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ENHANCEMENT OF CHOLESTEROL ESTERIFICATION IN SERUM BY AN EXTRACT OF GROUP-A STREPTOCOCCUS*

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SUMMARY

Extracts of certain Group-A streptococci, in minute amounts, markedly increase the rate and extent of cholesterol esterification occurring in human serum held at 37°. This enhancement of esterification depends on a factor in the extracts that produces opalescence in serum through a specific degradation of high-density (α-1-)lipoprotein. Results of experiments in which streptococcal extract was incubated with heated or aged serum, or with fresh serum in the presence of various esterification inhibitors, suggest that the streptococcal factor potentiates, rather than supplements, the normal process of ester formation. Taking advantage of the enhanced esterification occurring in the presence of the streptococcal factor, an apparent conversion of lecithin to lysolecithin has been shown to accompany cholesterol esterification *in vitro* and to be, in all probability, an integral part of the esterification reaction in serum.

The present findings, and those of others, lead us to conclude that the direct transfer of a single fatty acid from lecithin to cholesterol constitutes the major mechanism of cholesterol esterification *in vitro* in human serum; the rate and extent of ester formation being limited by the availability of lipoprotein-bound lecithin. The streptococcal factor, in promoting the degradation of high-density serum lipoprotein, evidently enhances esterification by making bound lecithin more accessible to the serum enzyme involved in fatty acid transfer.

INTRODUCTION

Human and animal sera held at 37° show a marked fall in free cholesterol without change in total cholesterol. SPERRY[1], who first reported this observation in 1935, concluded on the basis of a large number of experiments that cholesterol was being esterified under the influence of a serum enzyme. Although production of fatty acid esters of cholesterol in incubated serum has been confirmed[2,3], the precise mechanism of ester formation remains unknown. The esterifying enzyme in human serum, unlike esterases from other biological sources[4-9], is unable to bring about direct combination of cholesterol and fatty acids in artificial emulsion[6]. Cholesterol esterification in serum is undoubtedly a more complex process accompanied by phospholipid degradation[10] and by an alteration in the electrophoretic behavior of serum lipoproteins[11].

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Furthermore, esterification *in vitro* is inhibited by a wide variety of unrelated agents including heat[1], bile salts[12], bromoacetate[10], organic phosphates[13], saponin[14], and red blood-cell lysates[1]. Normally, cholesterol esterification in incubated human serum is a relatively slow reaction, requiring about 3 days to reach its maximum[15]; rarely more than 60 % of the original free cholesterol is converted to the ester during this period. Relatively few substances have been reported to increase the normal rate or extent of esterification[16,17]. The present work is concerned with a new enhancing agent, of bacterial origin, whose action seems to throw considerable light on the mechanism of cholesterol ester formation *in vitro*.

It has been known for many years that extracts of certain Group-A streptococci contain an enzyme-like substance capable of producing opalescence in human and animal sera[18,19]. Previous work in our laboratory has revealed this opalescence to be the result of a partial degradation of high-density (α-1-)lipoprotein in which specific lipids are set free[20]. In this early study, employing stored serum and relatively short periods of incubation (2–6 h), little or no change in the serum lipids themselves was detected. However, later experiments clearly indicated that incubation of fresh human serum with small amounts of streptococcal extract for periods exceeding 4 h results in a profound augmentation of cholesterol esterification. An investigation of this newly discovered activity of streptococcal extracts is the subject of the present communication.

METHODS

Sera

Blood was obtained from apparently healthy young adults after an overnight fast. The aseptically recovered serum was stored at 4° and, unless otherwise indicated, used within 2 weeks of bleeding.

Streptococcal extracts

Extracts were prepared from the Type-12 Group-A streptococcus (Strain 2RP196) employed in our previous experiments. The procedure generally followed that of KRUMWIEDE[19] with special cultural conditions[20] used to provide massive growth of the organism. Stock extracts, which had a protein concentration of approx. 0.3 %, were stored at −20°.

Incubation mixtures

Test mixtures usually comprised 18 parts by volume of serum, 1 part of 0.2 % merthiolate, and 1 part of appropriately diluted streptococcal extract or diluent (0.08 M NaCl buffered with 0.067 M phosphate at pH 7.0). In experiments involving the addition of other reagents to serum–extract or serum–buffer mixtures, 2 parts of the serum were replaced by an equivalent volume of reagent, or by diluent in the case of controls. Suitable corrections were made in calculations of lipid content to account for dilution, and all lipid values are presented in terms of original serum.

Opalescence measurement

Opalescence is recorded as $\Delta A_{540\,m\mu} \times 10^3$ between serum–extract mixtures and concurrently incubated serum–buffer controls. Samples in 1:5 dilution were read in a Beckman DU spectrophotometer using standard cuvettes having a light path of 1 cm.

Lipid determinations

The method of SPERRY AND WEBB[21] was used to measure free and total cholesterol. Lipid-phosphorus was determined by a modification of the STEWART AND HENDRY procedure[22]. All determinations were carried out in duplicate.

Lipid chromatography

Silicic acid-impregnated paper was prepared according to the procedure outlined by MARINETTI[23]. Test and control serum mixtures, applied directly to the paper[24] in 25-μl quantities, were chromatographed at 23–25° by an ascending technique using diisobutyl ketone–acetic acid–H_2O (40:20:3, v/v/v) for resolving phospholipids and *n*-heptane–diisobutyl ketone (96:6, v/v) for separating neutral lipids. Chromatograms were stained with Rhodamine 6G and photographed in ultraviolet light.

RESULTS

Demonstration of enhanced esterification

As indicated in Table I, human sera incubated 24 h in the absence of streptococcal extract show cholesterol esterification ranging from 13 to 54%, depending on the source of the sample and the time of storage before testing. In most cases the same sera incubated with a small amount of streptococcal extract become opalescent and show approximately twice as much esterification as in the absence of the extract. The last two sera are notable exceptions. In neither was opalescence produced nor the spontaneous esterification increased in the presence of the extract. As reported previously[20], the sera of about 25–30% of normal adults are refractory to the opalescence-producing principle and have been presumed by KRUMWIEDE[19] to contain

TABLE I

INFLUENCE OF STREPTOCOCCAL EXTRACT ON CHOLESTEROL ESTERIFICATION IN REPRESENTATIVE HUMAN SERA

Incubation mixtures comprised 1.8 ml serum, 0.1 ml extract (1:4–1:10 dilution depending on potency) or buffer and 0.1 ml of 0.2% merthiolate. All sera were tested within 4 weeks after collection. Per cent depletion of unesterified cholesterol is taken as per cent conversion to the ester since total cholesterol in every case was unchanged following incubation. Extract-induced opalescence was observed 2 h after start of incubation (prior to the occurrence of appreciable esterification).

Donor	*Total cholesterol (mg %)*	*Unesterified cholesterol (mg %)*			*Esterification (%)*		*Extract-induced opalescence*
		Before incubation	*After incubation for 24 h at 37° with:*				
			Buffer	*Extract*	*Buffer*	*Extract*	
He.	136	33	22	5	33	85	+
Ad.	163	39	27	11	31	72	+
De.	191	51	42	33	18	35	+
Gi.	238	54	33	17	39	69	+
Le.	125	24	11	2	54	92	+
Tr.	149	35	23	9	34	74	+
Wi.	187	42	22	7	48	83	+
Ba.	195	43	36	36	16	16	o
Ro.	175	46	38	38	17	17	o

antibody to the streptococcal factor. The above findings suggest a direct relationship between the capacity of extracts to induce opalescence in serum by degradation of high-density lipoprotein and their ability to augment esterification.

Kinetics

The time-course of cholesterol esterification in the presence and absence of streptococcal extract (Fig. 1) illustrates that the initial rate of esterification in the presence of the streptococcal extract is more than double that found in its absence; *e.g.* the degree of esterification at 24 h in the extract-treated sample is equivalent to that attained at 72 h without the extract. It is also apparent that the effect of the extract is mainly manifested during the period of maximal opalescence production.

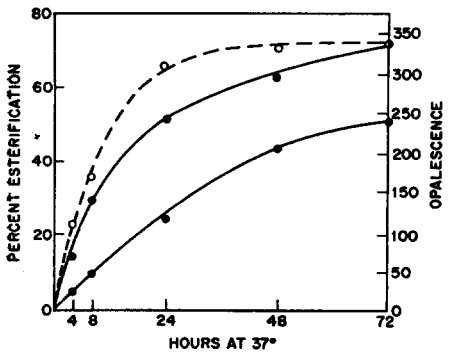

Fig. 1. Time-course of cholesterol esterification in human serum incubated at 37° in the presence and absence of streptococcal extract. The development of opalescence in the serum–extract mixture is also shown, with the points of maximal opalescence and esterification in the presence of the extract arbitrarily made to coincide. Incubation mixtures contained streptococcal extract or buffer in a final dilution of 1:200. At each interval a 2 ml sample was taken; opalescence was measured with 0.5 ml and the remainder immediately frozen until all samples were accumulated for cholesterol determinations. Initial concentration of unesterified cholesterol was 41 mg %. O--O, opalescence in the presence of extract; ●—●, esterification in the presence of extract; ◉—◉, esterification in the absence of extract.

After 24 h, esterification in the presence and absence of streptococcal extract appear to proceed at an equal rate.

Effect of extract concentration

The concept that the same principle in streptococcal extracts is responsible both for opalescence production and enhanced cholesterol esterification is supported by the general parallelism observed between these two effects as a function of extract concentration. In the experiment summarized in Fig. 2, both effects were detected in mixtures containing extract diluted beyond 1:2500, were maximal over a broad range of equivalent concentrations and were markedly inhibited at high extract concentration. With the particular extract used in this experiment, no enhanced esterification and only minimal opalescence was observed in the mixture containing extract in a final concentration of 1:10.

Previous studies[20] have indicated that, although opalescence is caused by lipids (principally cholesterol esters) liberated from high-density lipoprotein, the release of cholesterol esters is not suppressed at high extract concentration as is opalescence. Thus, with concentrated extract, the enhancement of esterification apparently correlates more with opalescence than with lipid release. The reason for this is currently being investigated.

Inhibitors

Various physical and chemical agents known to arrest normal cholesterol esterifi-

cation *in vitro* were tested for their effects on esterification and opalescence production in incubated serum–extract mixtures. The influence of repeated freeze–thawing and lyophilization of serum was also studied since, conceivably, these treatments could modify either or both effects through their ability to alter the state of serum lipoproteins[25,26].

In general (Tables II and III), all agents that prevented normal esterification also prevented esterification in the presence of the streptococcal extract, suggesting that the basic mechanism of esterification in the presence and absence of extract is identical. The same agents also depressed or prevented the development of opalescence, indicating again that at least part of the opalescence produced in extract-treated fresh human serum is intimately related to esterification. The validity of these

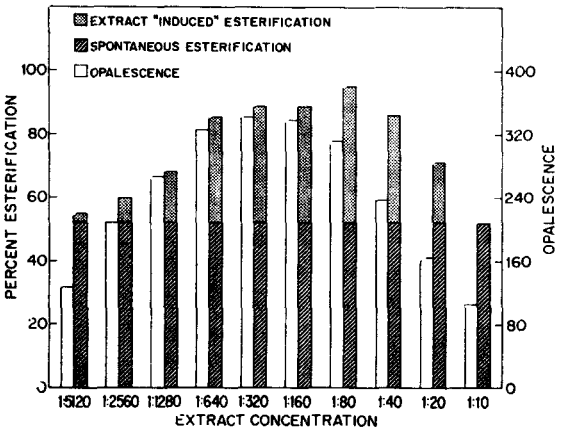

Fig. 2. Cholesterol esterification and opalescence production in human serum as a function of extract concentration. Mixtures containing 1.7 ml of serum, 0.2 ml of appropriately diluted extract and 0.1 ml of 0.2 % merthiolate, were incubated at 37° for 22 h. Value for spontaneous esterification was obtained from concurrently incubated serum–buffer mixture. Opalescence was measured at the completion of incubation. Initial concentration of unesterified cholesterol was 24 mg %.

TABLE II

EFFECT OF PHYSICAL AGENTS ON THE CAPACITY OF HUMAN SERUM TO SHOW CHOLESTEROL ESTERIFICATION AND OPALESCENCE PRODUCTION ON INCUBATION WITH STREPTOCOCCAL EXTRACT

Mixtures containing 1.8 ml serum, 0.1 ml streptococcal extract (diluted 1:4) or buffer and 0.1 ml of 0.2 % merthiolate were incubated at 37°. Concentrations of unesterified cholesterol before incubation were as follows: Le., 25 mg % prior to storage, 23 mg % after storage; Wi., 37 mg %. Opalescence produced with extract was measured at the completion of incubation.

Donor	*Treatment of serum before incubation*	*Esterification (%) after 24 h incubation with:*		*Opalescence produced with extract*
		Buffer	*Extract*	
Le.	None (fresh serum)	40	73	382
	Heated at 56° (30 min)	0	0	231
	Stored 6 months at 4°	0	0	210
Wi.	None	41	86	409
	Frozen and thawed 25 ×	48	74	363
	Lyophilized and reconstituted	49	66	243

conclusions gains from the fact that the effective agents appear to have acted in several different ways. Heating and aging of serum probably owe their effectiveness more to enzyme denaturation than to modification of the substrate-containing lipoproteins; *e.g.* free cholesterol in heat-inactivated serum can still be esterified upon addition of fresh serum[15,27]. Saponin, cholate, and glycocholate may be considered as modifiers of lipid–protein relationships[28] as they altered the electrophoretic

TABLE III

EFFECT OF CHEMICAL AGENTS ON CHOLESTEROL ESTERIFICATION AND OPALESCENCE PRODUCTION IN EXTRACT-TREATED HUMAN SERUM

Mixtures comprised 1.6 ml human serum (collected 48 h prior to testing), 0.1 ml streptococcal extract (diluted 1:4) or buffer, 0.1 ml of 0.2% merthiolate and 0.2 ml of appropriately diluted chemical agent. Incubation temperature, 37°. Concn. of unesterified cholesterol before incubation, 67 mg %. The opalescence produced in the serum–extract mixture was measured at the completion of incubation. Alteration of lipoproteins by added reagent was judged by change in electrophoretic mobility of β-lipoprotein, tested in the absence of extract. Cholate and glycocholate produced an apparent increase in the mobility of β-lipoprotein while the lipids in saponin-treated serum failed to migrate from the origin.

Agent added to serum–extract and serum–buffer mixture	*Esterification (%) at 24 h in serum incubated with:*		*Opalescence produced in serum–extract mixture*	*Lipoproteins altered by added reagent*
	Buffer	*Extract*		
None (control)	28	64	195	
Saponin (1.0%)	7	9	2*	+
Sodium cholate ($9 \cdot 10^{-3}$ M)	7	0	6	+
Sodium glycocholate ($8 \cdot 10^{-3}$ M)	0	0	0	+
Sodium bromoacetate ($6 \cdot 10^{-3}$ M)	0	0	119	0
Sodium iodoacetate ($7 \cdot 10^{-3}$ M)	0	0	113	0
DFP ($5 \cdot 10^{-4}$ M)	0	0	93	0

* Samples treated with saponin were markedly opalescent in the presence or absence of streptococcal extract. Value represents difference in opalescence between serum–extract and serum–buffer mixture.

distribution of serum lipids. Bromoacetate, iodoacetate, and DFP caused no visible change in the electrophoretic character of serum lipoproteins; they presumably inhibit some essential enzyme in the esterification process. The chemical inhibitors that changed the lipoprotein pattern obliterated opalescence; those without visible effect on lipoproteins caused an incomplete inhibition of opalescence (as did heating or aging of serum).

That DFP probably exerts its inhibitory effect in serum–extract mixtures by acting on a serum enzyme, and not on some principle in the streptococcal extract, was apparent from a supplementary experiment. Thus, extract separately treated with DFP (at the concentration effective in serum–extract mixtures) retained its full capacity to induce opalescence and to enhance esterification when incubated with serum*. This result, coupled with those obtained with heated and aged serum,

* The streptococcal factor is thus differentiated from the DFP-sensitive β-naphthyl acetate esterase of Group-A streptococci reported by STOCK *et al.*[29]. Although β-naphthyl acetate esterase was detected in trace amounts in several of our streptococcal preparations, its presence was not related to the ability of the extracts to enhance cholesterol esterification in serum. Furthermore, several concentrated streptococcal culture filtrates rich in β-naphthyl acetate splitting enzyme, kindly furnished by DR. A. STOCK, proved incapable of producing opalescence in serum or augmenting cholesterol esterification.

strongly suggests that the added esterification observed in serum–extract mixtures is catalyzed by a serum enzyme. Lyophilization and repeated freeze–thawing of serum, which did not depress normal esterification but did decrease the extent of its enhancement, as well as opalescence production in the presence of the extract, might be visualized as interfering with the mechanism of enhancement rather than with the esterification process itself. This is presumably brought about by some modification of high-density lipoprotein upon which the streptococcal factor acts.

Phospholipid changes accompanying esterification

Although it has been generally accepted that lecithin serves as a source of fatty acid for cholesterol ester formation in incubated serum, there has been some disagreement regarding the actual mechanism. LEBRETON AND PANTALEON[30] proposed that both fatty acid units of lecithin are liberated by the hydrolytic action of a lecithinase, then combined with cholesterol under the influence of an esterase. GLOMSET *et al.*[3], however, have presented evidence that free fatty acids are not involved in the cholesterol esterification reaction; they suggest that lecithin and other preformed fatty acid esters of serum are direct donors of fatty acids through a process of transesterification. In our laboratory, preliminary examination of serum–buffer and serum–extract mixtures incubated at 37° for 24 h failed to reveal any correlation between extent of esterification and decrease in lipid phosphorus.

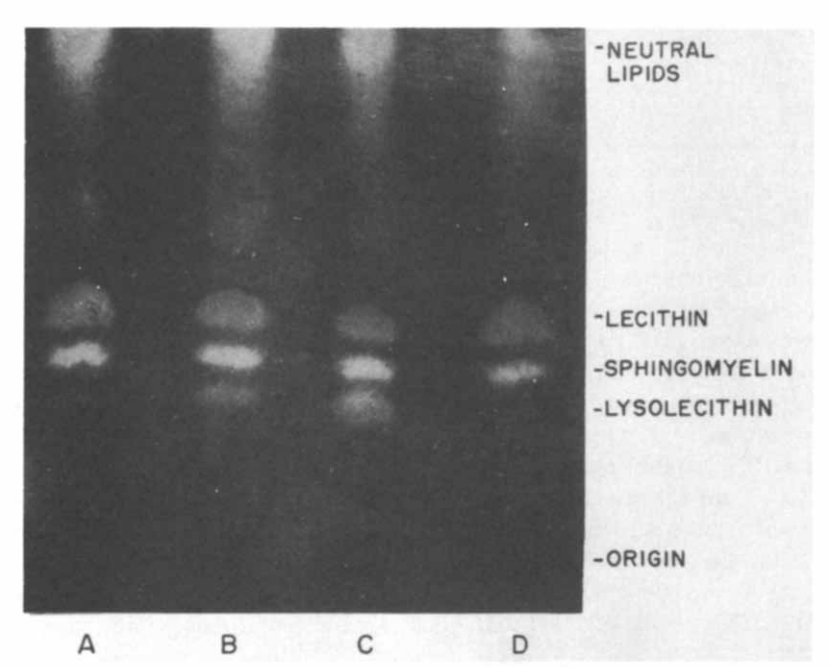

Fig. 3. Chromatogram showing phospholipid changes accompanying cholesterol esterification. Chromatography was carried out for 5 h at room temperature using, as solvent, diisobutyl ketone–acetic acid–H_2O (40:20:3, v/v/v). A, serum plus buffer, not incubated (no esterification); B, serum plus buffer, incubated at 37° for 22 h (42% esterification); C, serum plus 1:80 streptococcal extract, incubated at 37° for 22 h (95% esterification); D, heated (56°, 30 min) serum plus 1:80 streptococcal extract, incubated at 37° for 22 h (4% esterification).

To examine the relationship between lecithin-breakdown and enhancement of esterification in the presence of streptococcal extract, incubated serum–buffer and serum–extract mixtures (showing normal and enhanced esterification, respectively) were chromatographed along with appropriate controls on silicic acid-impregnated paper. A loss of lecithin and an accumulation of lysolecithin was clearly evident (Fig. 3) in serum incubated with buffer or extract (however, it is not known whether α- or β-lysolecithin is formed). Both changes were more pronounced in the presence of the streptococcal extract. Their linkage to cholesterol esterification is indicated by the finding that mixtures incubated under conditions inhibitory to cholesterol esterification (see Tables II and III) yielded chromatographic patterns indistinguishable from the unincubated control serum. The failure to detect conversion of lecithin to lysolecithin in heated or aged serum incubated with extract (see pattern D of Fig. 3) indicates that the streptococcal factor does not directly effect this conversion, but rather serves to potentiate a normal process mediated by a serum enzyme.

DISCUSSION

The foregoing experiments have established a definite correspondence between the ability of extracts of certain Group-A streptococci to produce opalescence in serum through lipoprotein degradation and the newly discovered capacity of these extracts to enhance the spontaneous esterification of cholesterol that occurs in human serum held at 37°. Any attempt to explain this correspondence must take into account the nature and origin of the substrates involved in the esterification reaction as well as the specificity of the streptococcal factor for high-density (α-1-)lipoprotein[19,20]. Since the addition of synthetic lecithin to serum greatly increases the extent of esterification[17], whereas added cholesterol is without effect[1,6], the availability of serum lecithin likely is the condition limiting the rate and extent of spontaneous cholesterol esterification. Lecithin in serum is bound within lipoprotein complexes and evidently is not entirely available for reaction. On incubation of serum alone, a slow spontaneous denaturation of lipoproteins perhaps makes the bound lecithin increasingly accessible to a serum enzyme involved in fatty acid transfer. On incubation of serum with the streptococcal factor, the relatively rapid degradation of high-density lipoprotein (which carries more than half of the serum lecithin[21]) is believed to augment the availability of lecithin and thereby enhance esterification.

Evidence has been presented to show that lecithin to lysolecithin conversion accompanies cholesterol esterification *in vitro* and indeed seems to be part of the esterification mechanism. ETIENNE AND POLONOVSKI[32] had reported the accumulation of lysolecithin in incubated serum without relating their findings to cholesterol esterification. We find that in the presence of the streptococcal extract increased esterification and increased lysolecithin formation go hand in hand; moreover, that the conversion of lecithin to lysolecithin does not occur under conditions which arrest esterification. These results clearly oppose LEBRETON AND PANTALEON'S concept of cholesterol esterification in serum[4] entailing hydrolytic release of both fatty acids of lecithin and their subsequent combination with cholesterol under the influence of a second enzyme. The simplest mechanism consistent with our results is that a single enzyme mediates the direct transfer of a fatty acid of lecithin to cholesterol yielding the sterol ester and lysolecithin. GLOMSET *et al.*[3], who first

suggested that cholesterol esterification in plasma was catalyzed by a fatty acid transferase, believe that net esterification may involve the coupled action of the transferase and a lysolecithinase. Although some of the formed lysolecithin does apparently undergo subsequent hydrolysis to glycerylphosphorylcholine[32,33], there is nothing to indicate that this secondary release of fatty acid is necessary for cholesterol esterification. GLOMSET and his group also proposed that the fatty acid transferase in plasma is capable of transferring fatty acids from triglycerides, as well as from phospholipid, to cholesterol. Triglyceride breakdown was not detected in the present study, nor in a previous study by ETIENNE AND POLONOVSKI[2], suggesting that lecithin (and possibly phosphatidyl ethanolamine, which has not been studied) is the prime, if not the sole, direct source of fatty acid for cholesterol ester formation in incubated human serum.

Although phospholipase A (EC3.1.1.4), which converts lecithin to lysolecithin by removing a single fatty acid, has been detected in human serum[34], it is probably not involved in cholesterol esterification. Serum phospholipase A resists heating at 60° for 30 min whereas cholesterol esterification and the accompanying phospholipid changes in serum are abolished by such treatment. Furthermore, the addition to serum of *Crotalus adamanteus* venom, rich in phospholipase A, prevents, rather than augments, cholesterol esterification (studies to be published).

Release of preformed cholesterol esters from high density (α-1-)lipoprotein by the streptococcal factor, and subsequent conversion of a significant portion of the lecithin to lysolecithin by the serum enzyme mediating cholesterol esterification, should yield a partly degraded lipoprotein having a density greater than ordinary high-density lipoprotein (*i.e.* greater than 1.21 g/ml), containing little or no cholesterol and an unusually high proportion of lysolecithin. The fact that a lipid–protein complex having these characteristics is ordinarily present in human serum[35,36] may be an indication that the streptococcal factor acting on fresh serum can reproduce in the test tube a vital process in lipoprotein metabolism.

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