Purification and Characterization of an Induced Plasma Lipoprotein that Inhibits Streptolysin O.* (28619)

ROBERT ROWEN (Introduced by Edward J. Hehre)
Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York City

Intravenous administration of streptolysin O preparations in sublethal amounts renders mice refractory to a subsequently administered lethal dose of the same material(1). The induced resistance becomes evident within a matter of hours, is of relatively short duration (less than 40 hours), and does not rest on an immunological basis. Rowen and Bernheimer(2) showed that plasma of refractory mice (RMP) markedly inhibits the *in vitro* hemolytic action of streptolysin O and that the inhibitory activity resides in a lipoprotein fraction of the plasma. Partial purification of the inhibitor was achieved by ultracentrifugal flotation of low density lipoproteins in plasma raised to an approximate density of 1.06 by the addition of salt. More recent experiments(3) have shown that purification can be improved considerably if flotation is carried out at normal plasma density. The current report presents the modified procedure for recovering streptolysin O inhibitory lipoprotein from RMP, and an analysis of the purified material with a view to establishing some correlation between chemical structure and biological activity.

Materials and methods. For production of streptolysin O inhibitory plasma, Swiss Webster mice (Carworth Farms) weighing 16-18 g were given an initial intravenous injection of ¼ of an LD_{50} of partially purified and reduced streptolysin O, followed at 18-20 hours by a second injection of one LD_{50}. Using a syringe rinsed in 2% heparin, the mice were bled directly from the heart 15-18 hours after the second injection. The RMP and normal mouse plasma (NMP) used in the present study represent pools from groups of 25-75 mice. All plasma was stored at —20°C. The procedure employed for *in vitro* titration of inhibitory activity, based on inhibition of streptolysin O hemolysis, has been described in detail elsewhere(2); under the conditions of the titration, 1 unit of inhibitor will neutralize 2 out of 3 hemolytic units of the toxin.

Plasma fractionations were carried out at 4°C in a Spinco model L ultracentrifuge with a 40.3 rotor according to the scheme outlined in Fig. 1. Individual fractions were recovered with the aid of a tube-slicing device. The non-protein solution density of mouse plasma was determined by pycnometry after removing all but trace amounts of protein and lipoprotein by ultracentrifugation.

The micromethod of Scheidegger(4) was used for immunoelectrophoretic analysis of fractions. Patterns were devoloped for 3 days at 4°C, and the washed and dried slides stained for protein with naphthol blue black and for lipid with oil red O. The antiserum employed was from a single rabbit immunized with 4 intravenous injections of RMP (0.5-1.0 ml) per week for 8 weeks.

Floatable fractions of NMP and RMP were analyzed for free and total cholesterol by the modified Schoenheimer-Sperry procedure(5). Lipid phosphorus content, determined by a method similar to that of Stewart and Hendry (6), was multiplied by a factor of 25 to obtain the approximate phospholipid concentration. Estimates of protein content were made with the Folin-Ciocalteu phenol reagent(7) using a protein standard of known nitrogen content. The technic described by Marinetti and Stotz(8) was employed for chromatographic resolution of lipids on silicic acid impregnated paper.

Results. The newly modified procedure for separating streptolysin O inhibitor from RMP and the results of a typical fractionation are outlined in Fig. 1. In the example cited, a 350-fold purification (in terms of units of inhibitor per mg protein) was achieved in a single step, without loss, by ultracentrifugal flotation at normal plasma density. Since the solution density of mouse plasma (exclusive of the contribution of proteins and lipoproteins) was found to be 1.006

* This work was supported by a grant from New York Heart Assn.

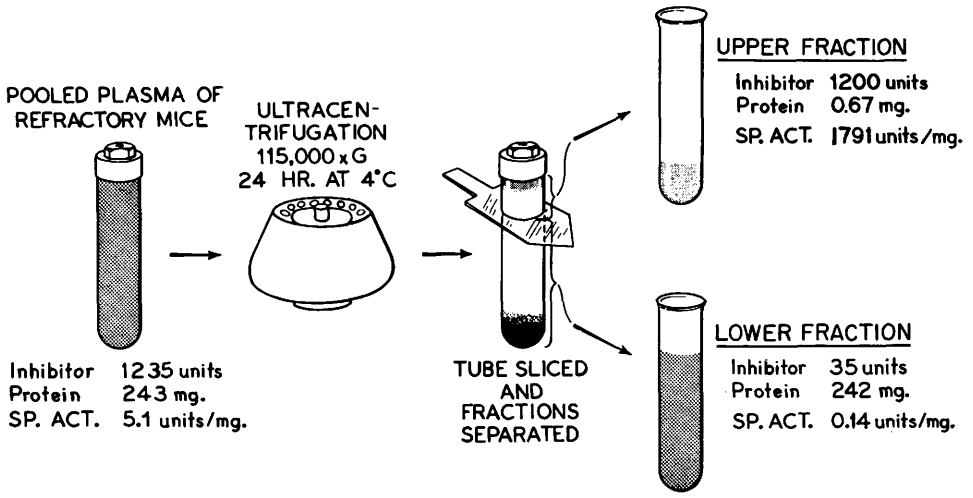

FIG. 1. Procedure for recovery of streptolysin O inhibitor from refractory mouse plasma, including results of a typical fractionation.

TABLE I. Analysis of Floatable Fractions from Refractory and Normal Mouse Plasma.*

	Floatable fraction from 5 ml plasma					
	Streptolysin O inhibitor		Protein	Phospholipid	Cholesterol	
Source of plasma	Units	% recovery	(mg)	(mg)	Free (mg)	Esterified† (mg)
Refractory mice (I)	1200	97	.44	1.97	.50	.44
" " (II)	650	90	.45	1.51	.46	.35
Normal mice	40	>90	.33	1.30	.37	.09
" " (contaminated plasma‡)	600	98	.38	1.66	.33	.31

* All fractions were concurrently analyzed.
† Represented by difference between free and total cholesterol.
‡ *Pseudomonas sp.* isolated.

at 23°C, the floatable inhibitor must have a density below that value. Although nearly all of the inhibitory activity is recovered in the floatable fraction (Table I), a small percentage is always retained in the residual plasma associated with a lipoprotein in the density range 1.006-1.063.

Fig. 2 shows the immunoelectrophoretic patterns of floatable and residual fractions, recovered from RMP (A) and NMP (B) by the above procedure, developed with antiserum to RMP. The effectiveness of the separation is clearly evident. Although concentrated 4-fold with respect to the original plasma, the floatable fractions show only some antigenically active lipoprotein with an electrophoretic mobility similar to albumin. Other plasma proteins, including normally-occurring lipoproteins localized in the regions of α_1-globulin and pre-albumin (ρ region),† are completely retained in the residual plasma. The only readily apparent difference between the floatable fractions of NMP and RMP disclosed by immunoelectrophoresis is the greater abundance of lipoprotein in the latter fraction. Whether or not the inhibitor-rich RMP fraction contains an antigenic component peculiar to it is uncertain.

In spite of the extensive purification achieved by ultracentrifugation, it was to be expected that floatable fractions of RMP

† As the antiserum employed failed to react with the more rapidly migrating lipoprotein, this plasma component is not represented in the patterns. Its presence was determined by staining the slides immediately following electrophoresis.

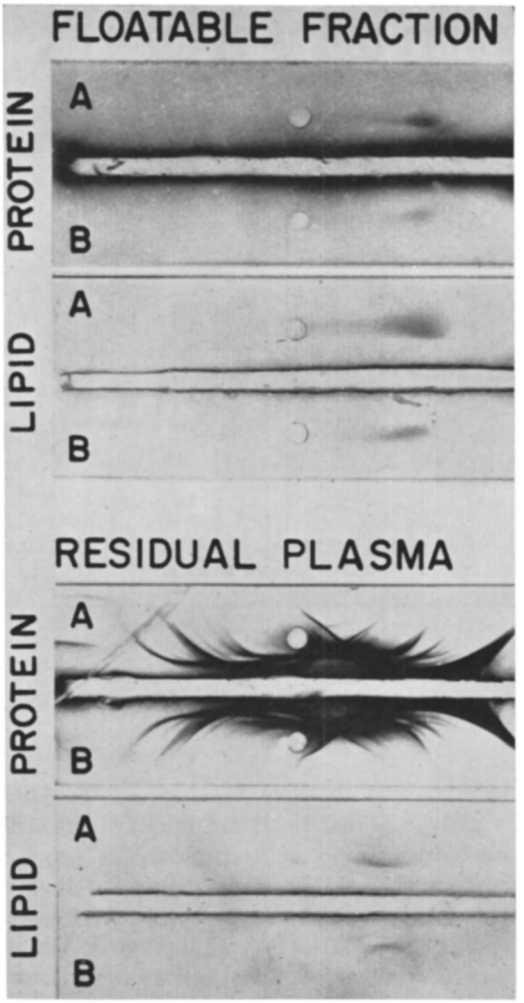

FIG. 2. Immunoelectrophoretic patterns of fractions recovered after ultracentrifugation, developed with rabbit antiserum to RMP. A = fraction derived from RMP; B = fraction from NMP. Floatable fractions are concentrated 4-fold with respect to the original plasma.

would contain normally-occurring chylomicrons and very low density lipoproteins in addition to inhibitor. Hence, to further characterize the inhibitor, differences were sought between the floatable fractions of RMP and comparable fractions from NMP. Chemical analyses of each (Table I) indicate the presence of an extremely high proportion of lipid relative to protein. The data further show that increased streptolysin O inhibitory activity is accompanied by elevated values for protein as well as lipid. Although no direct proportionality was found between inhibitory activity and the concentration of any of the constituents measured, the values for esterified cholesterol most closely parallel the ability of the fractions to inhibit the hemolytic action of streptolysin O; esterified cholesterol in the floatable fractions from inhibitory plasma was approximately 3-5 times higher than normal.

The accidental contamination of a sample of NMP with a species of *Pseudomonas,* which rendered the plasma markedly inhibitory to streptolysin O, provided an opportunity to carry out a supplementary experiment. (Nonspecific increases in antistreptolysin O titer resulting from bacterial contamination and various *in vitro* treatments of serum have been reported by others(9).) The contaminated plasma was fractionated and the floatable fraction analyzed concurrently with the NMP and RMP fractions considered above. The results, which are included in Table I, show that the inhibitory material of contaminated plasma is also floatable at normal plasma density. Protein in the recovered fraction was elevated only slightly, but phospholipid and esterified cholesterol values were comparable to those found for RMP fractions. In contrast to the RMP fractions, unesterified cholesterol in the fraction from contaminated plasma was somewhat lower than normal.

The lipids of RMP, NMP and their floatable fractions were compared by chromatography on silicic acid impregnated paper. As shown in Fig. 3 the lipids found in the floatable fractions are those characteristic of intact plasma; no unusual lipid was detected in whole RMP or its floatable fraction. Chromatograms (A) in which neutral lipids are separated indicate that triglycerides, which make up a sizable portion of the floatable lipids of RMP and NMP, are present in higher concentration in the former, as are cholesterol and cholesterol esters. Phospholipid chromatograms (B) show that the floatable fraction from RMP has more lecithin, sphingomyelin and lysolecithin than the comparable fraction from NMP.‡ That whole unfractionated RMP contains more abundant

‡ Chromatograms were not tested for "cephalins".

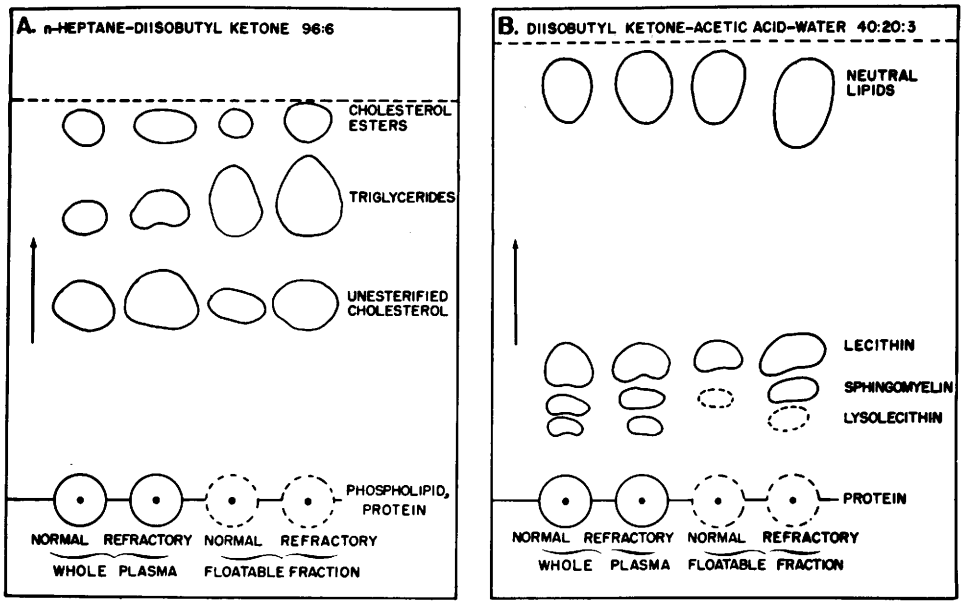

FIG. 3. Chromatographic resolution on silicic acid impregnated paper of A, neutral lipids and B, phospholipids of whole NMP, whole RMP and the floatable fraction of each. Floatable fractions were collected in one-fourth the volume of original plasma. Volume of plasma or concentrated fraction applied to paper was 25 μl. Chromatograms stained with rhodamine 6G and viewed under UV. Solid outlines indicate strong fluorescence; broken outlines faint fluorescence.

lipid material than whole NMP is also shown in the chromatograms of Fig. 3; this has been confirmed by chemical analysis.

Discussion. The prime asset of ultracentrifugal floatation as a means of separating streptolysin O inhibitor from mouse plasma is the extensive purification achieved under conditions least likely to alter normal or abnormal plasma lipoproteins. Consistent with its low density, the material recovered by floatation shows a sufficiently high proportion of lipid to protein to allow substantial exposure of the lipid moiety. A constituent such as cholesterol, a well-known inhibitor of streptolysin O(10), may thus be so oriented as to permit interaction with the toxin; cholesterol in ordinary plasma lipoproteins is prevented from exerting its inhibitory effect. The marked elevation of esterified cholesterol in inhibitor-rich fractions of mouse plasma is of special interest since little is known of the effect of sterol esters on streptolysin O. The possibility that certain cholesterol esters in floatable fractions of RMP may directly inhibit the toxin is currently being investigated.

Although past(2) and present experiments indicate that the floatable inhibitor in RMP is closely related to, if not identical with, an inhibitory lipoprotein present in smaller amount in NMP, practically nothing is known of the mechanism by which streptolysin O stimulates the production of inhibitor. Preliminary studies indicate that treatment of mice with streptolysin O causes a generalized increase in all major classes of plasma lipoproteins. This suggests that *in vivo* inhibitor production is more complex than the mere modification or degradation of existing plasma lipoproteins which presumably accounts for increased streptolysin O inhibitory activity following certain *in vitro* treatments of plasma (*e.g.*, bacterial contamination; freeze-drying; treatment with dilute acids or bases, etc.).

The existence in mice of a non-immunological protective mechanism against the lethal action of streptolysin O directs attention to a virtually unexplored area of host resistance, namely, the possible influence of serum lipoproteins on the activity of a variety of cytolytic toxins. The poisonous quality of streptolysin O, streptolysin S, pneumolysin,

clostridial lecithinases, and perhaps the majority of hemolytic toxins, may depend on their ability to disrupt the delicate lipid-protein relationships within membranes of living mammalian cells. Specific lipoproteins, like the experimentally induced streptolysin O inhibitor in mouse plasma, or the naturally-occurring streptolysin S inhibitor in human and animal sera(11,12), may, by way of similarity to some structural sub-unit of the susceptible membrane, combine with and competitively inactivate such toxins.

Summary. A simple technic is described for separating streptolysin O inhibitor from the plasma of mice made refractory to the lethal effect of streptolysin O. Results of fractionation and subsequent analysis indicate that the bulk of the inhibitory activity of refractory mouse plasma is associated with a lipoprotein of extremely low density (<1.006) containing abundant lipid relative to protein. All classes of serum lipids are represented in the inhibitor-rich fraction; their concentrations, as well as the concentration of protein, are significantly greater than values obtained for comparable fractions from normal mouse plasma. Esterified cholesterol shows the greatest increment and seems most closely to parallel the inhibitory activity. It is suggested that the capacity of the induced lipoprotein to inhibit streptolysin O depends on an abundant content and unique orientation of lipids that are capable of interacting with the toxin.

The technical assistance of Mrs. Janet Martin and Mrs. Harriet Kaplan is gratefully acknowledged.

1. Bernheimer, A. W., Cantoni, G. L., *J. Exp. Med.*, 1947, v86, 193.
2. Rowen, R., Bernheimer, A. W., *J. Immunology*, 1956, v77, 72.
3. Rowen, R., *Bacteriol. Proc.*, 1962, p80.
4. Scheidegger, J. J., *Intern. Arch. Allergy and Appl. Immunol.*, 1955, v7, 103.
5. Sperry, W. M., Webb, M., *J. Biol. Chem.*, 1950, v187, 97.
6. Stewart, C. P., Hendry, E. B., *Biochem. J.*, 1935, v29, 1683.
7. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry*, Charles C Thomas, Springfield, 1961, p557.
8. Marinetti, G. V., Stotz, E., *Biochim. Biophys. Acta*, 1960, v37, 571.
9. Bernheimer, A. W., in *Streptococcal Infections*, Columbia U. Press, New York, 1954, p19.
10. Hewitt, L. F., Todd, E. W., *J. Path. Bact.*, 1939, v49, 45.
11. Humphrey, J. H., *Brit. J. Exp. Path.*, 1949, v30, 365.
12. Stollerman, G. H., Bernheimer, A. W., MacLeod, C. M., *J. Clin. Invest.*, 1950, v29, 1636.

Received June 24, 1963. P.S.E.B.M., 1963, v114.

Cytochemical Observations on Intranuclear Inclusion of Feline Viral Rhinotracheitis Virus. (28620)

ROBERT A. CRANDELL* AND DAVID F. HERSEY† (Introduced by S. S. Kalter)
Armed Forces Institute of Pathology, Washington, D. C.

The sequence of the development of inclusion bodies in cultures of feline renal cells infected with feline viral rhinotracheitis virus has been reported(1). In hematoxylin and eosin preparations, the earliest discernible change was stippling of the nuclei with basophilic chromatin. Later, these chromatin granules appeared as fine particles dispersed on a light-staining background of nuclear substance. These granules and the nucleoli eventually marginated and the nuclear material contracted, forming an acidophilic inclusion surrounded by a clear halo.

Histochemical methods have been used to demonstrate the presence of desoxyribonucleic acid in animal tissue and cell cultures

* Present address: USAF Epidemiological Laboratory, Lackland Air Force Base, Texas.

† Present address: Science Information Exchange, Washington, D.C.