl. Optimum pH of enzymatic activity was at pH 8.2, and 80% of the activity was lost by heating at 50°C for 2 min and 70°C for 30 sec. After perfusion, LPL activity in the heart disappeared. Total activity appearing in the circulating solution was nearly equal to total activity of heart tissue which was extractable with ammonium buffer solution.

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Conversion of Mevalonic Acid-2-C^{14} to Biliary Cholesterol and Cholanic Acids in the Guinea Pig.* (26777)

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Since mevalonic acid was discovered(1) numerous studies have been conducted to show the conversion *in vitro* of this compound to cholesterol(2,3). Important intermediates in the metabolic pathway appear to be phosphomevalonic acids, pyrophosphates of isopentenyl and dimethylallyl alcohol, geraniol, farnesol and nerolidol, as well as squalene and lanosterol(3). Cholesterol, in turn, is the major precursor of bile acids. In comparison to many studies on conversion of mevalonic acid to cholesterol *in vitro,* few investigations have been reported on *in vivo* conversion to cholesterol(4) or to bile acids. The present study concerns *in vivo* conversion of mevalonic acid-2-C^{14} to labeled cholesterol and bile acids in guinea pig.

Materials ad methods. Each of 25 guinea pigs of either sex, weighing 140-180 g, received, intravenously, 3.26 μc (0.772 mg) mevalonic acid-2-C^{14} in 0.10 ml 0.15 molar NaCl. Four hours later, all animals were sacrificed and the contents of their gall bladders pooled. Bile acids and cholesterol were then isolated, identified and the C^{14} content determined by the following procedures.

The 4.20 ml of pooled bile were hydrolyzed in 1 N NaOH for 5 hours at 110°C, extracted with ethyl ether and further analyzed on a column of hydrophobic kieselguhr using chloroform:heptane (9:1) as stationary phase and 58% aqueous methanol as mobile phase(5). Aliquots of the original sample of pooled biles, of the ether extract, and of the fractions of the effluent from the column were applied to aluminum planchets and their C^{14} content determined by a windowless flow counter with accumulation of sufficient counts for 1% standard error.

Results. The pooled bile contained 2.2% of injected C^{14}. Of this activity, 99.3% was recovered in ether extract after hydrolysis. Most of the C^{14} applied to the column was found in the fractions having the mobility of 3α, 7α-dihydroxycholanic (59.7%) and 3α-hydroxy, 7-ketocholanic acid (28.5%). After collection of 150 ml of effluent, the column was eluted with $CHCl_3$, and 11.8% of C^{14}

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