THE RELEASE OF CHOLESTEROL ESTERS FROM SERUM LIPOPROTEINS BY EXTRACTS OF CERTAIN GROUP A STREPTOCOCCI*

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In the course of studies on hemolytic streptococci derived from human sources, Ward and Rudd (1) in 1938 encountered certain Group A strains which gave rise to a marked opalescence when grown in a medium rich in horse serum. The opalescence, which the authors believed to be a result of enzymic denaturation of serum protein, could be reproduced in rabbit, mouse, or human sera by Seitz filtrates of serum-broth cultures of the active strains. The factor responsible for opalescence was shown to be heat-labile and inhibited by KCN. Krumwiede (2), investigating this phenomenon a number of years later, found the opalescence-producing principle to be a cellular component of Group A streptococci, extractable from washed cells with the aid of concentrated solutions of urea. Using these extracts, she showed by means of paper electrophoresis that opalescence is accompanied by a particular alteration in the electrophoretic distribution of serum lipids; *i.e.*, by a shift in lipid from the region of α_1-globulin to the region of β-globulin or the origin. No change in the concentration of the several classes of serum lipids was detected, nor was any change observed in the electrophoretic pattern of the serum proteins. Krumwiede suggested that the extracts contained an enzyme, termed "lipoproteinase," which initiated opalescence by splitting α_1-lipoprotein into its lipid and protein moieties. Rowen and Bernheimer (3) subsequently found that an extract prepared by Krumwiede partially inactivated a streptolysin O inhibitor of lipoprotein nature found in mouse plasma following the intravenous administration of sublethal quantities of streptolysin O.

Although the available evidence indicates that a factor produced by certain Group A streptococci can alter serum lipoproteins, to date there has been no report concerning the precise chemical nature of this alteration. The present investigation is an initial effort to elucidate the mechanism of action of the streptococcal factor that causes serum to become opalescent.

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Materials and Methods

Streptococcal Extracts.—Extracts were prepared from a strain (2RP196) of Group A streptococcus Type 12 originally isolated from a patient with rheumatic fever. This strain was selected on the basis of preliminary tests made upon more than 100 Group A strains, most of which were kindly furnished by Dr. Alan W. Bernheimer; it sedimented rapidly upon centrifugation and yielded extremely potent extracts. The organisms were grown at 37°C in 5 liter carboys of fresh beef heart infusion broth buffered at pH 7.8–8.0 and containing 4 per cent glucose, 0.2 per cent potassium bicarbonate, and 0.8 mg per cent phenol red. The glucose and bicarbonate were sterilized by Seitz filtration and added to the broth just prior to inoculation. Carboys of the complete medium, warmed to 37°C, were inoculated in the early morning with 500 ml of a fresh overnight serum-broth culture of strain 2RP196, and incubated at 37°C for 8 to 10 hours with frequent neutralization of the acid formed using 5 N NaOH. Grown cultures were placed at 4°C overnight prior to harvesting and extracting the bacteria. The separated streptococci (20 to 40 gm wet weight) from each 5 liter batch of culture were washed several times with cold 0.15 M NaCl and extracted with 150 to 200 ml of 40 per cent urea in M sodium acetate at pH 7.0. The extraction and subsequent concentration of the extract generally followed the procedure outlined by Krumwiede (2). As a final step, extracts were dialyzed either against 0.1 M sodium acetate or 0.067 M phosphate buffer containing 0.08 M NaCl at pH 7.0, and stored at −20°C. Based on a crude opalescence titration procedure, extracts stored in this way appeared to retain full activity for several years.

Sera.—Fresh horse serum without preservative was generously supplied by Miss A. Walter of the Bureau of Laboratories, New York City Department of Health. Human serum was obtained aseptically from bleedings of apparently healthy young adults following an overnight fast. All sera were kept at 4°C until use (usually within 2 months) and every precaution taken to maintain strict asepsis during their storage and utilization.

Ultracentrifugation.—Ultracentrifugal fractionations of sera, to be more fully described in the experiments to follow, were carried out at 16–18°C in a Spinco model L preparative ultracentrifuge utilizing a 40.3 rotor. Material separated by flotation was recovered with the aid of a Spinco tube-slicing device.

Measurement of Opalescence.—Opalescence was measured as the difference in optical density at 540 mμ between an incubated serum-extract mixture and a concurrently incubated serum-diluent control. In cases where opalescence of test mixtures was pronounced, suitable dilutions in saline were examined. Readings were made in a Beckman DU spectrophotometer using standard cuvettes having a light path of 1 cm.

Chemical Determinations.—Free and total cholesterol were determined by the modified Schoenheimer-Sperry procedure (4); values for cholesterol esters were obtained by multiplying the difference between free and total cholesterol by 1.68 (5). Phospholipid was estimated from lipid phosphorus measured by a method similar to that of Stewart and Hendry (6); a factor of 25 was used to convert the lipid phosphorus to phospholipid. Total lipid was measured gravimetrically after Folch extraction and purification of the lipid extract (7). Although specially constructed, extremely light weighing-shells were used to minimize error, the lower limit of total lipid concentration that could be determined with reasonable accuracy was 10 mg per cent. A micro-Kjeldahl procedure (8) was used for total nitrogen.

Paper Electrophoresis.—To observe the electrophoretic distribution of lipids in serum or serum fractions, samples were prestained with acetylated Sudan black B following the procedure recommended by McDonald and Bermes (9); a slight modification was introduced to increase staining efficiency; *i.e.*, several additions of the alcoholic dye solution were made to the serum or fraction, with removal of alcohol *in vacuo* between each addition. 70 μl of each prestained sample was applied to a strip of Whatman 3MM paper and subjected to electrophoresis in a Spinco Durrum-type cell, using veronal buffer at pH 8.6 and 0.05 ionic strength, and a constant current of 3 ma at room temperature for 6 hours.

Lipid Chromatography.—Lipids were chromatographed directly on silicic acid–impregnated paper without solvent extraction of the samples, according to the method of Marinetti and Stotz (10). Twenty-five μl of each sample, containing 4 to 55 μg of total lipid, was spotted on the paper. Ascending chromatograms were developed at 23°C with *n*-heptane:diisobutyl ketone (96:6 *v/v*) for 1 hour (for non-phospholipids), and with diisobutyl ketone:acetic acid:water (40:20:3 *v/v*) for 4 hours (for phospholipids). The chromatograms were stained with rhodamine-6-G and examined for fluorescence under ultraviolet light. To detect "cephalins," chromatograms were sprayed with a freshly prepared solution of ninhydrin in acetone-lutidine (9:1 *v/v*).

EXPERIMENTAL

The Separation of Low Density Products Formed through the Action of Streptococcal Extracts on Serum

Proceeding on the premise that the opalescence produced in serum by streptococcal extracts might be due to low density lipids or lipid-protein complexes liberated from their normal associations with serum lipoproteins, an experiment was undertaken to determine whether such products could be separated from extract-treated sera by ultracentrifugal flotation at normal serum density.

TABLE I

Recovery by Ultracentrifugal Flotation of Material Responsible for Opalescence in Sera Incubated with Streptococcal Extract

Source of serum	Degree of opalescence*		Recovery
	Unfractionated serum-extract mixture‡	Isolated low density fraction	
	optical density units	*optical density units*	*per cent*
Horse, No. 1-87	1.01	1.01	100
Horse, No. 1-88	1.09	1.07	98
Human, Gro	1.07	0.98	92
Human, Ger	1.15	1.08	94

* Difference in optical density at 540 mμ between each mixture or fraction and the corresponding buffer control.

‡ Mixtures incubated at 37°C for 24 hours; final dilution of extract, 1:200.

Samples of several human and horse sera were incubated for 24 hours at 37°C with streptococcal extract in a dilution (1:200) known to provide a high degree of opalescence. After measurement of the opalescence produced, 5 ml of each mixture was placed in a capped 6.5 ml lusteroid centrifuge tube with the aid of a hypodermic syringe and 23 gauge cannula. Tubes were filled to capacity with 0.15 M NaCl, plugged, and centrifuged at 40,000 RPM for 18 hours. Upon completion of the run, each tube showed 3 distinct zones: an uppermost thin milky layer, a clear intermediate zone representing about two-thirds of the total volume, and a lower straw-colored zone of sedimented protein. Tubes were sliced about 1 cm below the top layer and, by several washings of the upper section of the tube with saline, the floating material was quantitatively recovered in the same volume (5 ml) as that of the original mix-

ture placed in the tube. The opalescence of each recovered low density fraction was measured against a control preparation derived from a serum-buffer mixture that had been incubated and fractionated in parallel with the corresponding serum-extract mixture. In addition, the low density fraction from one of the incubated extract-serum mixtures (horse 1-88) was subjected to paper electrophoresis after prestaining for lipoprotein. Included in the same run were prestained samples of the unfractionated test and control mixtures containing this particular serum.

As shown by the data in Table I, ultracentrifugal flotation at normal serum density provides effective separation of the products responsible for opalescence. Thus, the floatable fraction exhibited 92 to 100 per cent of the opal-

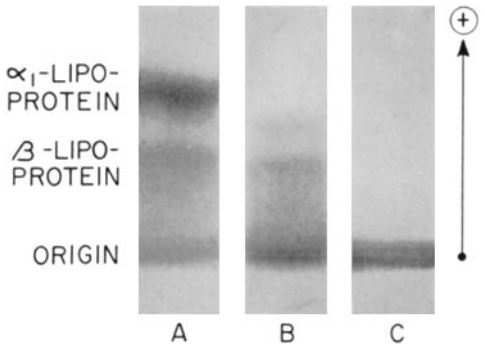

FIG. 1. Lipid patterns following paper electrophoresis of A, horse serum 1-88 incubated at 37°C for 24 hours with buffer (no streptococcal extract); B, same horse serum incubated with streptococcal extract (unfractioned mixture); C, ultracentrifugally derived opalescent fraction of B. All samples were prestained with acetylated Sudan black.

escence of the unfractionated reaction mixtures while the infranatant fraction was, in all cases, crystal clear. This result is consistent with the concept that lipoidal products are responsible for opalescence in extract-treated sera. That the streptococcal extract affects α_1-lipoprotein of horse serum, and that ultracentrifugal flotation provides a clean-cut isolation of low density lipoidal products is evident from the lipid-stained paper electrophoretic patterns of Fig. 1. Following incubation of horse serum with the extract there is complete disappearance of the α_1-lipoprotein band with a concomitant accumulation of sudanophilic material in the region of the origin, and the appearance of a faint new band of lipoidal material in the region between α_1- and β-lipoprotein. The ultracentrifugally separated opalescent fraction, concurrently subjected to electrophoresis, shows material at the origin with a strong affinity for the Sudan stain; it otherwise appears free of the normal lipid-containing components of the serum. The absence from this fraction of the new mobile component of the extract-treated serum, noted above, suggests that this component may represent a high density product of extract action. Electro-

phoretograms of floatable fractions separated from control sera incubated without extract do not show non-migrating lipoidal material.

Chemical Characterization of the Low Density Reaction Products

Examination of the fraction recovered by ultracentrifugal flotation seemed to offer an excellent opportunity to identify and measure one or more of the products of the action of the streptococcal extracts on serum. It was necessary to consider, however, that the recovered fraction might also contain traces of normally occurring low density lipoproteins (11), and possibly products of lipoprotein degradation arising independently of the action of the streptococcal extract (12). Hence, in obtaining information concerning the chemical nature of the low density reaction products, concurrent analyses were made as routine of fractions prepared from sera incubated with and without extract.

Fresh samples of one human and two horse sera were cleared of material floatable at normal serum density by preliminary ultracentrifugation under conditions identical with those used for the recovery of low density reaction products. The small amount of floatable material in each case was discarded and the remaining serum brought to the original volume with 0.15 M NaCl. The total cholesterol and phospholipid contents of these sera, in mg per 100 ml were 77.8 and 155.6 for horse 1-55; 62.6 and 123.4 for horse 1-75; 164 and 194.4 for the human serum. Twenty ml of each of these prepared sera were incubated at 37°C with 0.2 ml of streptococcal extract in 0.1 M sodium acetate, or with 0.2 ml of the acetate solution as control; test and control mixtures were at pH 7.3–7.5. Opalescence, measured on small aliquots of test mixtures, was nearly maximal by 3 hours. After 6 hours at 37°C definitive determination of opalescence was made, and duplicate 5 ml samples of all incubated mixtures fractionated by ultracentrifugation as in the preceding experiment. After tube sectioning, the upper fraction in each tube was quantitatively recovered and adjusted to a volume of 5 ml. The prepared fractions were analyzed for total lipid, unesterified and total cholesterol, lipid phosphorus, and total nitrogen. Direct protein analyses were not carried out because, in trials using several commonly employed methods, the opalescence and fatty nature of the fraction recovered from extract-treated sera had been found to interfere with such analyses. Unesterified fatty acids were not measured since previous tests had indicated their virtual absence from the ultracentrifugally recovered products.

Table II shows the composition of the low density fractions obtained from the two samples of horse serum and the sample of human serum incubated in the presence and absence of the streptococcal extract. As the fractions from each extract-treated serum and its control were concurrently prepared and analyzed, the difference may be taken as a valid representation of the low density products released through the action of the extract. Cholesterol esters represented the major reaction product, and actually comprised 85 to 90 per cent of the total low density lipid released. Moreover, the liberated cholesterol accounted for a sizeable part of the total cholesterol present in the original sera: 18 per cent in the case of the human serum, and 75 and 92 per cent for the horse sera. Unesterified cholesterol was in no instance detected in the low density product fractions of the incubated mixtures. On the other hand,

some phospholipid was apparently released through the action of the extract; the amount (4.5 to 12.8 mg per cent) was relatively small, representing 7 to 12 per cent of the total low density lipid released, or 2 to 10 per cent of the total serum phospholipid. Although the values for total lipid are of limited accuracy, they nevertheless tend to indicate that esterified cholesterol and phospholipid probably are the only lipids present in significant concentration among the low density products. A small amount of lipid unaccounted for in the low density products from the human serum may represent released triglyceride,

TABLE II

Effect of Streptococcal Extract on Serum as Reflected by Changes in the Chemical Composition of the Fraction Obtained by Ultracentrifugal Flotation at Normal Serum Density

		Incubated mixture	Opalescence*	Components of low density fraction (mg per 100 ml native serum)				
				Total lipid	Cholesterol esters	Unesterified cholesterol	Phospholipid	Non-lipid nitrogen‡
I	(*a*)	Horse serum 1-55 plus extract	0.86	111	97.9	0§	17.4	5.5
	(*b*)	Horse serum 1-55 plus diluent	0.00	3 (<10)	0.0	0	7.7	5.5
	(*a-b*)	(Low density products)	0.86	*ca* 108	97.9	0	9.7	0.0
II	(*a*)	Horse serum 1-75 plus extract	1.03	107	97.1	0	21.4	7.9
	(*b*)	Horse serum 1-75 plus diluent	0.00	0 (<10)	0.0	0	8.6	7.0
	(*a-b*)	(Low density products)	1.03	*ca* 107	97.1	0	12.8	0.9
III	(*a*)	Human serum, Der., plus extract	0.93	60	50.7	0	18.5	4.8
	(*b*)	Human serum, Der., plus diluent	0.00	0 (<10)	0.0	0	14.0	4.8
	(*a-b*)	(Low density products)	0.93	*ca* 60	50.7	0	4.5	0.0

* Expressed as optical density units.

‡ Total nitrogen minus phospholipid nitrogen (assuming N/P in phospholipid to be unity).

§ Not detected; probably less than 1 mg per 100 ml.

but the insensitivity of the total lipid determination makes this uncertain. The lack of any appreciable difference in non-lipid nitrogen between the fractions derived from extract-treated and control sera indicates the probable absence of protein from the low density reaction products.

Although cholesterol esters represented the major lipid component of the opalescent fraction of each of the treated sera, the amount released relative to the degree of opalescence differed from serum to serum. A uniform proportionality was also lacking between opalescence and the amount of phospholipid released through extract action.

In an experiment designed to supplement the above chemical analyses, the lipids in the floatable fraction of extract-treated and control sera were examined chromatographically using silicic acid–impregnated paper and two solvent systems, A and B, capable respectively of resolving phospholipids and

neutral lipids. Fractions were prepared in the same manner as for the chemical analyses except that each was recovered in a volume one-half that of the original serum. 25 μl samples were directly chromatographed, along with appropriate reference lipids, and the chromatograms examined for fluorescence under ultraviolet light after staining with rhodamine-6-G.

The resulting chromatograms (Fig. 2) show the resolution of lipids contained in the low density product fraction of two different extract-treated sera (horse and human), as well as the absence of detectable lipid in the fractions from the corresponding serum-buffer controls. With solvent system A, the low density products of the treated horse and human sera are seen to consist of abundant neutral lipid plus a relatively small amount of phospholipid migrating with the velocity of lecithin. The patterns obtained with solvent system B established that all, or nearly all, of the neutral lipid consists of cholesterol esters. Triglycerides appear to be absent from the low density products of both sera, while a trace of unesterified cholesterol is detectable in the fraction from the treated human serum. (Free cholesterol was never discerned among the floatable products of treated horse sera, nor consistently found with other samples of human serum exposed to the streptococcal extract.) The fluorescent material evident at the origin in the chromatograms of fractions of both extract-treated sera with solvent system B doubtless includes the minor phospholipid component noted above. Though not illustrated in Fig. 2, chromatograms of the same fractions, developed in solvent system A and sprayed with ninhydrin, failed to reveal any phosphatidyl ethanolamine or phosphatidyl serine. The area of sample application did show faint ninhydrin staining of equal intensity with product and control fractions, presumably due to traces of protein or polypeptide. The chromatographic studies thus substantiate the chemical analyses in implicating esterified cholesterol as the prime low density product released by the action of the extract on serum lipoproteins. In addition, they affirm that phospholipid is also liberated in small amount, and indicate that the latter may be a single substance, namely lecithin.

Although unesterified cholesterol is usually not detected among the low density reaction products, one cannot be certain it is not released by extract action. Because of its relatively high density (*ca* 1.06), free cholesterol, unless associated with other lipids, would not be recoverable by flotation at the unadjusted solution density of serum. Freely dispersed cholesterol in minute amounts is, however, extremely inhibitory to the hemolytic action of streptolysin O (13) and, if present in significant quantity in extract-treated sera, would be expected to increase the streptolysin O inhibitory activity of these sera. Streptolysin O inhibitor titrations were carried out by a very sensitive procedure (3) on several samples of human and horse sera before and after treatment with extract. Elevated titers could not be demonstrated in the extract-treated sera, suggesting that if cholesterol is released as such, it must

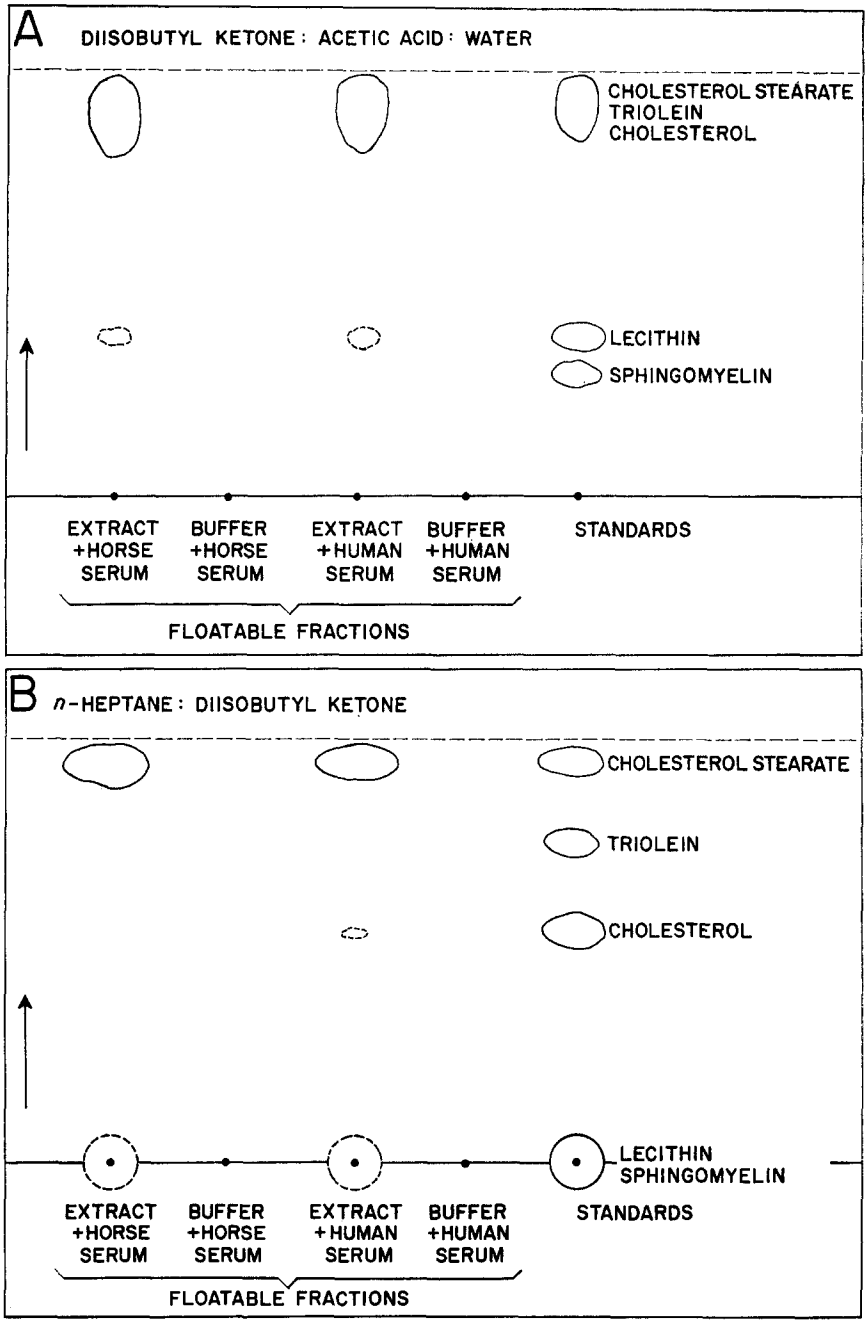

FIG. 2. Chromatographic resolution on silicic acid–impregnated paper of phospholipids (solvent system A) and neutral lipids (solvent system B) in fractions separated by ultracentrifugal flotation from incubated serum-extract and serum-buffer mixtures. Incubations were at 37°C for 6 hours; final dilution of extract 1:100. Chromatograms stained with rhodamine-6-G were examined under ultraviolet light. Solid outlines indicate pronounced fluorescence; broken outlines, faint fluorescence seen best on moist chromatograms.

undergo rapid recombination. Indeed, as will be described in a later report (14), streptococcal extracts can augment the process of cholesterol esterification that occurs spontaneously at a very slow rate when fresh human serum is kept at 37°C (15).

Factors Influencing the Rates of Opalescence Development and Cholesterol Release in Extract-Treated Serum

In order to further characterize the interaction of the streptococcal extract and serum, kinetic studies were carried out under varied conditions of pH, extract concentration, and incubation temperature. Since one major purpose was to discover the relationship, if any, between the development of opalescence and the release of cholesterol, these two effects were compared in all cases. Measurement of released cholesterol at specific time intervals was made possible by the finding, in preliminary experiments, that the activity in extract-serum mixtures is arrested by acidification to pH 4.0. Horse serum was chosen as "substrate" for these studies because the bulk of its cholesterol is able to be liberated by the action of the extract; moreover, incubated horse serum–buffer mixtures rarely contain any cholesterol floatable under the conditions used to recover low density reaction products. Specially stabilized serum was used throughout to prevent the spontaneous increases in pH that might otherwise occur on incubation through the loss of CO_2 (16).

Effect of pH.—

A large sample of horse serum was first stabilized by repeated adjustment to pH 6.5 with N HCl, with removal of released CO_2 under partial vacuum at room temperature after each adjustment. This serum, which remained at pH 6.5 after being subjected to reduced pressure for 1 hour, finally was filtered through sintered glass of fine porosity. Five 40 ml portions were individually adjusted to a particular pH in the range 4.0 to 8.0 by the addition of N HCl or N NaOH, and the small volume increments equalized with 0.15 M NaCl. 20 ml of serum at each pH was mixed with 5 ml of a 1:20 dilution of streptococcal extract in 0.2 M buffer (acetate for pH 4.0 and 5.0; phosphate for pH 6.0 and 7.0; tris for pH 8.0), and incubated at 37°C. A control mixture containing buffer in place of diluted extract was prepared at each pH and incubated concurrently. Aliquots were taken from test and control mixtures after 1 and 2 hours at 37°C for measurement of opalescence (1 ml)[1] and cholesterol release (5 ml). Samples for the latter determination were immediately adjusted to pH 4.0, and ultracentrifuged as described above for the recovery of low density reaction products. By appropriately accounting for dilution, the amount of esterified cholesterol recovered by floatation could be expressed in terms of 100 ml of the original serum. As cholesterol was found only in the fractions from serum-extract mixtures, no correction for the control was necessary in calculating the amount released through extract action.

[1] For convenience, optical density was measured without changing the pH of the mixtures. Control experiments indicated that bringing all samples to a common pH would not have altered the results.

The data in Fig. 3 show that the interaction of streptococcal extract and horse serum proceeds effectively over the range of pH 5.0 to 8.0, but is scarcely detectable at pH 4.0. In the pH 5.0–8.0 reaction mixtures, the opalescence produced and cholesterol released during the 1st hour of incubation parallel one another, with both effects maximal at pH 6.0.[2] During the 2nd hour of incubation, however, the rate of opalescence development in each reaction mixture falls off sharply compared with the rate of cholesterol release. The divergence is greatest in the mixture (pH 6.0) with the highest initial reaction

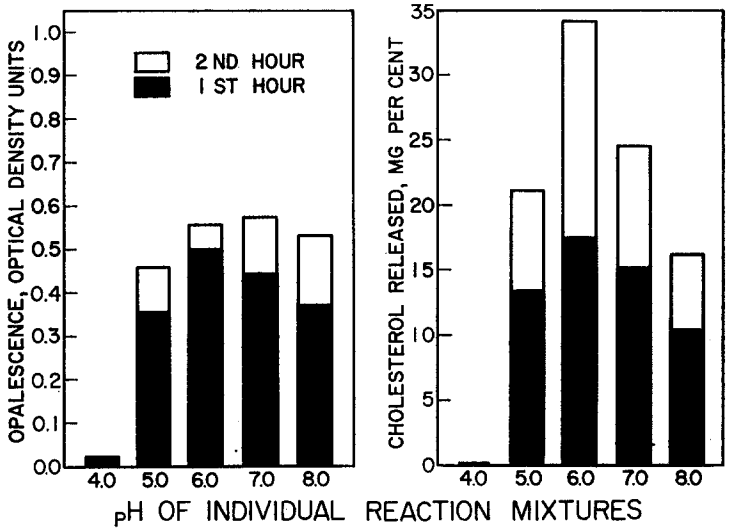

FIG. 3. Opalescence production and cholesterol release as a function of pH. Determinations were made after incubation of individual mixtures at 37°C for 1 and 2 hours. Final dilution of extract in all cases, 1:100.

rate where, during this period, the amount of released cholesterol is doubled while opalescence is increased only about 10 per cent. After 2 hours of incubation a higher degree of opalescence is, in fact, attained at pH 7.0 than at pH 6.0.

In a supplementary test in which a sample of the same serum was incubated with the extract in a final dilution of 1:10 instead of 1:100, the divergence at pH 6.0 was even more pronounced. Cholesterol release in this case was essentially complete (33.1 mg per cent) after 1 hour of incubation, whereas, the opalescence produced amounted to only 0.30 optical density units, as compared to 0.50 for the corresponding mixture (see Fig. 3) with the more dilute extract.

[2] Experiments with mixtures differing by only 0.4 pH unit indicate that the true optimum is closer to pH 5.8 than to pH 6.0.

This result recalls Krumwiede's finding of diminished opalescence at high extract concentration despite maximal alteration in the electrophoretic distribution of serum lipids.

Effect of Extract Concentration.—Since the relationship between opalescence and cholesterol release is evidently profoundly influenced by extract concentration, the progress of the two induced changes in serum with time was systematically compared at several different concentrations of streptococcal extract.

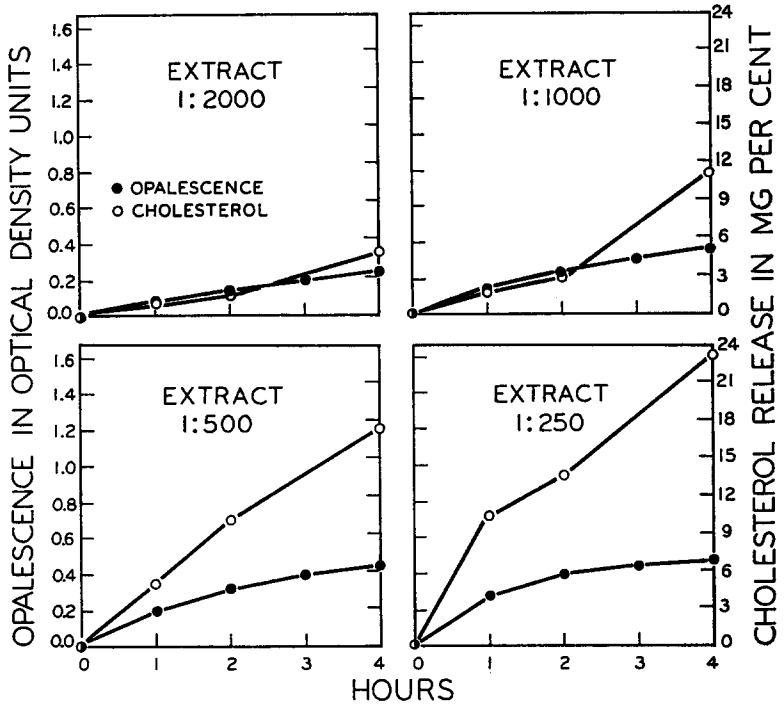

FIG. 4. Time course of opalescence production in comparison to cholesterol release as a function of extract concentration. Serum-extract mixtures (pH 5.8) were incubated at 37°C.

The experimental procedure followed that of the previous experiment except that all serum-extract mixtures were incubated at pH 5.8,[2] and the final concentration of extract was varied from 1:250 to 1:2000. At 0 time, and after several intervals of incubation at 37°C, opalescence was measured and a portion of each reaction mixture immediately adjusted to pH 4.0 for subsequent estimation of released cholesterol.

The results (Fig. 4) indicate that opalescence production and cholesterol release do not follow a completely parallel course in any of the reaction mixtures. The deviation between the two effects appears earlier and is more pronounced as the concentration of extract is increased. With the higher concentrations of extract, cholesterol release continues at a high rate after opalescence has levelled

off considerably. The implications of these and other data pertaining to the discrepancy between opalescence production and cholesterol release will be considered in the Discussion.

Effect of Temperature.—The initial rates of opalescence production and cholesterol release were further compared as a function of incubation temperature. Experimental conditions were chosen (optimal pH, high extract concentration) so as to provide sufficient cholesterol release for accurate measurement at an early period in the reaction.

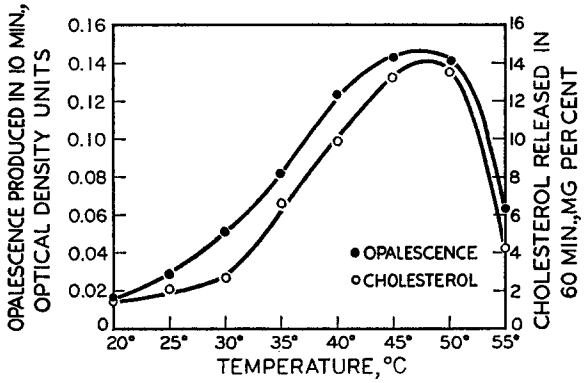

FIG. 5. Comparison of initial rates of opalescence production and of cholesterol release a a function of incubation temperature. All mixtures were at pH 5.8 and contained streptococca extract in a final dilution of 1:250.

Replicate mixtures comprising 20 ml of stabilized horse serum at pH 5.8 and 5 ml of 1:50 streptococcal extract buffered at the same pH were incubated at different temperatures, from 20°–55°C. Released cholesterol was measured at 1 hour and opalescence at 10 minute intervals during the hour. A preliminary plot of the opalescence values *versus* time showed some departure from linearity after the first 20 minutes in mixtures incubated above 40°C. Therefore, values for an earlier 10 minute interval were used to represent the initial rates of opalescence production.

It is evident from the temperature-activity curves in Fig. 5 that the initial rates of opalescence production and cholesterol release are temperature-dependent, and run roughly parallel in mixtures incubated over the range 20°–55°C. From 20°–30°C opalescence production appears more responsive than cholesterol release to temperature change. However, the rates for both effects show an equally sharp increase (about 1.5-fold) between 35° and 40°C; an optimum between 45° and 50°C; and a precipitous decline at 55°C. These similarities, as well as those noted in the experiment involving alterations in pH, are evident at concentrations of extract where opalescence development and cholesterol release do not follow a parallel course throughout an extended reaction period (see Fig. 4). Thus, it would seem that the two effects are in

fact closely related and, though subject to secondary modification, are probably expressions of a single basic reaction. All of the data are consistent with the idea that this underlying reaction is enzymic in nature with clearly defined pH and temperature optima.

DISCUSSION

That human and animal sera may be optically clear despite a relatively high lipid content is owing to the intimate association of these lipids with specific serum proteins. Theoretically, opalescence could result from denaturation or degradation of the lipoprotein complexes with exposure or release of the water-insoluble lipids. The present experiments provide direct chemical evidence that extracts of certain Group A streptococci cause opalescence in serum by setting free particular lipids from one or more serum lipoproteins. These liberated lipids, recoverable by ultracentrifugal flotation at normal serum density, consist for the most part of cholesterol esters accompanied by a small amount of phospholipid (probably lecithin). Visual inspection of lipid-stained electrophoretograms of extract-treated sera suggests that these lipids come from α_1-lipoprotein. This is supported by unpublished experiments (17) employing ultracentrifugation at elevated solution densities (18) to fractionate the lipoproteins of extract-treated human serum. These experiments indicate that the floatable products are derived almost exclusively from high density ($d > 1.063$) lipoprotein—which corresponds to α_1-lipoprotein. The present findings disagree, however, with the concept of total or indiscriminate release of lipids from α_1-lipoprotein suggested by Krumwiede (2). The low density product fractions of extract-treated sera do not contain the full variety of lipids in α_1-lipoprotein, nor do they have a cholesterol/phospholipid ratio characteristic of any serum lipoprotein (19, 20). The disappearance of sudanophilic material from the α_1-lipoprotein region on electrophoretograms of extract-treated sera is not evidence for the complete depletion of lipid from α_1-lipoprotein; it could be the result of partial loss of lipid with decreased mobility of the residue.

Although the opalescence produced by extract-serum interaction is caused by specific low density lipids released from lipoprotein, the extent of opalescence development and the amount of the major lipid released (esterified cholesterol) do not correspond perfectly. If liberated cholesterol esters are responsible for opalescence, factors in addition to concentration must be involved, for example, factors influencing the state of lipid aggregation. The finding that the discrepancy between the two effects is greatest at high extract concentration suggests that the available extracts may contain some substance interfering with the full expression of opalescence. Notwithstanding the imperfect parallelism between opalescence production and cholesterol release, the two phenomena appear to be closely related, and brought about by a specific enzymic

alteration of a limited class of serum lipoproteins. One possibility is that the alteration is due to the direct action of a streptococcal enzyme or "lipoproteinase." Krumwiede reached this conclusion although she could show no effect of the extracts on crude preparations of α_1-lipoprotein from horse serum unless serum albumin was present. In several exploratory experiments, we likewise have detected little or no action of the extracts on ultracentrifugally or electrophoretically isolated serum lipoproteins, or on commercially prepared Cohn fractions of human and bovine serum.[3] Some subtle alteration during purification might have rendered these lipoproteins resistant to enzymic attack. However, one must also consider the possibility that serum (or crude preparations of serum albumin) may contain a lipoprotein-degrading enzyme in zymogen form activated by a streptococcal factor in a manner analogous to the activation of plasminogen by streptokinase (21). Alternatively, serum may supply some substance required to activate a streptococcal proenzyme or to bind some product of extract action; *e.g.*, in the way that serum albumin binds liberated fatty acids in the clearing of lipemic sera by lipoprotein lipase (22). Considering the inability to choose between the various possibilities at present, Krumwiede's designation of the streptococcal factor as a "lipoproteinase" seems premature.

Although the mechanism of action of the streptococcal extracts is not known, the reaction itself appears unique. The opalescence brought about in certain animal sera by α-toxin of *Clostridium welchii* differs markedly in that α-toxin hydrolyzes the lecithin and sphingomyelin components of serum lipoproteins (23), as a consequence of which a broad spectrum of lipids, as well as protein, is found among the floatable products (24). Alterations in serum lipoproteins produced by certain Gram-negative bacteria, proteolytic enzymes, or acids and alkalies (13, 25) also differ from those produced by streptococcal extracts since the pronounced elevations in streptolysin O inhibitory activity caused by the former agents are not evident in sera treated with streptococcal extract. The prominent and disproportionate release of esterified cholesterol through extract action doubtless is related to the manner in which cholesterol esters are held within the substrate lipoproteins. If, because of the non-polar nature of the sterol esters, weak forces of the van der Waals variety are involved (26), an enzymically induced lesion in some part of the lipoprotein, not necessarily involving the cholesterol esters themselves, might cause a disorganization of the complex with a "spilling out" of esterified cholesterol. This could especially be the case if, as suggested by Gould (27), cholesterol esters are held within lipoproteins as the "guest" portion of an inclusion complex. The experimental fact that a small amount of phospholipid always accompanies the esterified cholesterol set free by extract-serum interaction may be a clue to the possible

[3] "α-lipoprotein" and "β-lipoprotein," Pentex Corporation, Kankakee, Ill.

site of enzymic attack. Conceivably, since phospholipids have both polar and non-polar groups, the phospholipid recovered in the low density product fraction may have originally served as a "link" between cholesterol esters and protein within the lipoprotein complex. Enzymic rupture of the bond between such phospholipid and the protein could fully account for the experimental findings and would appear to be a reasonable working hypothesis. Through better understanding of the action of the streptococcal factor and of the exact nature of the lipoprotein degradation products, valuable information might accrue regarding the structures of at least certain serum lipoproteins. The predominant action of the extracts on high density lipoprotein already indicates that these lipoproteins have some basic structural feature different from lipoproteins of lower density. Furthermore, the close similarity in composition of the low density product fractions of extract-treated human and horse sera suggests that high density lipoproteins of different mammalian species, although not identical, have some specific structural elements in common with each other.

As cholesterol occupies a vital position in many physiological and pathological processes, any biological system capable of liberating bound cholesterol from serum (and perhaps tissue) lipoproteins deserves close attention. The streptococcal system is of fundamental significance in this respect, even if only as a model for other cholesterol-releasing systems that may exist in nature. Of possible relevance is the fact that human serum regularly contains, in small amount, a lipoprotein fraction of density greater than 1.21 that has been characterized as having little or no cholesterol (28). It is tempting to ask whether this represents α_1-lipoprotein from which cholesterol has been set free by a cholesterol-releasing system similar to that involving the streptococcal factor.

Finally, the significance of the streptococcal factor in relation to the pathogenesis of human Group A streptococcal infections merits evaluation. In one survey carried out in our laboratory, examination of approximately 100 strains of Group A streptococci from human sources revealed at least 25 strains able to produce opalescence in horse serum. In a different type of survey, a substance capable of inhibiting opalescence by streptococcal extracts, and presumed to be antibody (2), was detected in the sera of 25 to 30 per cent of apparently healthy adults, suggesting prior contact of these individuals with the streptococcal factor or some immunologically related material. Thus, it seems likely that in the course of some streptococcal infections the opalescence-producing factor is elaborated *in vivo* where it may exert a deleterious effect on certain serum lipoproteins. Experiments in progress demonstrate that opalescent plasma can be produced in experimental animals by the parenteral administration of either streptococcal extracts or the living streptococci from which they are derived.

SUMMARY

A chemical explanation has been provided for the production of opalescence in human and animal sera by extracts of certain Group A streptococci. Opalescence results from the selective liberation of specific lipids from α_1-serum lipoprotein. The released lipids have been quantitatively separated through the relatively simple technique of ultracentrifugal flotation, and subsequently analyzed by chemical and chromatographic means. Esterified cholesterol constitutes by far the major low density reaction product, accounting for 85 to 90 per cent of the total lipid released. A small amount of phospholipid (apparently limited to lecithin) was the only other lipid consistently found in low density product fractions.

Comparative kinetics of opalescence development and cholesterol release under varying conditions of pH, extract concentration, and temperature reveal that the two effects do not run exactly parallel but are, nevertheless, probably closely related manifestations of a specific enzymic degradation of serum lipoprotein. It has been suggested that enzymic action may occur at a site within the lipoprotein remote from the cholesterol esters themselves. Although the over-all reaction appears to be mediated by an enzyme present in many Group A streptococci, it is uncertain whether this enzyme acts directly on lipoprotein or acts by causing the activation of a serum enzyme which in turn attacks the lipoprotein.

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