Role of Serum Albumin in Lipemia Clearing Reaction. (20579)

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Earlier publications from this Laboratory (1,2) have described investigations into the mechanism of the "lipemia clearing reaction" wherein a clearing factor, produced *in vivo* by the intravenous administration of heparin, clears elementary lipemia or artificial oil emulsions *in vitro* in the presence of normal plasma. The cofactor supplied by the normal plasma was termed "coprotein". The preparation of a partially purified, presumably "coprotein"-free, clearing factor which could be used to assay for "coprotein" activity has been reported(2). Attempts were therefore made to purify "coprotein" by the alcohol fractionation methods of Cohn, *et al.*(3), as well as by classical ammonium sulfate procedures, in which it became increasingly evident that the albumin-rich fractions contained considerable "coprotein" activity. Since it was impossible to separate this activity from the serum albumin, experiments were undertaken to clarify the effect of purified albumin itself on the clearing reaction.

Methods. In the first experiments to be described, the source of clearing factor was fresh whole plasma from a dog that was anesthetized with nembutal, treated with 2.5 mg/kg heparin intravenously, and bled after 5 minutes. The purified clearing factor was prepared by the method referred to above(2). Two sources of albumin were employed: commercially available bovine Fraction V (Armour Laboratories), and a human albumin twice crystallized with mercury by the method of Hughes(4). No difference was observed in the behavior of the 2 albumin preparations in these experiments. A coconut oil emulsion supplied by Dr. Douglas Frost of Abbott Laboratories, diluted to 0.5% fat content, was used as substrate. The buffer diluent described previously(2) was employed, and all reaction mixtures contained sufficient quantities of the disodium salt of ethylenediaminetetraacetic acid to prevent the formation of insoluble calcium soaps. Reactions were run at 37°C in a waterbath, and their progress followed by optical density readings in the Coleman Junior Spectrophotometer at a wave length of 500 mμ.

TABLE I. Effect of Added Albumin in the Clearing System.

Clearing system	Decrease in optical density after 10 min.	60 min.
Heparinized D.p* alone	.012	.027
D.p + 0.1 cc 5% a*	.020	.088
D.p + 0.2 cc 5% a	.021	.088

* D.p = dog plasma; a = albumin.

Observations. Effect of added albumin in the clearing system. In a 1 cc test system containing 0.07 cc of clearing factor-containing dog plasma and 0.1 cc of coconut oil substrate, the addition of 0.1 cc of a 5% solution of albumin produced a moderate increase in the initial rate of clearing, and a marked increase in the extent of the reaction after one hour. Further additions of albumin produced no further effect within the one-hour period of observation. Table I illustrates this effect. In another type of experiment, 0.1 cc of dog plasma was allowed to act on 0.1 cc of coconut oil in a total volume of 1 cc for 135 minutes, until the rate of clearing was markedly reduced. At this time, 0.1 cc of 5% albumin was added, resulting in a greater than 3-fold increase in the rate of clearing.

Effect of fatty acid in the clearing system. Sodium oleate was added in varying amounts to a 1 cc clearing system containing 0.1 cc dog plasma, 0.2 cc 5% albumin, and 0.1 cc coconut oil substrate. Increasing inhibition of the clearing reaction was observed with increasing amounts of oleate. In Fig. 1 is shown the extent of clearing after one hour as a function of the amount of oleate added (expressed as moles of oleate per mole of albumin present, assuming that the dog plasma also contains approximately 5% albumin.) It is evident

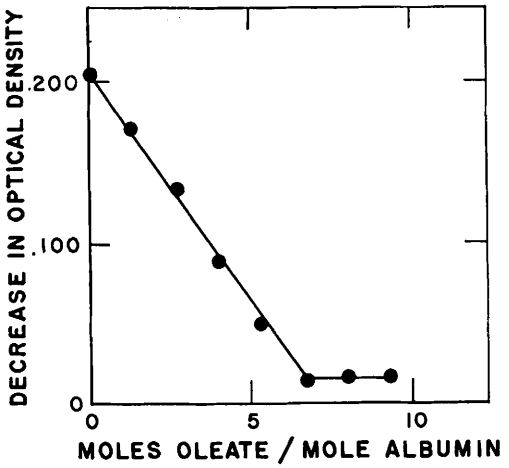

FIG. 1. Inhibition of lipemia clearing reaction by oleate.

that maximum inhibition, the reaction being virtually completely stopped, is reached when 6 to 7 moles of oleate have been added for each mole of albumin present. Sodium palmitate was found to produce inhibition in similar fashion, while glycerol in concentrations up to M/300 was without noticeable effect.

Effect of albumin and serum on purified clearing factor. In a system containing purified clearing factor and an optimal quantity of albumin, the addition of a small amount of normal serum or plasma will greatly accelerate the clearing reaction. Table II outlines a typical experiment to illustrate this effect. Each tube contained 0.1 cc of a 2% solution of purified clearing factor, 0.1 cc of coconut oil substrate, and enough buffer to make a total of 1.0 cc. It is evident that the greatest clearing was obtained in the tube containing normal serum.

Discussion. An impressive body of evidence has been accumulated showing that serum albumin, at physiologic pH values, has a considerable affinity for the anions of various organic acids, including the fatty acids(5-7). Unpublished data of Dr. H. A. Saroff indicate that at pH 4.3, each mole of albumin will bind approximately 2 moles of palmitate ion, while at pH 6.5 it can bind 6 moles per mole. Davis and Dubos(6) find a binding capacity for oleate of about 9 moles per mole of albumin (pH not stated). Cohn, Hughes, and Weare (8) indicate that, as it is prepared from plasma, albumin contains up to one mole of fatty acid per mole of protein. In our inhibition experiments, therefore, a total of 7 to 8 moles of fatty acid per mole of albumin was probably present at the point of complete inhibition of the clearing reaction.

TABLE II. Effect of Albumin and Serum on Purified Clearing Factor.

Cofactor supplied to purified clearing factor	Decrease in optical density after 1 hr	2 hr
0	.004	.009
.2 cc 5% albumin	.046	.069
.2 cc 5% albumin + .005 cc normal serum	.109	.159

It has been found(2,9,10) that during the clearing reaction, fats are hydrolyzed to liberate fatty acids. That fatty acids can inhibit the clearing reaction is shown by these experiments. It seems highly likely, therefore, that serum albumin enters the reaction by binding and thereby removing the fatty acids evolved.

The authors are not aware of published experiments concerned with the possible role of albumin in other serum-catalyzed lipolytic reactions. The highly purified preparations of serum esterase reported by Surgenor and coworkers(11,12) did not appear to lose activity with the progressive removal of albumin, suggesting that this enzyme, at least, can function in the absence of albumin.

Whole normal serum, in minute amounts, considerably enhances the effect of albumin in a purified clearing system, indicating that it provides a second cofactor which is of importance in the clearing reaction. Our present data are not adequate to decide whether this factor is an essential reactant or catalyst, or merely an accelerator. With better purification of the clearing factor it should be possible to resolve this problem. It seems logical meanwhile to reserve the term "coprotein" for this second, as yet unidentified, material in serum. Preliminary experiments indicate that it is thermolabile and non-dialyzable, hence probably protein in nature.

The discovery that two cofactors, albumin and coprotein, play simultaneous roles in the clearing reaction casts doubt on the reliability of our earlier studies on the fractionation and properties of "coprotein" (1,2). Further ex-

periments to isolate and characterize coprotein are in progress.

Summary. Data are presented to show that purified serum albumin enhances the lipemia clearing reaction, probably by binding the fatty acids produced which, if not removed, are capable of inhibiting the reaction. Evidence that albumin is not the only cofactor in the clearing reaction is presented.

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Biosynthesis of C^{14}-Labeled Benzylpenicillin. (20580)

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Although phenylacetic acid is one of the well-established precursors of benzylpenicillin, only limited attention has been given to the metabolic changes which occur when the acid is utilized by a penicillin-producing fungus. For this reason we investigated the problem in more detail. The incorporation of phenylacetic acid-l-C^{14} into benzylpenicillin has been achieved and is reported together with observations which indicate other possible pathways of phenylacetic acid metabolism.

Experimental and results. Phenylacetic acid-l-C^{14} was synthesized by the method of Dauben *et al.**(1). Two separate preparations had specific activities of 15.29 x 10^5 and 30.15 x 10^9 c.p.m. per mole.† The acid of lower activity was used in this investigation. One hundred and sixty ml of semisynthetic medium‡ in 500 ml baffled Erlenmeyer flasks were inoculated with the young (24-36 hour) vegetative mycelium of *Penicillium chrysogenum,* BC-65, Upjohn, and incubated on a rotary shaker at 25-27°C. After incubating for 24 hours, potassium phenylacetate-l-C^{14}, equivalent to 375 μg of the free acid/ml was added. At the end of the fermentation period (96 hours) the pH had risen from 5.5 to 8.4. The amount of penicillin produced was 867 μg/ml, while the control with no phenylacetic acid contained 311 μg of penicillin/ml. The harvested dry mycelium from the fermentation with the added precursor amounted to 11.2 mg/ml. A 200 ml portion of the fermented beer was acidified (pH 2.2) with 25% H_2SO_4 and extracted with amyl acetate. The combined amyl acetate extracts in turn were extracted with saturated phosphate buffer (pH 6.8). The buffer was acidified (pH 2.2) and extracted with ether. After the

* The $BaC^{14}O_3$ used was purchased from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

† Measurements of radioactivity were made on the solid neutralized samples with a thin-window counting tube and autoscaler manufactured by Tracerlab, Inc., Boston, Mass. The values reported are corrected for infinitely thin samples.

‡ The medium consisted of: lactose 50 g, glucose 5 g, corn steep liquor solids 17 g, $CaCO_3$ 3 g, $FeSO_4 \cdot 7H_2O$ 0.004 g, Na_2SO_4 0.5 g; H_2O to 1000 ml; pH 5.5.