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ABSTRACT

Further studies have been made on the heparin-catalyzed enzyme of human plasma that liberates free fatty acid from lipoprotein triglycerides. It has been found that the S_f6 lipoprotein of the human is not a substrate for the enzyme, that the tributyrinase activity of serum is not enhanced significantly after heparin injection, and that free fatty acid inhibits the reaction. Such substances as adenosine triphosphate and Coenzyme A, sulfhydryl reagents, aerobiosis, anaerobiosis (with nitrogen), and the antioxidant α-naphthol are without effect. The inhibition of fatty acid release by diisopropylfluorophosphate suggests that the enzyme is an esterase.

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INTRODUCTION

Recently, it was demonstrated that incubation of egg lipoprotein or human S_f20–400 lipoprotein with plasma of a human given an intravenous injection of 100 mg sodium heparin (Lederle) results in hydrolysis of the lipoprotein triglycerides with concomitant release of fatty acids,[1, 2, 3]. The increase in acidity of the incubation mixture was demonstrated by infrared analysis of a lipid extract separated into various components by a modification of the chromatographic techniques developed by Borgstrom[4]. The release of fatty acid was confirmed subsequently by others.[5, 6] In this report we wish to present additional data on the subject. The effects described were not observed when serum to which heparin had been added in vitro was incubated with a lipoprotein substrate. Plasma obtained from a human subject 15 to 30 minutes after administration of heparin was in every case capable of bringing about liberation of fatty acid from suitable substrates.

Materials and Methods

Preheparin serum, postheparin plasma, and egg lipoprotein were obtained by methods described in the previous communication,[3]; human S_f20–400 lipoproteins were obtained by the method of Lindgren, Elliott, and Gofman.[7] In general, 1 to 2 ml of diluted egg lipoprotein (elp) or undiluted human lipoprotein was incubated with 2 or 3 ml serum or plasma at 37°C for periods ranging from 3 to 8 hours. The incubations were performed with parallel sets of samples, one set containing preheparin serum, the other postheparin plasma from the same individual. Extraction of lipids from the incubation mixture was accomplished by the methanol–ethyl ether procedure indicated previously.[3]

1. B. Shore, Educational Proceedings of the Permanente Hospitals 2, 174, November, 1952.
2. A.V. Nichols, N.K. Freeman, B. Shore, and L. Rubin, Circulation 6, 437, (1952).
3. B. Shore, A.V. Nichols, N.K. Freeman, Proc. Soc. Exp. Biol. and Med., 83, 216, (1953), "Evidence for Lipolytic Action by Human Plasma Obtained After Intravenous Administration of Heparin," University of California Radiation Laboratory Report No. UCRL-2204, May, 1953.
4. B. Borgstrom, Acta Physiol. Scand. 25, 101, 111 (1952).
5. R.K. Brown, E. Boyle, C.B. Anfinsen, J. Biol. Chem. 204, 423, (1953).
6. D.S. Robinson, J.E. French, Quart. J. Exp. Physiol. 38, 233, (1953).
7. F.T. Lindgren, H.A. Elliott, and J.W. Gofman, J. Phys. Colloid Chem. 55, 80 (1951).

For analysis of the total lipid extract from an incubation mixture to determine triglyceride, fatty acid, and in some instances other lipids as well, a lipid separation was carried out by means of a silicic acid adsorption column in the manner described by Borgstrom[4] and discussed in the previous communication.[3] After their separation, triglyceride and fatty acid were usually determined by two-component infrared spectrophotometric analysis.

The Beer-Lambert law may be written in a generalized form for i components: $A = \ell \sum_i a_i c_i$, where A is the observed optical density (absorbance), ℓ the cell length, a the absorptivity of component i, and c the concentration of component i. Measurement of optical density at 5.8 and 5.9 microns determined fatty acid and triglyceride. In general, the infrared measurements themselves were the same as mentioned previously.[3,8] In some cases, the fatty acids were separated from triglycerides by extraction of the fatty acids with alkaline 50% ethanol-water and re-extraction of the acids after acidification.[4] The acids were then titrated or their infrared absorption at 5.9 microns was measured.

Experimental

Action of postheparin plasma on S_f6 lipoprotein

Because the triglycerides of human $S_f20-400$ lipoproteins are extremely susceptible to hydrolysis, which is accompanied by fatty acid release, it seemed of interest to see whether the human S_f6 lipoprotein, of triglyceride content 10% of its total lipid, could serve as a substrate for the postheparin plasma. A sample of S_f6 lipoprotein, prepared according to previously published methods,[7] that contained 20.2 mg triglyceride was incubated for 8 hours at 37.5°C with 3 ml postheparin plasma. The amount of fatty acid found after incubation (1 mg) agreed closely with that present in the system before incubation (0.9 mg); incubation of human $S_f20-400$ lipoprotein containing 22.0 mg triglyceride with the same plasma resulted in the release of 5.4 mg fatty acid.

Effect of heparin injection on serum tributyrinase activity

That serum possesses lipolytic activity towards tributyrin has been noted by many workers.[9] Consequently comparison was made between the tributyrinase activity of serum and that of postheparin plasma. In experiments carried out by the method of Alper[9] with an incubation time of 2 hours, 3.74, 3.70, and 3.79 ml 0.05 N sodium hydroxide were required to titrate the fatty acids liberated from tributyrin as a result of the action of postheparin plasma; 3.60, 3.54, and 3.59 ml were required in the incubation with preheparin serum.

Inhibition of lipolysis by fatty acid

Since the plasma of an individual who (even though warned against this practice) had eaten a very lipemic breakfast before heparin injection contained a considerable amount of fatty acid, experiments were performed to investigate

8. N.K. Freeman, F.T. Lindgren, Y.C. Ng, A.V. Nichols, J. Biol. Chem., 203, 293 (1953).
9. C. Alper, Standard Methods of Clinical Chemistry, Academic Press Inc., 1953, Vol. 71.

the role of free fatty acid in the triglyceride lipolysis. After incubation of 1 ml e/p with 3 ml postheparin plasma for 4 hours at 37°C, the initial level in the system (at zero time) of 9.5 mg fatty acid (of which 90% was contributed by the plasma) rose to 10.4 mg after 4 hours and 11.0 mg after 6 hours. Plasma from a fasting individual, after it had been incubated with the same substrate, produced a 6 mg rise after 4 hours.

Effects of various substances on fatty acid release

The effects of such substances as adenosine triphosphate (ATP) and Coenzyme A (CoA), diisopropylfluorophosphate (DEP), oxygen (aerobiosis), nitrogen (anaerobiosis), α-napthol, p-chloromercuribenzoate, o-iodosobenzoate, and iodoacetate on fatty acid release from lipoprotein triglycerides are indicated in the table.

Discussion and Summary

Statistical evaluation of the lipid separation and identification methods is not complete, but on the basis of column recoveries, instrumental error, etc., we believe that they are accurate to within about 10%. To a considerable extent we have relied on the reproducibility in several experiments, and on comparison with controls, as criteria of the validity of the observed differences. The data presented indicate that the triglycerides of the $S_f 6$ lipoprotein, in contrast to those of the $S_f 20-400$ lipoproteins, are not good substrates for fatty acid release by postheparin plasma. The tributyrinase activity of serum is not significantly altered after heparin injection. The data also indicate a significant inhibition of lipolysis of triglycerides by fatty acid itself. The inhibitory powers of free fatty acid, as well as the general role of fatty acid and albumin in the reaction, are discussed more thoroughly elsewhere.[10] Such sulfhydryl inhibitors as p-chloromercuribenzoate, o-iodosobenzoate, - and iodoacetate are without effect. Aerobiosis and the antioxidant α-naphthol are without effect, but anaerobiosis with nitrogen produces at most a 10% increase in fatty acid release.

The standard free energy of hydrolysis of most alcohol–fatty acid esters ranges from −3,000 to +3,000 calories. Such a hydrolysis or esterification should be easily reversible unless reaction occurs under conditions that alter radically the component concentrations, e.g., at an interface. Since in our system at most 25% of the available triglyceride was hydrolyzed to fatty acid, it was thought that presence in the incubation mixture of ATP and CoA might result in the formation of substances whose hydrolysis could liberate large amounts of energy to the medium, resulting either in significantly heightened or lessened hydrolysis. The observed 10% diminution of hydrolysis does not argue strongly for this hypothesis and, in view of the catalysis of the fatty acid liberation by substances of such strong negative charge as heparin, it is possible that the strong negative charges on the ATP and CoA molecules, rather than their "high-energy" nature, may be responsible for the appearance of less fatty acid.

Aldridge[11] has shown that the esterases of serum may be classified into two distinct types (A and B) by their sensitivity to organophosphorus inhibitors, the B-type esterase being inhibited by low concentrations and the A-type esterase being resistant. Our data indicate that DFP (diisopropylfluorophosphate) is extremely effective in preventing fatty acid release from triglycerides, and

10. F. T. Lindgren, doctoral dissertation, Univ. of Calif., 1954.
11. W. N. Aldridge, Biochem. J. 53, 110, 117, (1953).

Release of Fatty Acid from Lipoprotein Triglycerides after Incubation with Preheparin Serum or Postheparin Plasma and Various Compounds at 37°C for 4 Hours.

Incubation Constituents	mg Fatty Acid Increase	mg Triglyceride Decrease[a]
3 ml postheparin plasma (Posthp), 2 ml egg lipoprotein (elp)	12.8, 12.5, 12.5	13.6, 13.9, 13.6
3 ml preheparin serum 0.02 mg sodium heparin (Prehs), 2 ml egg lipoprotein	0.0, 0.3, 0.3	0.0, 0.2, 0.3
3 ml posthp, 2 ml elp, DFP ($1x10^{-4}M$)[b]	0.4, 0.5, 0.7	0.4, 0.5, 0.6
3 ml prehs., 2 ml elp, DFP ($1x10^{-4}M$)[b]	0.4, 0.3, 0.2	0.5, 0.3, 0.2
3 ml posthp, 2 ml elp, oxygen	12.3, 12.4, 12.9	13.7, 13.6, 14.1
3 ml prehs, 2 ml elp, oxygen	0.2	0.3
3 ml posthp, 2ml elp, nitrogen	13.4, 13.5	14.7. 14.5
3 ml prehs, 2 ml elp, nitrogen	0.4	0.2
3 ml posthp, 2 ml elp, naphthol ($1x10^{-3}M$)	12.7, 12.9	13.6, 13.8
3 ml prehs, 2 ml elp, naphthol ($1x10^{-3}M$)	0.3	0.4
3 ml posthp, 2 ml elp, p-chloromercuri-benzoate ($1x10^{-3}M$)[c]		13.5, 13.7, 15.1
3 ml prehs, 2 ml elp, p-chloromercuri-benzoate ($1x10^{-3}M$)[c]		0.4
3 ml posthp, 2 ml elp, o-iodosobenzoate ($1x10^{-3}M$)[c]		13.4, 13.8, 13.7
3 ml prehs, 2 ml elp, o-iodosobenzoate ($1x10^{-3}M$)[c]		0.3
3 ml posthp, 2 ml elp, iodoacetate ($1x10^{-3}M$)[c]		13.4, 13.9, 13.7
3 ml prehs, 2 ml elp, iodoacetate ($1x10^{-3}M$)[c]		0.2

3 ml posthp, 1 ml elp	8.2, 8.4, 8.3	9.1, 9.2, ,9.5
3 ml prehs, 1 ml elp	0.2, 0.1	0.2, 0.3
3 ml posthp, 1 ml elp, cysteine ($8x10^{-3}$M)	8.3, 8.5, 8.5	9.0, 9.5, 9.2
3 ml prehs, 1 ml elp, cysteine ($8x10^{-3}$M)	0.2, 0.4	0.0, 0.4
3 ml posthp, 1 ml elp, cysteine ($8x10^{-3}$M) ATP ($1x10^{-3}$M), CoA ($1x10^{-3}$M)	7.3, 7.5, 7.7	8.4, 8.5, 9.0
3 ml prehs, 1 ml elp, cystein ($8x10^{-3}$M) ATP ($1x10^{-3}$M), CoA ($1x10^{-3}$M)	0.2, 0.3	0.4, 0.4
3 ml posthp, 1 ml elp, ATP ($1x10^{-3}$M), cysteine ($8x10^{-3}$M)	7.9, 8.1	8.6, 9.0
3 ml prehs, 1 ml elp, ATP, cysteine ($8x10^{-3}$M)	0.3, 0.4	0.2, 0.4
3 ml posthp, 1 ml elp, CoA ($1x10^{-3}$M), cysteine ($8x10^{-3}$M)	8.0, 8.0	8.8, 8.7
3 ml prehs, 1 ml elp, CoA ($1x10^{-3}$M), cysteine ($8x10^{-3}$M)	0.3, 0.4	0.1, 0.3

[a] No significant decrease in phospholipids or cholesteryl esters. The first values listed in the columns of triglyceride decrease and fatty acid increase were obtained in the same experiment, the second values in the same experiment, and so on.

[b] Incubated with serum or plasma for 20 minutes at 25°C before addition to elp.

[c] Incubated with serum or plasma for 120 minutes at 25°C before addition to elp.

this sensitivity to DFP suggests that the heparin-activated enzyme is a B-type esterase.

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