shown to be interchangeable in replacing L-phenylalanine for *L. casei* 280-16A, but not interchangeable in functioning as a D-α-hydroxy acid source for this organism. It was concluded that *L. casei* 280-16A cannot racemize phenyllactic acid, and it was suggested that utilization of L- and D-phenyllactic acids in place of L-phenylalanine for this organism is mediated by separate (L- and D-) phenyllactic dehydrogenases.

1. Eiduson, S., Camien, M. N., Dunn, M. S., *Arch. Biochem.*, 1950, v29, 302.
2. Malin, R. B., Camien, M. N, Dunn, M. S., *Arch. Biochem. Biophys.*, 1951, v32, 106.
3. Eiduson, S., Dunn, M. S., *ibid.*, 1952, v35, 146.
4. Camien, M. N., Dunn, M. S., *ibid.*, 1956, v60, 452.
5. ———, *J. Biol., Chem.*, 1953, v201, 621.
6. ———, *ibid.*, 1954, v211, 593.

Received September 19, 1958. P.S.E.B.M., 1958, v99.

Studies Indicating Inactivation of Post-Heparin and Endogenous Human Plasma Lipoprotein Lipase During Triglyceride Lipolysis.* (24394)

H. ENGELBERG
Cedars of Lebanon Hospital, Los Angeles

Circumstanstial evidence has been obtained (1,2) which suggested that endogenous plasma lipemia clearing factor (lipoprotein lipase) was inactivated or became unstable as triglyceride lipolysis occurred. Further investigation was important since considerable data now indicate that lipoprotein lipase normally functions in clearing of post-alimentary fat from the bloodstream. However, investigators have been unable to demonstrate endogenous lipoprotein lipase in plasma of most healthy individuals. This fact is less puzzling, however, in view of data here presented which strongly support the concept that plasma lipoprotein lipase loses its activity during the lipolytic process. In addition other studies will be shown which indicate that heparin is a component of the enzyme.

Methods. Three sources of lipoprotein lipase were used: 1. fasting post-heparin human plasma; 2. fasting human plasma containing endogenous lipemia clearing activity; 3. citrate eluates(3) of tricalcium phosphate adsorbates of 1 or 2. Thus little or no neutral fat was available for lipolysis until it was added *in vitro*. The fat substrate used was human low-density lipoproteins.† The following approach was adopted: aliquots of plasma or of citrate eluates were placed in 2 tubes incubated at 37°C for 2 hours. Lipoproteins were added in excess (.2 ml/ml plasma, at which concentration the substrate does not effect rate of reaction(4)) to tube 1 at the start of first and second hours of incubation, and to tube 2 only at start of second hour. Thus any possible inactivating effect of incubation at 37°C was identical for both sets of tubes. Bovine albumin was also added to tube 1 for the second hour in some experiments to guarantee adequate fatty acid acceptor, although it was probably not necessary as the plasma itself contained ample albumin. Infranatant serum‡ was used as source of acceptor protein and necessary ions in the citrate eluate studies. In several experiments oleic acid was added to tube 2 as a control together with lipoproteins since release of unesterified fatty acids in tube 1 during first hour could possibly inhibit subsequent lipolysis. Aliquots of plasma were removed from each tube every 15

* Supported by Grant of Nat. Heart Inst.

‡ Infranatant serum obtained after ultracentrifugation contains no low density lipoproteins (supplied by Inst. of Medical Physics).

† Supplied by Inst. of Medical Physics, Belmont, Calif. through the courtesy of Mr. D. Spector. Lipoproteins were obtained by ultracentrifugation from sera of individuals with high concentration of Standard Sf 12-400 lipoprotein. They represent a 5-fold concentration of lipoprotein resuspended in their original sera, and predominately consist of Sf 12-400 and higher lipoprotein classes whose chief constituent is neutral fat.

TABLE I. Glycerol Release upon Incubation of Fasting Post-Heparin Plasma and Plasma Containing Endogenous Lipoprotein Lipase with Added Human Low-Density Lipoproteins.

Patient	Source of lipoprotein lipase	Preparation in tube	Glycerol in μmoles/ml Incubation time at 37°C 1st hr 0	15	30	45	60	2nd hr 0	15	30	45	60
A.H.	Fasting plasma 10′ after 25 mg heparin I.V.	Tube 1: 1 ml plasma, .2 ml LP* added at start of 1st and 2nd hr	2.6	2.9	3.1	3.4	3.5	3.5	3.5	3.5	3.5	3.6
		Tube 2: *Idem.* LP added at start of 2nd hr	2.7	2.7	2.7	2.7	2.7	2.7	2.8	3.0	3.4	3.5
H.E.	Citrate eluate of $Ca_3(PO_4)_2$ adsorbate of plasma 10′ after 25 mg heparin I.V.	Tube 1: .5 ml eluate, 1 ml infranatant serum. .2 ml LP added at start of 1st hr. .2 ml LP and .2 ml BA† added at start of 2nd hr	1.5	2.1	3.6	3.7	3.7	3.7	3.7	3.7	3.7	3.7
		Tube 2: .5 ml eluate, 1 ml infranatant serum. .2 ml LP and .2 ml oleic acid added at start of 2nd hr	1.5	1.5	1.5	1.5	1.5	1.5	3.6	3.7	3.7	3.8
		Tube 3: 1 ml supernatant plasma after $Ca_3(PO_4)_2$ adsorption, .2 ml LP	1.7	1.7	1.7	1.7	1.7					1.7
		Tube 4: .5 ml eluate, 1 ml infranatant serum	1.3	1.3	1.3	1.3	1.3					1.3
H.E.	Fasting plasma 10′ after 10 mg heparin I.V.	Tube 1: 1 ml plasma, .2 ml LP added at start of 1st hr, .2 ml LP and .2 ml BA at start of 2nd hr	2.0	2.2	2.3	2.4	2.6	2.6	2.6	2.6	2.6	2.6
		Tube 2: 1 ml plasma, .2 ml LP and .2 ml oleic acid added at start of 2nd hr	2.0	2.0	2.0	2.0	2.0	2.0	2.2	2.3	2.4	2.7
		Tube 3: 1 ml plasma, no added LP	1.9				1.9					1.9
‡ N.J.	Fasting plasma with endogenous lipemia clearing activity	Tube 1: 1 ml plasma. .2 ml LP at start of 1st and 2nd hr	2.0	2.3	2.3	2.4	2.5	2.5	2.6	2.6	2.6	2.6
		Tube 2: 1 ml plasma. .2 ml LP and 0.1 ml oleic acid at start of 2nd hr	1.8	1.8	1.9	1.8	1.8	1.8	2.0	2.3	2.4	2.5
		Tube 3: 1 ml supernatant plasma after $Ca_3(PO_4)_2$ adsorption. .2 ml LP	1.8	1.8	1.8	1.8	1.8					
I.S.	Citrate eluate of $Ca_3(PO_4)_2$ adsorbate of plasma with endogenous lipemia clearing activity	Tube 1: .5 ml citrate eluate. 1 ml infranatant serum. .2 ml LP at start of 1st and 2nd hr, BA at start of 2nd hr	1.2	1.3	1.3	1.4	1.4	1.4	1.4	1.3	1.4	1.4
		Tube 2: .5 ml citrate eluate. 1 ml infranatant serum. .2 ml LP at start of 2nd hr	1.2	1.2	1.2	1.2	1.2	1.2	1.1	1.3	1.4	1.45
		Tube 3: 1 ml infranatant serum. .2 ml LP	1.2				1.2					
		Tube 4: .5 ml citrate eluate. 1 ml infranatant serum. No LP	1.1				1.2					1.2

* LP—Human low-density serum lipoproteins in 5-fold concentration resuspended in their original serum.

† BA—Bovine albumin.

‡ The findings in this patient are typical of 5 individuals in whom the same experiment was done.

TABLE II. Free Fatty Acid Release upon Incubation of Olive Oil Emulsion and Human Pancreatic Lipase.

Preparation in tube	Free fatty acids in meq/l, Incubation time at 37°C							
	1st hr				2nd hr			
	0	15′	30′	60′	0	15′	30′	60′
Tube 1: 7.5 ml 5% olive oil emulsion, 1.5 ml 1% $CaCl_2$, 1.5 ml .05% human pancreatic lipase	8.8	10.3	11.5	15	15	17	15.4	13.5
Tube 2: Idem. Additional 7.5 ml olive oil emulsion and 1.5 ml $CaCl_2$ added at start of 2nd hr	8.5	11.4	15.5	16	25.1	36	39.3	41

minutes, and analyzed in duplicate for glycerol(5). In some cases lipoproteins were added to supernatant plasma after tri-calcium phosphate adsorption and then glycerol release was determined. The effect of heparin added *in vitro* to supernatant plasma after $Ca_3(PO_4)_2$ adsorption of post-heparin or endogenously active plasma was also observed. Lipemia clearing activity of the original plasma, the $Ca_3(PO_4)_2$ supernatant, and the supernatant plus heparin was determined as previously outlined(6) using optical density technics.

Results. The findings in studies in which post-heparin plasma and plasma containing endogenous lipoprotein lipase activity were used as a source of enzyme are shown in Table I. The results are similar using both sources of enzyme except for the decreased magnitude of lipolysis with endogenously active plasma. In all experiments glycerol release occurred in tube 1 only during the first hour of incubation although additional fat substrate and fatty acid acceptor (bovine albumin) were provided at start of second hour. In tube 2 no triglyceride lipolysis occurred during the first hour (since lipoproteins were not added until the second hour of incubation), whereas the rate of glycerol release during the second hour was similar to that of tube 1 during the first hour. Addition of free oleic acid to tube 2 did not inhibit lipolysis. In several instances the supernatant plasma after $Ca_3(PO_4)_2$ adsorption was used and there was absence or marked reduction of glycerol release as compared to plasma aliquots before adsorption. When infranatant serum was incubated together with citrate eluate, no release of glycerol occurred. Table II shows, in contrast to findings with lipoprotein lipase, that when fat splitting due to pancreatic lipase has occurred, the provision of more fat substrate and acceptor ($CaCl_2$) results in additional lipolysis.

In 10 individuals post-heparin plasma lipemia clearing activity was determined by optical density technics before and after $Ca_3(PO_4)_2$ adsorption, and after addition of heparin *in vitro* to the supernatant plasma. In 4 of 10 individuals this clearing activity, previously removed or reduced by gel adsorption, was partially restored by heparin *in vitro*. The data in one subject, representative of all 4, are shown in Table III (patient N.B.). When heparin was added in smaller or larger amounts (.2 mg% or .002

TABLE III. Post-Heparin and Endogenous Lipemia Clearing Activity before and after $Ca_3(PO_4)_2$ Adsorption, and Effect of Heparin.

Patient	Age	Sex	Plasma sample	Optical density decrease in 1 hr with incubation at 25°C
N.B.	41	♀	After 25 mg heparin I.V.	20
			Supernatant after $Ca_3(PO_4)_2$ adsorption	0
			After addition of .002 mg heparin to 1 ml supernatant	5
M.M.	76	♀	Control (no heparin injected)	7
			" + .002 mg heparin	7
			" + TEPP	2
			Supernatant after $Ca_3(PO_4)_2$ adsorption	3
			After addition of .002 mg heparin to 1 ml supernatant	6

mg%), the findings were less clear. Similar studies were performed in 51 patients with endogenous plasma lipemia clearing activity, but addition of heparin to supernatant plasma after $Ca_3(PO_4)_2$ adsorption restored clearing activity in only 2 instances. The data in one of these 2 subjects are also shown in Table III (patient M.M.). In this individual no increase in clearing activity occurred when heparin was added *in vitro* to plasma before adsorption, and endogenous activity was partially inhibited by tetraethylpyrophosphate which inhibits the post-heparin enzyme but has no effect on pancreatic lipase(7).

Discussion. Data presented in Table I show that when excess substrate was present during the first hour, additional lipolysis did not occur during the second hour although more lipoproteins were added. Various possible explanations come to mind. It has been suggested that post-heparin lipemia clearing activity may be inactivated by prolonged incubation at 37°C. This was not pertinent here, however, since lipolysis in the second tube, which had been incubated for the same time as tube 1, proceeded normally during the second hour, after addition of fat substrate. Although probably sufficient albumin was present in plasma or infranatant serum itself, addition of albumin to tube 1 in several studies during the second hour of incubation ruled out any lack of fatty acid acceptor as a factor limiting further triglyceride lipolysis. Since free fatty acids may limit the reaction (8), oleic acid was added to tube 2 in some experiments as a control, and no inhibition of lipolysis occurred. Finally $Ca_3(PO_4)_2$ adsorption removed enzymatic activity, thereby establishing that it was due to lipoprotein lipase(3,6). Thus it would appear reasonable to conclude that lipoprotein lipase loses its activity or dissociates during the lipolytic process. Table II presents data for one enzyme and cannot be used as evidence for this generalization.

It has been found that passage of post-heparin rat plasma through a column of De-Adidite F, a strongly basic anion exchange resin, removes heparin from plasma(9). The lipemia clearing enzyme system is not destroyed thereby, but its stability is markedly reduced. Addition of small quantities of free heparin to the effluent restores stability of the plasma. These results suggested the studies presented in Table III. Heparin added *in vitro* to supernatant plasma after $Ca_3(PO_4)_2$ gel adsorption (which removes heparin) restored lipemia clearing activity in 4 of 10 post-heparin plasma, and in 2 of 51 plasmas containing endogenous lipoprotein lipase. Apparently adsorption of apoenzyme by the gel or resin may not be as complete as adsorption of the heparin component. When apoenzyme is still present in the supernatant plasma or effluent, simple addition of heparin *in vitro* restores enzyme activity or stability. It is not surprising that this occurs more frequently when larger amounts of lipoprotein lipase are present, as in post-heparin plasma, than it does after adsorption of plasma containing endogenous activity. Several other conclusions would also seem to follow from these observations, particularly those using endogenously active plasma. It appears that the bond between heparin and apoenzyme is easily broken. Furthermore the results provide evidence that heparin is a component of human plasma lipoprotein lipase. A similar conclusion in rats was suggested by partial inactivation of tissue lipoprotein lipase by bacterial heparinase(10).

It is tempting to theorize, since lipoprotein lipase does not split simple triglycerides but acts only upon neutral fat of lipoproteins(11), that the heparin component of the enzyme combines with the protein moiety of the lipoprotein molecule. This effects contact between the triglyceride of the lipoprotein and the apoenzyme of lipoprotein lipase, and lipolysis takes place. At the same time, however, the bond between apoenzyme and heparin has been disrupted and further lipoprotein lipase activity is absent or unstable. This concept is admittedly speculative, but it is consonant with the results obtained.

Perhaps of more importance at this time, however, is the demonstration of lipoprotein lipase inactivation during lipolysis. Thus failure to demonstrate it in plasma of most individuals at any one time does not argue against its role in removal of fat from blood. Furthermore, at least in regard to circulating

lipoproteins and chylomicra, there is less need to place its site of activity at the cell membrane. It appears that plasma lipoprotein lipase is stimulated by fat intake(2,12), is destroyed by passage through the liver(13), and is inactivated in the process of lipolysis. Thus the quantity of demonstrable circulating enzyme at a given moment is a resultant of its stimulation and release on the one hand, and its destruction and inactivation on the other. It is therefore not surprising that lipoprotein lipase can only occasionally be demonstrated in the blood since physiologically it is probably slowly released in moderate amounts in response to lipemia, and it dissociates as lipolysis occurs.

Summary. Evidence has been obtained by *in vitro* studies that human post-heparin and endogenous plasma lipolytic activity (lipoprotein lipase) are inactivated during the process of triglyceride lipolysis. Addition of heparin to supernatant plasma after removal of lipoprotein lipase activity by $Ca_3(PO_4)_2$ adsorption occasionally restores its lipemia clearing activity. This indicates that heparin is a component of the enzyme, and that its dissociation from the apoenzyme readily occurs.

1. Engelberg, H., *Metabolism,* 1958, v7, 172.
2. ———, *J. Appl. Physiol.,* 1958, v12, 292.
3. Nikkila, E. A., Pesola, R., *Acta Chem. Scand.,* 1956, v10, 144.
4. Engelberg, H., *Cir. Research,* 1958, v6, 266.
5. Lambert, M., Neish, A. C., *Canad. J. Res.,* 1950, v28, 83.
6. Engelberg, H., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 394.
7. ———, *J. Biol. Chem.,* 1956, v222, 601.
8. Shore, B., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 73.
9. Robinson, D. S., *Biochem. J.,* 1956, v62, 188.
10. Korn, E. D., *Science,* 1957, v124, 489.
11. ———, *ibid.,* 1954, v120, 399.
12. Engelberg, H., *J. Applied Physiol.,* 1957, v11, 155.
13. Jeffries, G. H., *Quart. J. Expl. Physiol.,* 1954, v39, 261.

Received June 17, 1958. P.S.E.B.M., 1958, v99.

Experimental Production of Polycythemia in Humans by Administration of Cobalt Chloride. (24395)

JOHN E. DAVIS AND JAMES P. FIELDS
University of Texas, College of Pharmacy, Austin

The erythropoietic stimulating action of cobalt was first demonstrated by Waltner and Waltner(1) who produced polycythemia in rats by administration of this element and its chloride. Since that time, polycythemia has been induced in many animals such as rabbit (2), dog(3,4), duck(5), chicken(6) and other animals(7), by continued administration of cobalt salts. Although it is logical to presume that cobalt can produce polycythemia in normal human beings, we have been unable to find data in the literature which actually demonstrate ability of cobalt to cause this condition in healthy people. Berk, Burchenal and Castle(8) have shown that cobalt exerted erythropoietic stimulating effect in some hospitalized patients, most of whom were anemic, but very seldom did their erythrocyte numbers approach polycythemic levels. We believe that the results of the investigation herein described constitute the first clear-cut demonstration of experimental production of polycythemia in normal humans by daily administration of a cobalt salt.

Procedure. Six apparently normal men, ranging 20 to 47 years in age, served as subjects. Red blood cell counts, hemoglobin percentages, leukocyte counts, reticulocyte percentages and thrombocyte counts were determined during pre-experimental control period as well as during experimental period of cobalt medication. Hemoglobin was estimated by the Sahli method. Reticulocyte and thrombocyte counts were made on wet mount preparations in which a drop of fresh blood on slide was covered with cover slip coated with film of brilliant cresyl blue stain. Erythrocyte counts were made with a Spencer