Inhibitory Effect of a Serum Protein on Development of Opalescence in Lipoproteins Altered by Lecithinase C.* (24277)

ELMA KRUMWIEDE (Introduced by Ann G. Kuttner)
Department of Pediatrics, N. Y. University-Bellevue Medical Center, N. Y. City

The production of opalescence by *Cl. welchii* in broth containing human sera was reported independently by Nagler(1) and by Seiffert(2). MacFarlane and Knight(3) demonstrated that the reaction was due to the presence of lecithinase C in the α toxin of *Cl. welchii* and that development of acid soluble phosphorus in human sera and egg yolk could be used as a measure of enzyme activity. In contrast to published data on the action of *Cl. welchii* toxin on human sera, reports of several observers on horse sera yielded contradictory results(4). The effect of lecithinase C on horse sera, therefore, was reinvestigated.

Methods and materials. Methods for lipid extraction, cholesterol determinations and electrophoretic studies have been described (5). Lipid phosphorus and acid-soluble phosphorus in trichloroacetic acid filtrates (9 parts trichloroacetic acid, 1 part test material) were determined by the method of Stewart and Hendry(6) as modified by Ahrens (personal communication). The specific gravity of material for ultracentrifugation was raised with a saturated solution of KBr in 8.5% NaCl. After 20 hours centrifugation at 40,000 rpm in a Spinco preparative ultracentrifuge, the infranates and supernates were removed with capillary pipettes and dialyzed against 8.5% NaCl to remove KBr. Crude α_1 lipoprotein fractions were prepared by a modification of the method of Macheboeuf(7). One volume of horse serum† and one volume of 8.5% NaCl were stirred with 2 volumes of a saturated solution of ammonium sulfate and filtered. The filtrate was adjusted to pH 4.5 with 1N HCl at 30°C. The precipitate which formed was collected by centrifugation. After washing with 8.5% NaCl to remove all ammonium sulfate, it was emulsified in 8.5% NaCl (50 ml/L of serum). 1N NaOH was added until material dissolved and the pH adjusted to 7.0 with 1N HCl. This serum fraction contained α_1 lipoproteins and a protein which was shown by ultracentrifugation and lipid analysis to be lipid-free, hereafter referred to as L-FP. Borate buffer which was used to dilute all material was prepared by adding sufficient M/20 borax to M/5 boric acid in M/20 NaCl to bring the pH to 7.0. Filtrates of *C. perfringens* (*Cl. welchii*)‡ used as a source of lecithinase C contained phosphate buffer which was removed by precipitation with $CaCl_2$. To 5 ml of filtrate, 1 ml of 0.5 M $CaCl_2$ and 4 ml of borate buffer, 1N NaOH was added until no further precipitate formed. After overnight refrigeration it was centrifuged and the precipitate discarded. The supernate contained traces of phosphorus and 77 mg% Ca. Lecithinase was not standardized(3). Preliminary studies showed that maximum amount of acid soluble phosphorus released from lipoprotein fractions with equal lipid phosphorus content was dependent on unknown factors as well as concentration of lecithinase. Therefore, the amount of lecithinase necessary for production of maximum hydrolysis was determined for each substrate by preliminary titration. In tests where development of opalescence only is reported description of these titrations is omitted. Merthiolate in a final concentration of 1-10,000 was added to all materials.

Results. Effect of lecithinase C on horse serum. One ml of horse serum and one ml of a 1-16 dilution of lecithinase C in borate buffer (pH 7.0) were incubated overnight at 37°C. The results are presented in Table I. There was no correlation between development of acid soluble phosphorus and production of opalescence in sera in which the phos-

* Supported by grant from Dept. of Health, P.H.S., N.I.H., Bethesda, Md.

† Normal horse serum was generously supplied by Bureau of Laboratories, Dept. of Health, N. Y. City.

‡ *C. perfringens* filtrates were kindly supplied by Lederle Laboratories.

TABLE I. Effect of Lecithinase C on Horse Sera.

Phospholipid hydrolyzed ASP* (%)	Appearance
0	clear
0	"
0	"
70	"
89	"
82	slightly opalescent
80	milky

* ASP = Acid soluble phosphorus.

Percentages were obtained by dividing ASP content by original lipid P content.

pholipids were hydrolyzed by lecithinase C. These results indicate that a number of variable factors influence the effect of lecithinase C on phospholipids of horse sera. Since horse sera contain relatively large amounts of α_1 lipoproteins, the effect of lecithinase C on crude horse serum α_1 lipoprotein fractions was studied.

Effect of lecithinase C on crude α_1 lipoprotein fractions. Ten crude horse serum α_1 lipoprotein fractions were incubated overnight at 37°C with varying concentrations of lecithinase C. Concentration of lipid phosphorus in the fractions varied from 0.040 to 0.080 mg/ml. Percentage of phospholipid subject to hydrolysis by lecithinase C in different fractions varied from 65-90%. Four of the 10 fractions remained clear and 6 developed traces of opalescence. There was no correlation between development of opalescence in lipoprotein fractions and sera from which they were prepared.

Electrophoretic patterns. Paper electrophoretic patterns(5) showing the effect of hydrolysis of phospholipids in a crude α_1 lipoprotein fraction by lecithinase C are shown in Fig. 1. The most marked changes in the electrophoretic patterns resulting from hydrolysis of phospholipids by lecithinase C are a decrease in amount of L-FP and a slight increase in electrophoretic mobility of the α_1 lipoproteins.

Trypsin digestion of crude α_1 lipoprotein fractions. Since free lipids do not generally move in an electrophoretic field, the electrophoretic patterns of the crude α_1 lipoprotein fraction treated with lecithinase C (Fig. 1) suggest that cholesterol and the diglyceride portion of hydrolyzed phospholipids remain bound to protein. To determine whether these lipids were protein-bound, the results of ether extraction of lipoprotein fractions before and after enzyme action were compared. Only traces of lipid could be extracted with ether before and after treatment with lecithinase C. To obtain further evidence that lipids were still bound to protein, the effect of ether extraction following hydrolysis of the proteins with trypsin was investigated. Three crude α_1 lipoprotein fractions were incubated overnight at 37°C with lecithinase C (1.5 ml), with 1-400 trypsin§ (2.0 ml) and both lecithinase and trypsin. Volumes were adjusted to 5 ml with borate buffer. The lipid content of the fractions, amount of phospholipid hydrolyzed and ether extractable lipids are shown in Table II. Hydrolysis of the proteins by trypsin liberated a high percentage of lipids, while hydrolysis of the phospholipids by lecithinase C liberated little or no lipid. Although no chemical analyses were done to identify the lipids in the fat globules seen after hydrolysis of both the phospholipids and protein, they appear to represent the diglyceride portion of the hydrolyzed phospholipids. Bragdon's method(8) for determination of neutral fat based on reduction of potassium dichromate by lipid carbon with subsequent correction for phospholipid and cholesterol content was used to determine diglyceride content. Low lipid phosphorus values precluded calculations of amount of dichromate reduced by phospholipids. Amount of dichromate reduced therefore is given as a crude measure of lipid content. These experiments showed not only that lipid-protein linkages were present after hydrolysis of the phospholipids by lecithinase C but also that more phospholipid could be hydrolyzed when the proteins were hydrolyzed by trypsin.

Ultracentrifugation. The α_1 lipoproteins and L-FP in 4 crude lipoprotein fractions were separated by ultracentrifugation at specific gravity 1.175. The supernate contained α_1 lipoproteins. The infranates of 3 contained

§ Salt free trypsin, Worthington Biochemical Co., Freehold, N. J. 25 mg in 10 ml distilled H_2O.

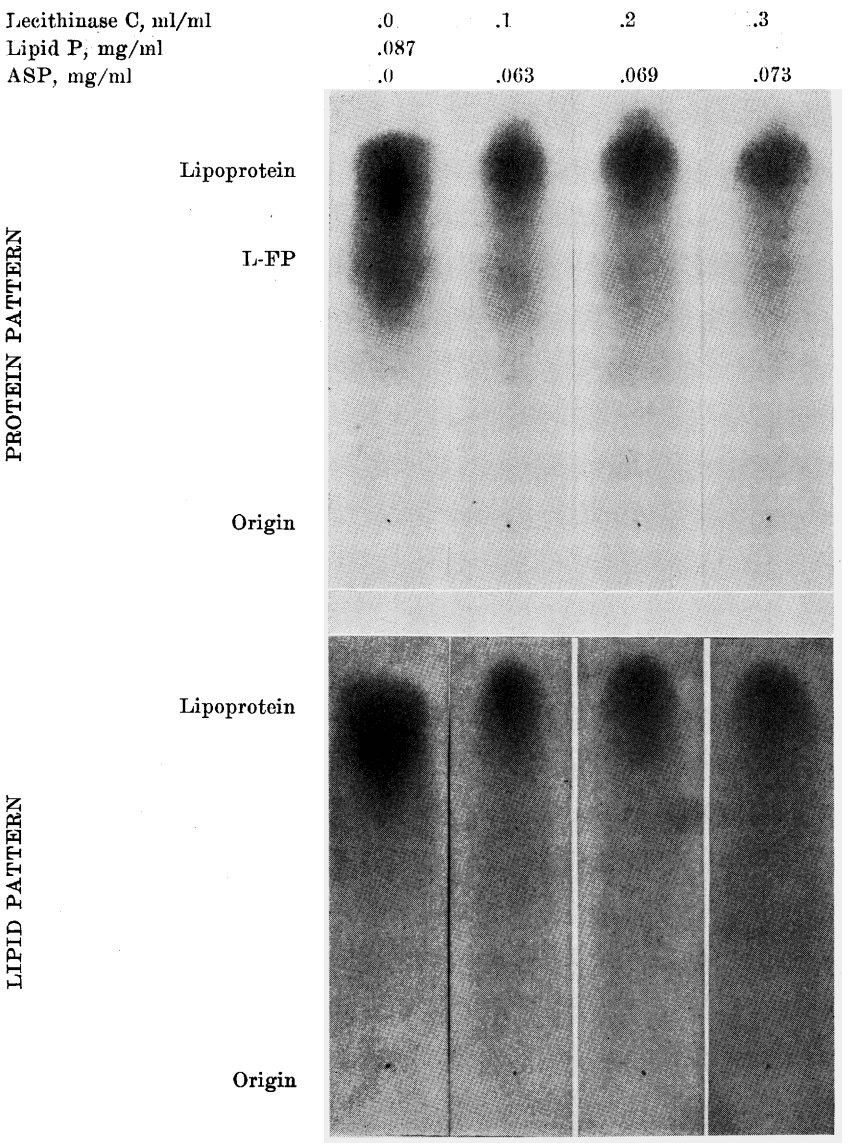

FIG. 1. Decrease in lipid-free protein accompanying hydrolysis of phospholipids in a crude α_1 lipoprotein fraction by lecithinase C. Final volume of each test was brought to 1.0 ml with borate buffer and incubated overnight at 37°C.

only L-FP. The infranate of the fourth contained L-FP and traces of albumin which had not been detected in electrophoretic patterns of the original material. The 4 α_1 lipoprotein fractions were incubated overnight with lecithinase C. Three became opalescent and a precipitate formed in the fourth. To determine whether lipids were still bound to protein 0.8 ml samples were incubated overnight with 0.2 ml of 1-200 trypsin. All developed a thick creamy appearance suggesting that lipids were freed when the protein was hydrolyzed.

Effect of L-FP on development of opalescence in lipoprotein fractions. An α_1 lipoprotein fraction was treated with lecithinase C after addition of decreasing amounts of the L-FP fraction containing albumin, L-FP markedly reduced precipitation and development of opalescence but did not influence

TABLE II. Lipids in Ether Soluble Form and Appearance of Crude α_1 Lipoprotein Fractions Treated with Lecithinase C, Trypsin, and Both Lecithinase and Trypsin.

Lot		Total lipid content before treatment	Lipids in ether soluble form — After treatment with — Control	Lecithinase	Trypsin	Lecithinase and trypsin	Increase in ASP Lecithinase	Lecithinase and trypsin
		mg/ml						
1.	Lipid P	.092	trace	trace	.055	trace	.084	.089
	Cholesterol	1.670	.16	.11	1.07	1.61		
	Dichromate reduced	85.7	5.0	4.6	33.3	41.9		
	Appearance before ether extr.		clear	clear	opalescent	visible fat		
2.	Lipid P	.088	trace	trace	.034	trace	.078	.081
	Cholesterol	1.710	.16	.16	1.4	1.4		
	Dichromate reduced	67.7	trace	trace	41.7	52.0		
	Appearance before ether extr.		clear	clear	opalescent	visible fat		
3.	Lipid P	.085	trace	trace	.077	trace	.071	.080
	Cholesterol	1.369	.186	.221	.945	.966		
	Dichromate reduced		5.0	11.7	44.3	46.7		
	Appearance before ether extr.		clear	clear	opalescent	visible fat		

liberation of acid soluble phosphorus. To rule out the possibility that L-FP might be exerting a non-specific effect, a freshly prepared α_1 lipoprotein fraction (sp. gr. 1.175) was treated with lecithinase C in the presence of 5% bovine albumin,‖ concentrated human serum albumin,¶ 5% casein, egg white and the L-FP fraction containing albumin. Each test contained 0.2 ml of lipoprotein (439 mg/ml lipid phosphorus), 0.1 ml of lecithinase C. 0.2 ml of test material and borate buffer to 1 ml. A control containing lipoprotein and lecithinase was included as were controls to determine effect of lecithinase on test materials. Additional controls of test materials and lipoprotein were set up. The results showed that the effect of L-FP on development of opalescence is specific. Only a trace of opalescence was observed in the presence of L-FP while marked opalescence and a precipitate were seen in tests containing only lipoprotein and lecithinase. Marked opalescence but no precipitate developed in the presence of egg white suggesting that egg white may contain L-FP.

Several pools of human plasma were fractionated. The yield of lipoprotein, which was smaller than that from horse serum, was greatly reduced when the material was washed sufficiently to eliminate albumin by removal of all ammonium sulfate. One human fraction containing visible fat globules and albumin as well as lipoprotein and L-FP was used in the following experiments. Fat globules were first removed by ultracentrifugation at specific gravity 1.075 and the α_1 lipoproteins then separated from albumin and L-FP at specific gravity 1.175. Opalescence and a precipitate were observed when human lipoprotein was incubated with lecithinase C. No precipitate formed and opalescence was markedly reduced by addition of either horse or human L-FP.

Standardization of L-FP on basis of nitrogen content. Human serum to which 4 concentrations of 3 albumin free lots of horse serum L-FP had been added were treated with lecithinase C. The results are shown in Table III. Development of opalescence was greatly reduced in presence of L-FP. Correlation between nitrogen content and suppression of opalescence was not absolute suggesting that the L-FP samples were not pure.

Influence of L-FP on development of opalescence in human sera. Nine human sera

‖ Bovine albumin powder, Armour Laboratories.

¶ Normal serum albumin (human) kindly supplied by American Red Cross.

TABLE III. Effect of L-FP on Development of Opalescence in Human Serum.

Lot L-FP	mg/ml L-FP nitrogen .26	.21	.16	.10	Lecithinase control	Serum control
I	±	±	±	2+	4+	—
II	±	±	±	+	4+	—
III	+	+	+	2+	4+	—

Each test contained 0.25 ml of serum (0.084 mg/ml lipid phosphorus), 0.05 ml of 1-5 lecithinase, and L-FP; lecithinase control, serum and lecithinase. All volumes were adjusted to 0.6 ml with borate buffer. Plus signs indicate degree of opalescence.

were treated with lecithinase C with and without addition of L-FP. Tests contained 0.5 ml of serum, 0.1 ml of lecithinase and 0.3 ml of L-FP (Lot I, final nitrogen content 0.16 mg/ml). L-FP was omitted from the lecithinase controls. All volumes were adjusted to 1.2 ml with borate buffer and incubated overnight at 37°C. The results are shown in Table IV. Addition of L-FP markedly reduced development of opalescence and milkiness resulting from hydrolysis of phospholipids by lecithinase C in 8 of 9 sera. Reduction of opalescence in serum (M.B.) which had high phospholipid and cholesterol values was only slight. To determine whether addition of larger amounts of L-FP would further reduce the milkiness in this serum, it was treated with lecithinase C after the addition of L-FP (Lot II, Table III). With a final nitrogen concentration 0.019 mg/ml this lot markedly reduced development of opalescence. Two sera (P.N. and P.B.) were incubated with lecithinase C after addition of human L-FP. No opalescence developed although as much phospholipid was hydrolyzed as in lecithinase treated controls.

Discussion. The development of opalescence or milkiness in sera treated with lecithinase C has generally been regarded as evidence of hydrolysis of phospholipids with subsequent liberation of lipids from lipoproteins (3,9,10). The work reported here was undertaken because of conflicting reports on development of opalescence in horse sera(4). It was found that hydrolysis of phospholipids in horse sera and crude horse serum lipoprotein fractions does not always lead to development of opalescence. Electrophoretic patterns of lecithinase treated crude lipoprotein fractions showed that L-FP (a lipid-free protein) present in the fractions is reduced when phospholipid is hydrolyzed. It was then shown that L-FP from both human and horse sera suppressed opalescence in whole human sera as well as in α_1 lipoproteins obtained by ultracentrifugation of crude horse and human fractions. Electrophoretic patterns also suggested that hydrolysis of the phospholipids altered but did not destroy the lipoproteins, *i.e.*, free the lipids from the protein. Further evidence of lipid-protein linkage after hydrolysis of the phospholipids in lipoproteins was obtained by ether extraction and trypsin digestion. The cholesterol and the diglyceride portion of the phospholipids remained bound to protein suggesting that phospholipids in α_1 lipoproteins are bound to protein through fatty acids by secondary valences(11,12). These findings appear to be at variance with those obtained by others. However, after high speed centrifugation to separate the "freed lipids" Crook (9) found that the fatty layer was ⅓ protein

TABLE IV. Effect of Horse Serum L-FP on Development of Opalescence in Human Sera Treated with Lecithinase C.

Serum	Lipid P, mg/ml in test	Effect of lecithinase C: Increase in ASP, mg/ml	Effect of lecithinase C: Opalescence	Effect of lecithinase C & L-FP: Increase in ASP, mg/ml	Effect of lecithinase C & L-FP: Opalescence
P.S.	.037	.028	4+	.029	± ?
P.B.	.033	.028	4+	.027	± ?
P.N.	.038	.031	3+	.029	±
M.S.	.034	.028	4+	.029	±
R.P.	.035	.031	4+	.030	+
L.L.	.034	.029	milky	.029	+
J.W.	.038	.031	very milky	.027	+
J.H.	.042	.036	*Idem*	.033	+
M.B.	.049	.043	"	.043	milky

and Peterman(10) found a 5% nitrogen loss in the infranate. These results as well as those reported here suggest that lipid-protein bonds in lipoproteins are not necessarily destroyed by the action of lecithinase C. They suggest rather that solubility of the lipoprotein is altered when phosphorylcholine is freed from the phospholipid.

Although MacFarlane and Knight(3) suggested that the integrity of the lipoprotein complex might be dependent on lecithin, they pointed out that development of turbidity is a secondary reaction. At pH 5.9 to 7.1 they found that rate of hydrolysis to give ASP and development of turbidity are almost parallel, while at pH 9.3 rate of hydrolysis is faster than development of turbidity. Subsequently Oakley and Warrack(4) found that development of turbidity in lecithovitallin altered by lecithinase C was suppressed by high concentrations of human sera. MacFarlane (personal communication) observed that development of turbidity but not hydrolysis of lecithin by *Cl. welchii* toxin was inhibited by high concentrations of normal human, guinea pig and rabbit sera. By simple ammonium sulfate fractionation this author was able to demonstrate that the inhibitor was present only in the "albumin" fraction of sera. Brown *et al.*(13) have reported a coprotein in plasma which may act as an acceptor substance in conjunction with clearing factor. Both coprotein and L-FP are present in the supernate when sera are precipitated by 50% saturation with ammonium sulfate and neither is destroyed by dialysis. Both appear to prevent opalescence in sera in which lipids have been altered, coprotein working in conjunction with clearing factor and L-FP in conjunction with lecithinase C. Neither the effect of L-FP on lipids treated with clearing factor nor the effect of coprotein on lipoproteins acted on by lecithinase C has been investigated. Further work is necessary before the relation if any between L-FP and coprotein can be established.

The role of L-FP in suppressing development of opalescence in lipoproteins acted on by lecithinase C was not fully investigated. Although it was shown to suppress opalescence in human sera containing β lipoproteins its effect on β lipoprotein fractions was not studied. It appears possible that L-FP becomes bound to diglyceride when phosphorylcholine is liberated and that it takes the place of the charged ions of phosphorylcholine in making the lipoprotein soluble. Some evidence has been obtained that there is competition between L-FP and calcium and that the inhibitory effect of L-FP is blocked by high concentrations of calcium (unpublished).

Summary. 1. Hydrolysis of phospholipids of horse serum is not always accompanied by development of turbidity. 2. Although α_1 lipoprotein fractions from horse sera become opalescent with or without development of a precipitate following hydrolysis of the phospholipids by lecithinase C, cholesterol and the diglyceride portion of phospholipids remain bound to protein. 3. L-FP (a lipid-free protein present in human and horse sera) suppresses development of opalescence in α_1 lipoprotein fractions and whole human sera in which phospholipids are hydrolyzed by lecithinase C.

1. Nagler, F. P. O., *Brit. J. Exp. Path.*, 1939, v20, 473.
2. Seiffert, G., *Z. f. Immun forsch,* 1939, v96, 515.
3. MacFarlane, M. G., Knight, D. C. J. G., *Biochem. J.*, 1941, v35, 884.
4. Oakley, C. L., Warrack, G. H., *J. Path. and Bact.*, 1941, v53, 335.
5. Krumwiede, E., *J. Exp. Med.*, 1954, v100, 629.
6. Stewart, C. P., Hendry, E. B., *Biochem. J.*, 1935, v29, 1683.
7. Macheboeuf, M., Rebeyrette, P., Discussion of the Faraday Society, 1949, No. 6, 62.
8. Bragdon, J. H., *J. Biol. Chem.*, 1951, v190, 513.
9. Crook, E. M., *Brit. J. Exp. Med.*, 1942, v23, 37.
10. Peterman, M. L., *J. Biol. Chem.*, 1946, v162, 37.
11. Dervichian, D. G., *Discussions of the Faraday Soc.*, 1949, No. 6, 7.
12. Chargaff, E., *ibid.*, 1949, No. 6, 118.
13. Brown, R. K., Boyle, E., Anfinsen, C. B., *J. Biol. Chem.*, 1953, v204, 423.

Received June 10, 1958. P.S.E.B.M., 1958, v99.