# Effect of phospholipase A treatment of low density lipoproteins on the dextran sulfate–lipoprotein interaction

TOSHIRO NISHIDA

The Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801

ABSTRACT The effect of phospholipase A on the interaction of low density lipoproteins of the $S_f$ 0–10 class with dextran sulfate was studied in phosphate buffer of pH 7.4, ionic strength 0.1, by chemical, spectrophotometric, and centrifugal methods. When low density lipoproteins that had been treated with phospholipase A were substituted for untreated lipoproteins, the amount of insoluble dextran sulfate–lipoprotein complex formed was greatly reduced. Hydrolysis of over 20% of the lecithin and phosphatidyl ethanolamine constituents of the lipoproteins prevented the formation of insoluble complex. However, even the lipoproteins in which almost all the phosphoglycerides were hydrolyzed produced soluble complex, which was converted to insoluble complex upon addition of magnesium sulfate. It is apparent that the lipoproteins altered extensively by treatment with phospholipase A retain many characteristic properties of native low density lipoproteins.

Fatty acids, but not lysolecithin, released by the action of phospholipase A interfered with the formation of insoluble complex; this interference was due to association of the fatty acids with the lipoproteins. With increases in the concentration of the associated fatty acids, the amounts of magnesium ion required for the conversion of soluble complex to insoluble complex increased progressively. Charge interaction is evidently of paramount importance in the formation of sulfated polysaccharide–lipoprotein complexes.

KEY WORDS phospholipase A · low density lipoproteins · dextran sulfate · insoluble complex · soluble complex · fatty acids · magnesium ion · lysolecithin · albumin

THE INTERACTION of sulfated polysaccharides with low density lipoproteins (LDL) is known to produce either soluble or insoluble complexes (1–6). The type of complexes formed is primarily dependent upon the structure of sulfated polysaccharides, the metal ions present, and the pH and ionic strength of the medium used (1, 7–9). Our previous study of the interaction of dextran sulfate (DS) with LDL indicated that, in the absence of divalent ions, the amount of free DS in DS–LDL mixtures is one of the decisive factors in the distribution of various molecular species formed by the DS–LDL interaction (10). Although the lipoprotein–polyanion interaction seems to be caused primarily by polar forces (1, 6), the exact nature of the interaction and the structural factors or configuration of the lipoproteins contributing to this interaction have not yet been clarified. The use of enzymatically, chemically, or otherwise modified LDL may provide valuable information concerning the mechanism of the interaction. It has recently been reported that prolonged action of proteolytic enzymes did not influence the ability of LDL to interact with amylopectin sulfate in the presence of calcium ion (11). The present communication describes the results of DS–LDL interaction as influenced by treatment with phospholipase A, whose involvement in the lysis or alteration of various cell membranes is well recognized (12–15).

## MATERIALS AND METHODS

The sodium salt of DS, with a sulfur content of 16.5% corresponding to 1.8 sulfate groups per hexose unit, was prepared from dextran with an average molecular weight of 180,000 according to the method of Ricketts (16), as previously described (10).

Human plasma LDL of the $S_f$ 0–10 class was isolated from pooled plasma in the presence of 0.05% EDTA according to the methods of Gillies, Lindgren, and Cason

Abbreviation: LDL, low density lipoproteins; DS, dextran sulfate.

(17) and Gofman et al. (18), as previously described (10). A typical LDL preparation used in this study contained 19.9% protein and 80.1% lipid on a dry weight basis as determined gravimetrically (19). Prior to incubation with phospholipase A, the LDL was dialyzed against phosphate buffer of pH 7.4, ionic strength 0.1, for 24 hr under nitrogen at 1°C with four changes of the external solution.

As a source of phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4), lyophilized venom from *Naja naja* was purchased from Ross Allen's Reptile Institute, Inc., (Silver Springs, Fla.). The enzyme was purified by the method of Ibrahim, Sanders, and Thompson (20) and assayed at 25°C by the method of Saito and Hanahan (21). The specific activities of the enzyme, expressed as μmoles of ovolecithin hydrolyzed per minute per mg of protein, were 80.9 and 57.6 for the purified preparation and the original lyophilized venom, respectively. Proteolytic activity was assayed essentially according to the method of Greenberg (22) with denatured hemoglobin (Worthington Biochemical Corporation, Freehold, N. J.) as substrate and found to be extremely low; the purified preparation and the original venom released $7.6 \times 10^{-4}$ and $5.4 \times 10^{-3}$ μmole of tyrosine, respectively, per mg of protein in 16 hr at 25°C. The protein content was determined by the method of Lowry, Rosebrough, Farr, and Randall (23) with crystalline bovine plasma albumin as a standard.

In the general procedure for the treatment of LDL with phospholipase A, mixtures containing 20 mg of LDL and various amount of the phospholipase A preparation in 1.8 ml of phosphate buffer of pH 7.4, ionic strength 0.1, were incubated for different periods of time at 25°C under nitrogen. After incubation, 0.2 ml of 1% EDTA in the same phosphate buffer was added to the incubation mixtures as an inhibitor of phospholipase A, and the mixtures were immediately cooled in an ice bath and stored in the refrigerator at 1°C. The inhibitory effect of EDTA is described in the text. In the initial phase of the study, LDL was isolated from the incubation mixtures as follows. The incubation mixtures were centrifuged at 79,490 *g* for 24 hr in 0.5 M phosphate buffer of pH 7.4, d 1.066, at approximately 4°C. The LDL floated to the top of the medium and was removed by aspiration, dialyzed at 1°C against phosphate buffer of pH 7.4, ionic strength 0.1, and diluted to the original volume of the incubation mixtures with the same phosphate buffer.

The amount of fatty acids released from the phospholipid components of LDL by phospholipase A treatment was determined by microtitration (24) of 0.5 ml aliquots of the incubation mixtures.

To determine the change in the distribution of phospholipid components of LDL after treatment with phospholipase A, we extracted the total lipids from 1 ml of the incubation mixtures with chloroform–methanol 2:1 (25) and dissolved them in 0.5 ml of chloroform. An aliquot of the chloroform solution was applied approximately 1.5 cm from the bottom of a 0.5 mm thick layer of Silica Gel G (20 cm × 20 cm) and chromatographed in chloroform–methanol–acetic acid–water 25:15:4:2. Spots were detected and lipid phosphorus was analyzed according to Parker and Peterson (26). As reference compounds for thin-layer chromatography, lysolecithin, sphingomyelin, and phosphatidyl ethanolamine were purchased from Applied Science Laboratories, Inc. (State College, Pa.), and lecithin was prepared from egg yolk (27) and purified by silicic acid column chromatography (28).

To study the effect of treatment of LDL with phospholipase A on the formation of insoluble DS–LDL complex, we took 0.08 ml aliquots of the incubation mixtures in graduated centrifuge tubes, added various amounts of DS in phosphate buffer of pH 7.4, ionic strength 0.1, and adjusted the volume to 1.5 ml with buffer. The contents of the tubes were mixed after each addition. After a 30 min standing period the tubes were centrifuged at 1250 *g* for 30 min at 20°C, and the maximum amount of supernatant fluid was removed with a syringe by slanting the tubes. In order to avoid solubilization of insoluble complex, we did not wash the precipitate. The protein content of the precipitate was determined by the method of Lowry et al. (23) with LDL of known protein content (19) as a standard.

The solubilization of preformed insoluble DS–LDL complex by phospholipase A was studied in the following manner. The insoluble complex was prepared in suspension in phosphate buffer of pH 7.4, ionic strength 0.1, by mixing DS and LDL in concentrations of 0.012 mg/ml and 0.40 mg/ml, respectively. 0.1 ml of the phosphate buffer containing various amounts of phospholipase A preparation was added to 3 ml of the DS–LDL mixture in a cylindrical absorption cell. The change in the optical density of the insoluble complex suspension was measured with a Cary spectrophotometer at 750 mμ for a period of 30 min.

Ultracentrifugal analyses of control and phospholipase A-treated LDL and the mixture of these lipoproteins with DS at a DS/LDL ratio of 0.03 were carried out at 20°C in phosphate buffer of pH 7.4, ionic strength 0.1, in a Spinco model E ultracentrifuge at 59,780 rpm with an acceleration time of 6 min, bar angle 55°.

## RESULTS

Formation of insoluble DS–LDL complex in phosphate buffer of pH 7.4, ionic strength 0.1, was greatly influenced by the DS/LDL weight ratio (Fig. 1, curve *1*),

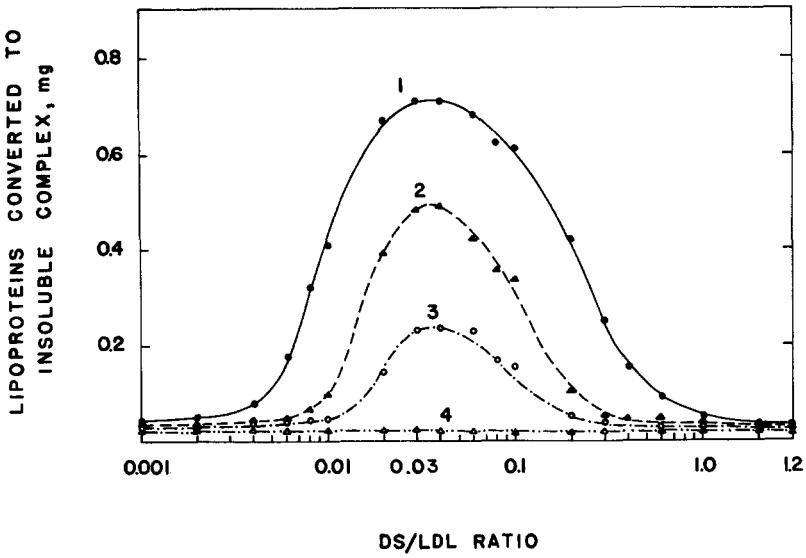


FIG. 1. Formation of insoluble DS–LDL complex as influenced by prior treatment of LDL with venom phospholipase A. Curve *1* shows the amount of untreated LDL that is converted to insoluble complex with changes in DS/LDL weight ratio. Curves *2*, *3*, and *4* show the alterations in insoluble complex formation with the use of phospholipase A-treated LDL samples; the fatty acid content increased 0.15, 0.30, and 0.66 μmole/ml, respectively (control LDL sample contained 0.04 μmole/ml). This corresponds to 7, 15, and 32% hydrolysis of the lecithin plus phosphatidyl ethanolamine components of LDL. The amount of LDL converted to insoluble complex was determined by analysis of the protein content of the complex sedimented by centrifugation.

in accordance with previous observations (10). The maximum degree of conversion of LDL to the insoluble complex occurred at a ratio of 0.03–0.04. When the LDL samples treated with phospholipase A were substituted for the untreated LDL samples, the equivalence ratio was the same, but the amount of insoluble complex formed was greatly reduced (curves *2* and *3*). After a certain degree of lipolysis, no soluble complex could be detected (curve *4*).

Addition of phospholipase A to the suspension of DS–LDL complex, prepared at a DS/LDL ratio of 0.03, caused a progressive decrease in the amount of insoluble complex during incubation (Fig. 2). The rate of clearing of the insoluble complex was greatly accelerated by increasing the concentration of phospholipase A preparation in the incubation medium. This clearing was, however, effectively inhibited by the presence of EDTA (Fig. 3); concentrations as low as 5 μg/ml nearly nullified the effect of phospholipase A on the insoluble complex prepared from 0.40 mg of LDL and 0.012 mg of DS per ml. It was noticed that the presence of EDTA at a concentration as high as 0.2% in the DS–LDL mixture did not interfere with the formation of insoluble complex, so that it could be conveniently added to stop the action of phospholipase A (29,30) when LDL samples of various degrees of hydrolysis were needed for the investigation of the factors influencing DS–LDL interaction.

The study of the conversion of phospholipase A-treated LDL into insoluble DS–LDL complex in the presence of 0.1% EDTA and at a DS/LDL ratio of 0.03 indicated that the formation of insoluble complex was markedly reduced by the use of 1% LDL samples containing more than 0.10 μmole of released fatty acids per ml (Fig. 4). Conversion was nearly prevented when the concentration of the fatty acid released reached 0.40 μmole/ml. Since phospholipase A specifically cleaves the ester linkage at the 2-position of phosphoglycerides, the amount in μmoles of fatty acids released from LDL represents the amount in μmoles of LDL phospholipids hydrolyzed by the enzyme. The total phospholipid content per ml of 1% LDL solution was found to be 2.91 μmoles, of which 1.91, 0.13, 0.76, and 0.11 μmoles were present as lecithin, phosphatidyl ethanolamine, sphingomyelin, and lysolecithin, respectively. Among these phospholipid constituents, lecithin and phosphatidyl ethanolamine are the only LDL components hydrolyzed by phospholipase A (31). Therefore, the release of 0.40 μmole/ml of free fatty acids by the treatment of 1% LDL solution with phospholipase A represents the hydrolysis of 13.8% of the total lipoprotein phospholipids, or 19.6% of the lecithin and phosphatidyl ethanolamine constituents of the lipoproteins.

It was suspected that the free fatty acids formed by the treatment of LDL with venom phospholipase A might be in association with the LDL, thus interfering with the formation of insoluble complex. If this were the case, the removal of the fatty acids from the LDL should prevent any appreciable decrease in the complex formation.

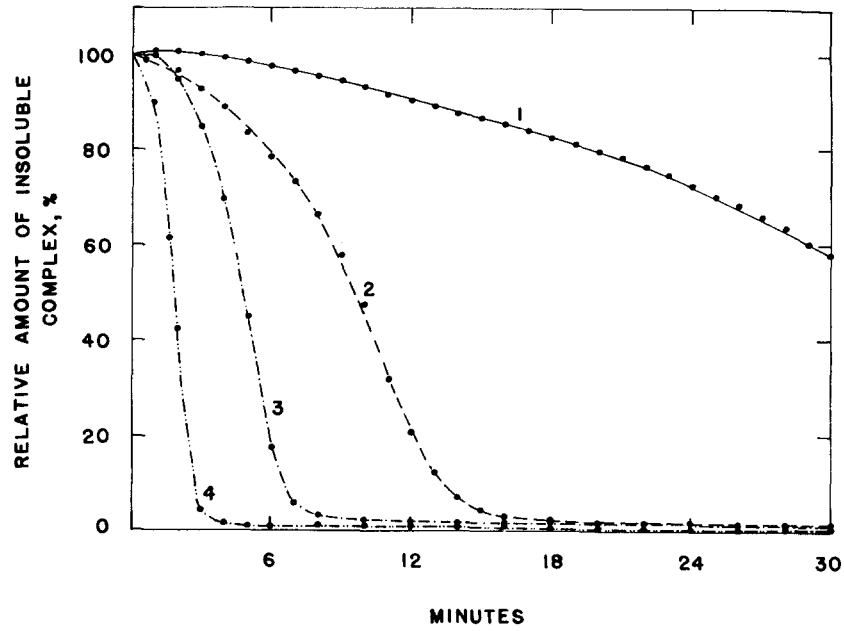


FIG. 2. Solubilization of suspended insoluble DS–LDL complex by the action of phospholipase A. The insoluble complex was prepared by mixing LDL and DS in final concentrations of 0.40 and 0.012 mg/ml, respectively. Curves *1*, *2*, *3*, and *4* show reductions with time in the relative amount of insoluble complex caused by the addition of 0.1 ml of enzyme solution containing 0.01, 0.03, 0.05, and 0.10 mg of phospholipase A preparation, respectively, to 3 ml of the suspension. The "relative amount of insoluble complex" on the ordinate is the optical density of the suspension as a percentage of the zero time value. The optical density of the control did not change during the incubation period of 30 min.

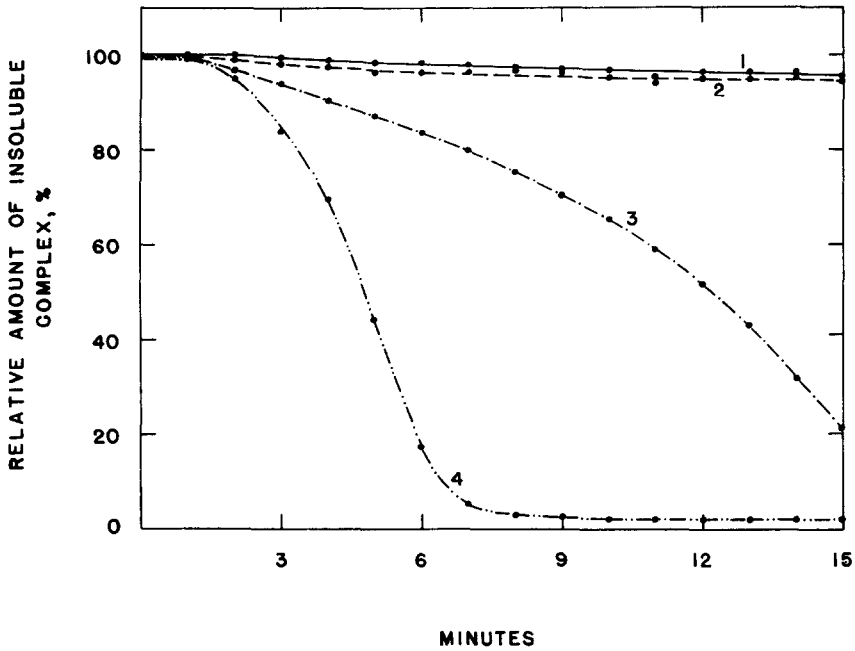


FIG. 3. Effect of EDTA on the solubilization of insoluble DS–LDL complex by phospholipase A. The insoluble complex was prepared in the presence of EDTA and then incubated with 0.05 mg of phospholipase A preparation. Curves *1*, *2*, *3*, and *4* show changes in the relative amount of insoluble complex in the presence of EDTA in final concentrations of 10, 5, 2, and 0 $\mu$g/ml, respectively.

This supposition was supported by the results obtained from the experiment in which 1% LDL samples were incubated with phospholipase A preparation at a concentration of 5 $\mu$g/ml in phosphate buffer of pH 7.4, ionic strength 0.1, in the presence and absence of 5% bovine plasma albumin, a known acceptor of free fatty

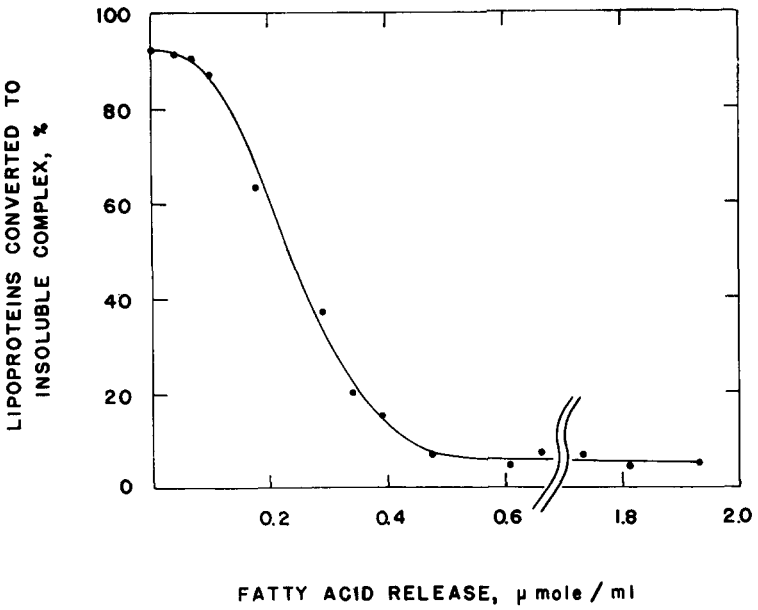


FIG. 4. Correlation of the conversion of LDL into insoluble complex with the amount of fatty acid released from the phospholipid components of LDL by treatment with phospholipase A. The fatty acid content of 1% control LDL was 0.06 μmole/ml. The percentages of LDL converted to insoluble complex were determined by protein analysis of the complex sedimented by centrifugation.

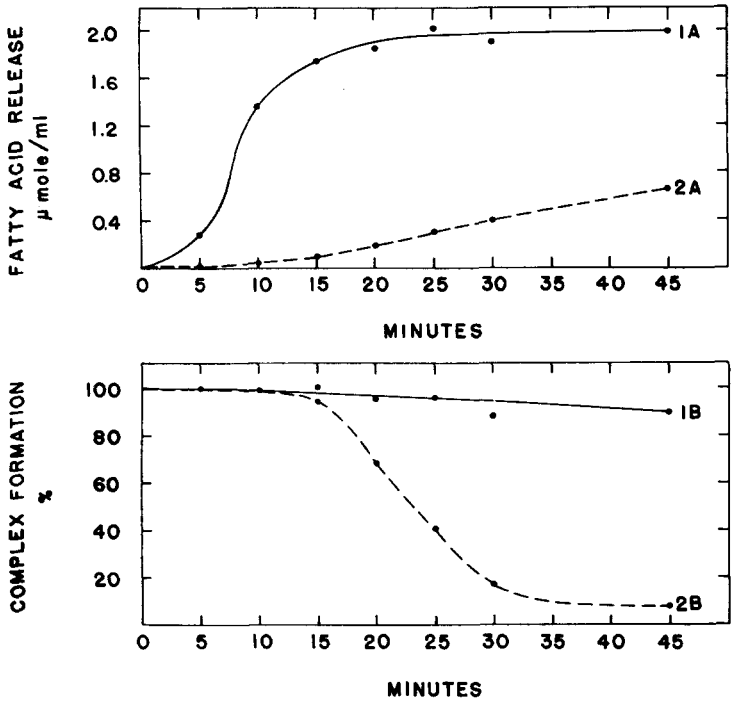


FIG. 5. Effect of albumin on the fatty acid release from phospholipid constituents of LDL incubated with phospholipase A and on the formation of insoluble DS–LDL complex. Curves *1A* and *2A* show the release of fatty acids upon incubation of 1% LDL solution with 5 μg of phospholipase A preparation per ml in the presence and absence of 5% bovine plasma albumin, respectively. Curves *1B* and *2B* show the conversion of LDL, incubated in the presence and absence of 5% albumin, respectively, to insoluble complex. The complex was formed at a DS/LDL weight ratio of 0.03. The 1% LDL without incubation contained 0.04 μmole of fatty acids per ml.

acids. Although the presence of albumin greatly enhanced the release of fatty acids (Fig. 5, curve *1A*) to the extent equivalent to hydrolysis of over 91% of the LDL phosphoglyceride components after 20 min incubation, the formation of insoluble complex at DS/LDL ratio of 0.03 was not appreciably reduced (curve *1B*). This was in

contrast to the LDL samples incubated with phospholipase A in the absence of albumin, where, even though the release of fatty acids was considerably less pronounced (curve *2A*), the insoluble complex formation progressively decreased with increasing fatty acid concentrations (curve *2B*).

To determine the extent of fatty acid removal by albumin, we centrifuged the LDL samples, incubated with phospholipase A in the presence of albumin in sodium chloride of d 1.063 at 114,400 *g* for 20 hr to allow both quantitative flotation of the LDL and sedimentation of the albumin. The determination of fatty acids in both top and bottom fractions indicated that the released fatty acids were almost entirely associated with the albumin fraction. However, when the LDL samples incubated in the absence of albumin and centrifuged in the same manner were analyzed, the released fatty acids were recovered almost completely in the LDL fraction. A similar result was obtained upon analysis of the fatty acids in both albumin and LDL fractions that were separated by the selective precipitation of the lipoproteins with DS and magnesium sulfate. Thus, it is apparent that albumin, by removing the released fatty acids, facilitated the formation of insoluble complex even from the LDL in which almost all of the phosphoglyceride components were hydrolyzed. Although the mere presence of albumin in the medium for insoluble complex formation might have exerted some effect on the DS–LDL interaction, this possibility was eliminated by the observation that the addition of albumin to mixtures of DS and control LDL with various DS/LDL ratios did not influence the degree of insoluble complex formation.

In view of the recent finding that plasma albumin is the main carrier of lysolecithin in the circulation (32), the distribution of lysolecithin in LDL and albumin fractions was determined for the lipoprotein samples incubated with venom phospholipase A in the presence of albumin. Thin-layer chromatography of the extracted lipids revealed that a major portion of the lysolecithin formed was retained in the reisolated LDL fraction (Fig. 6, sample 3), although a part of the lysolecithin was transferred to the albumin fraction (Fig. 6, sample 4). Upon analysis of the phospholipid phosphorus, the distribution of the lysolecithin in the lipoprotein and albumin fractions was found to be approximately 77% and 23%, respectively, of the total lysolecithin present in the incubation mixture. These results indicated that the albumin added to the incubation mixture preferentially removed the fatty acids but not the lysolecithin. Furthermore, the lyso compounds formed by the treatment of LDL with phospholipase A, unlike the released fatty acids, did not seem to influence the interaction of LDL with DS. This speculation was based on the

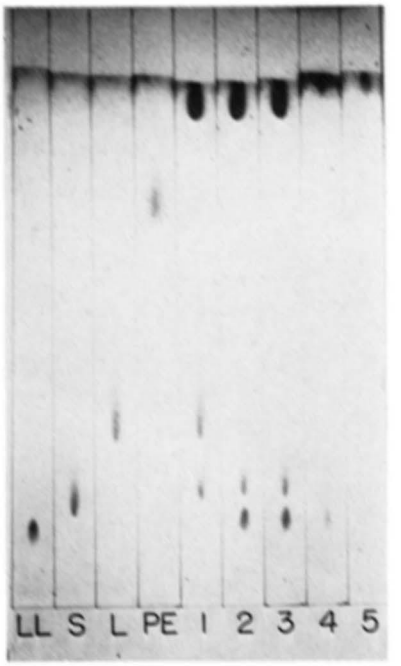


FIG. 6. Thin-layer chromatogram of phospholipids extracted from control LDL and from LDL treated with phospholipase A. 50 mg of LDL was incubated with 0.6 mg of phospholipase A preparation in the presence of 250 mg of bovine plasma albumin in 4.5 ml of phosphate buffer for 1 hr. After addition of 0.5 ml of 1% EDTA, the LDL and albumin fractions were reisolated by centrifugation at d 1.063. For thin-layer chromatography, the lipids extracted from 1 ml of 1% control LDL (sample 1), 1 ml of the incubation mixture containing 1% LDL and 5% albumin (sample 2), the LDL fraction (sample 3) and albumin fraction (sample 4) separated from 1 ml of the incubation mixture, and 1 ml of 5% albumin (sample 5) were dissolved in 0.5 ml of chloroform; 20 μl each of the chloroform solutions was applied to a Silica Gel G plate. LL, S, L, and PE designate 40 μg each of standard lysolecithin, sphingomyelin, lecithin, and phosphatidyl ethanolamine, respectively, applied to the plate. Solvent, chloroform–methanol–acetic acid–water 25:15:4:2; detection, sulfuric acid–dichromate spray.

following considerations. (*a*) The hydrolysis of approximately 20% of LDL lecithin and phosphatidyl ethanolamine components was sufficient to nullify the formation of insoluble complex in the absence of albumin; and (*b*) when LDL was incubated in the presence of albumin, even as much as 97% hydrolysis of the phospholipids did not significantly reduce the formation of insoluble complex in spite of the association of a major portion of the lyso-compounds with the LDL.

In order to determine the nature of the interaction between DS and LDL treated with phospholipase A, we subjected the mixtures at a DS/LDL weight ratio of 0.03 to ultracentrifugal analysis. The DS–untreated LDL mixture did not produce an observable boundary because the LDL was almost completely precipiated as insoluble complex (Fig. 7, A-3). However, the mixtures of DS and LDL in which 29% (A-1) and 94% (A-2) of the lecithin plus phosphatidyl ethanolamine com-

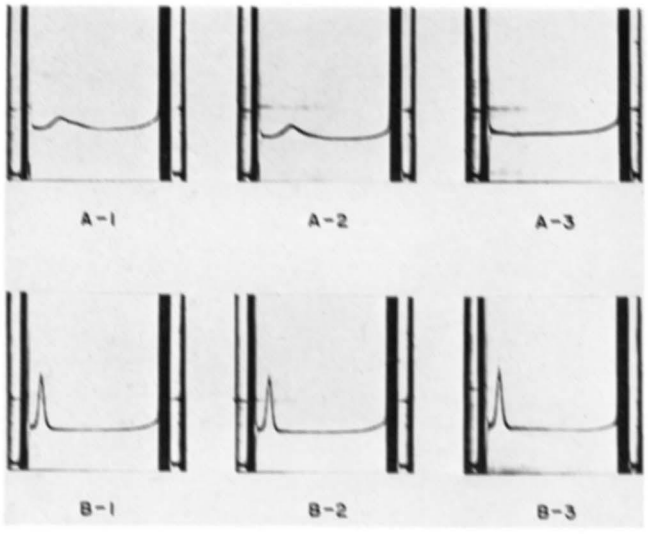


FIG. 7. Effect of prior treatment of LDL with phospholipase A on the sedimentation behavior of DS–LDL mixtures and LDL sample alone. LDL samples were treated with phospholipase A in the usual manner. Samples 1 and 2 are the treated LDL in which 29 and 94%, respectively, of the phosphoglyceride constituents were hydrolyzed; sample 3 is control LDL. A, sedimentation patterns of 0.0075% DS–0.25% LDL mixture; B, 0.25% LDL alone. The photographs were taken 14 min after reaching the maximum speed.

ponents were hydrolyzed by phospholipase A exhibited a boundary with an apparent sedimentation coefficient of approximately 14.5 (Table 1). Since this value was far greater than the sedimentation coefficient of free LDL, the boundary apparently represented the soluble DS–LDL complex. It seems that the fatty acids that were released by the treatment of LDL with phospholipase A and were still in association with LDL prevented the conversion of soluble complex to large insoluble molecular aggregates. Furthermore, the amount of the associated fatty acids influenced the heterogeneity of the soluble complex. The soluble complex present in the mixture A-1 (Fig. 7) was more heterogeneous than that in mixture A-2 and appeared to contain a higher molecular weight species.

TABLE 1 APPARENT SEDIMENTATION COEFFICIENTS OF LDL AND DS–LDL MIXTURES AS INFLUENCED BY PRIOR TREATMENT OF LDL WITH PHOSPHOLIPASE A

| Analyses | Phospholipase A-Treated Samples 1 | 2 | Control Sample |
|---|---|---|---|
| Phosphoglycerides hydrolyzed, % | 29 | 94 | 0 |
| Free fatty acids, μmoles/10 mg of LDL | 0.64 | 1.96 | 0.04 |
| Sedimentation coefficient | | | |
| 0.25% LDL | 6.1 | 6.4 | 6.0 |
| 0.0075% DS–0.25% LDL mixture | 14.5 | 14.4 | —* |

* No soluble complex was apparent, as can be seen in Fig. 7, A-3.

The addition of magnesium sulfate to the DS–phospholipase A-treated LDL mixture converted the soluble complex to the insoluble complex. The concentration of magnesium ion required increased with increasing concentrations of the fatty acids associated with the lipoproteins (Fig. 8); 0.08 ml of 1% LDL preparations containing 0.39, 0.66, and 1.54 μmoles/ml of fatty acids required magnesium sulfate concentrations of 3.9, 9.3, and 16.9 mM, respectively, for the conversion of 50% of the LDL into insoluble complex. In the presence of excess magnesium sulfate, the protein moiety of phospholipase A-treated LDL was almost completely recovered from the insoluble complex regardless of the extent of the phospholipid hydrolysis.

## DISCUSSION

Treatment of LDL with phospholipase A hydrolyzed as much as 97% of the lecithin and phosphatidyl ethanolamine components, and yet the LDL retained the ability to interact with sulfated polysaccharides. This was shown by (*a*) the formation of soluble complex upon interaction of the phospholipase A-treated LDL with DS and (*b*) almost complete conversion of the soluble complex to insoluble complex in the presence of excess magnesium sulfate. Furthermore, when fatty acids associated with the LDL were removed by albumin, the LDL interacted with DS in exactly the same manner as native LDL; the formation of insoluble complex did not require the presence of magnesium ion. It is thus apparent that 2-hydrolysis of almost all the phosphoglycerides in LDL leaves many properties of LDL intact.

The amount of insoluble DS–LDL complex formed at the equivalence region was greatly reduced with the LDL samples in which more than 5% of the lecithin and phosphatidyl ethanolamine components was hydrolyzed by treatment with venom phospholipase A. Upon hydrolysis of over 20% of the phospholipid components, the formation of insoluble complex was completely prevented. Since the fatty acids released by the treatment of LDL with phospholipase A were found to be associated with the lipoproteins and interfered with the formation of insoluble complex, an increase in the negative charge due to the bound fatty acids appears to have prevented the conversion of soluble complex to insoluble complex. This in turn suggests the nature of the interaction between DS and untreated LDL. In the equivalence region, which corresponds to a LDL/DS molar ratio of 5–6, small polymers of LDL molecules linked by DS bridges will initially be produced primarily by electrostatic attractions between positively charged groups of LDL and negatively charged sulfate groups of DS. Such electrostatic interactions will reduce the net surface polar charge of the lipoproteins and hence decrease the compatibility

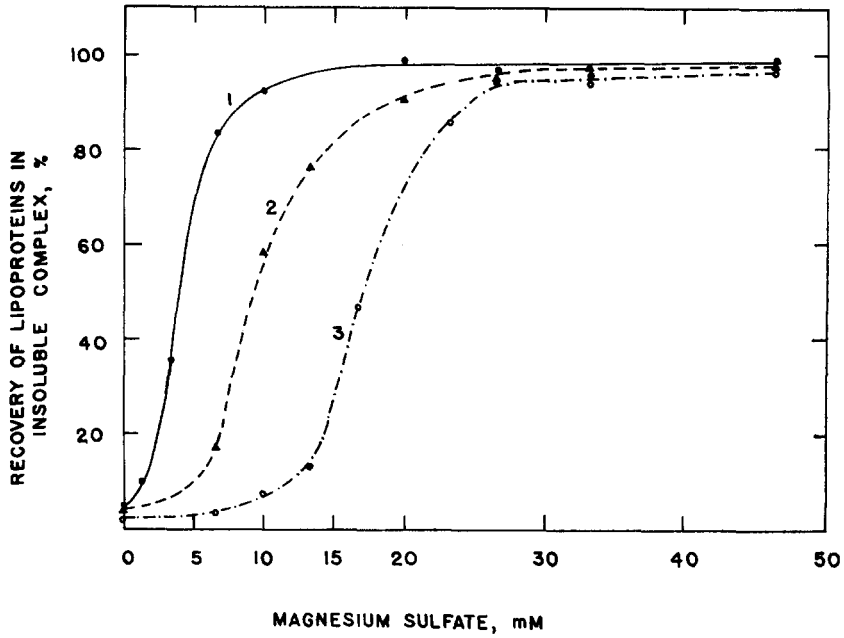


FIG. 8. Effect of magnesium sulfate on the conversion of phospholipase A-treated LDL to insoluble DS–LDL complex. LDL was treated with phospholipase A and 1% LDL preparations containing 0.39 (curve *1*), 0.66 (curve *2*), and 1.54 μmoles (curve *3*) of fatty acids per ml were obtained. To 0.08 ml of such solutions we added first 0.024 mg of DS dissolved in 1.2 ml of phosphate buffer and then various amounts of magnesium sulfate to the final concentrations shown. The total volume was 1.5 ml and the EDTA concentration, 0.01%. The recovery of LDL in the insoluble complex was determined by protein analysis of the complex sedimented by centrifugation.

of the small polymer with its aqueous environment and lead to the formation of large insoluble aggregates. On the other hand, in the phospholipase A-treated LDL, the aggregation of soluble complexes may be prevented by (*a*) the repulsion between negatively charged bound fatty acids present in adjacent soluble complexes and (*b*) the partial interference by the fatty acids with the electrostatic attraction between DS and LDL in the soluble complex itself, which increases the availability of polar groups for the solute–solvent interaction.

A previously formed insoluble DS–LDL complex was also shown to be converted to soluble complex by the treatment with phospholipase A. In this system, however, it was necessary to use approximately 20 times more enzyme preparation than that required for the same amount of LDL alone to obtain a comparable hydrolysis rate for the phospholipid components. The increased requirement for phospholipase A seemed to be due to the difference in the states of the substrates; in the insoluble complex, a considerable portion of LDL may not be readily accessible to the enzyme. It is conceivable that the phospholipid components of LDL at the surface of the insoluble complex may first be hydrolyzed by phospholipase A. The LDL molecules will then be detached from the insoluble complex together with the neighboring DS molecules as soluble complex, leaving more of the unhydrolyzed LDL of the insoluble complex exposed to phospholipase A. The continuous operation of this sequence seemed to cause complete conversion of the DS–LDL insoluble complex into soluble complex.

The presence of divalent ions in the mixture of sulfated polysaccharide and LDL is known to facilitate the formation of insoluble complex (1). Since, in the present study, the amount of magnesium sulfate required for the conversion of soluble complex to insoluble complex increased progressively with increasing amounts of fatty acids associated with LDL, the interaction of magnesium ions with the associated fatty acids was primarily responsible for the conversion. This interaction may involve the binding of magnesium ion to two molecules of fatty acids present in the same or different soluble complexes. In addition, magnesium ions may link the fatty acids of phospholipase A-treated LDL and the sulfate groups of the DS in soluble complex. In either case, charge destruction or neutralization of carboxylate ions by magnesium ions appears to promote the conversion of soluble complex to insoluble complex. It was further noted that, upon acidification of the mixture of DS and phospholipase A-treated LDL, the soluble complex was converted to insoluble complex. The protonation of the bound fatty acid carboxylate ions apparently eliminates the interfering effect of the bound fatty acids.

The removal of the bound fatty acids from phospholipase A-treated LDL in the presence of a large amount

of albumin is in agreement with the results obtained by Goodman and Shafrir (33). Since the presence of albumin facilitated the formation of insoluble complex, the bound fatty acids apparently masked some of the binding sites of the LDL and thus prevented an efficient association of the LDL with DS. A study is currently in progress in our laboratory to determine the relative contribution to the binding sites by the protein and lipid components of LDL.

The author wishes to thank Mrs. L. Mast, Miss J. Warfel, Mrs. E. Villarreal, and Miss S. Valentine for skillful assistance during the course of this study.

This investigation was supported by Grants HE 03063 from the National Institutes of Health, U.S. Public Health Service, and RN67-23 from the Illinois and Chicago Heart Associations.

*Manuscript received 14 March 1968; accepted 19 June 1968.*

## References

1. Cornwell, D. G., and F. A. Kruger. 1961. *J. Lipid Res.* **2:** 110.
2. Oncley, J. L., K. W. Walton, and D. G. Cornwell. 1957. *J. Am. Chem. Soc.* **79:** 4666.
3. Briner, W. W., J. W. Riddle, and D. G. Cornwell. 1959. *J. Exptl. Med.* **110:** 113.
4. Bernfeld, P., M. E. Berkowitz, and V. M. Donahue. 1957. *J. Clin. Invest.* **36:** 1363.
5. Anatoniades, H. N., J. L. Tullis, L. H. Sargeant, R. B. Pennell, and J. L. Oncley. 1958. *J. Lab. Clin. Med.* **51:** 630.
6. Bernfeld, P. 1958. *In* The Lipoproteins, Methods and Clinical Significance. P. Homburger and P. Bernfeld, editors. S. Karger, Basel. 24–36.
7. Bernfeld, P., J. S. Nisselbaum, B. J. Berkeley, and R. W. Hanson. 1960. *J. Biol. Chem.* **235:** 2852.
8. Bernfeld, P., V. M. Donahue, and M. E. Berkowitz. 1957. *J. Biol. Chem.* **226:** 51.
9. Boyle, E., and R. V. Moore. 1959. *J. Lab. Clin. Med.* **53:** 272.
10. Janado, M., and T. Nishida. 1965. *J. Lipid Res.* **6:** 331.
11. Bernfeld, P., and T. F. Kelley. 1964. *J. Biol. Chem.* **239:** 3341.
12. Condrea, E., Z. Mammon, S. Aloof, and A. De Vries. 1964. *Biochim. Biophys. Acta.* **84:** 365.
13. Blecher, M. 1967. *Biochim. Biophys. Acta.* **137:** 557.
14. Bradlow, B. A., and A. J. Marcus. 1966. *Proc. Soc. Exptl. Biol. Med.* **123:** 889.
15. Siliprandi, N. 1963. *In* Biochemical Problems of Lipids. A. C. Frazer, editor. Elsevier Publishing Co., New York. 346–351.
16. Ricketts, C. R. 1952. *Biochem. J.* **51:** 129.
17. Gillies, G. A., F. T. Lindgren, and J. Cason. 1956. *J. Am. Chem. Soc.* **78:** 4103.
18. Gofman, J. W., F. Lindgren, H. Elliott, W. Mantz, J. Hewitt, B. Strisower, and V. Herring. 1950. *Science.* **111:** 166.
19. Nishida, T., and H. Nishida. 1965. *J. Biol. Chem.* **240:** 225.
20. Ibrahim, S. A., H. Sanders, and R. H. S. Thompson. 1964. *Biochem. J.* **93:** 588.
21. Saito, K., and D. J. Hanahan. 1962. *Biochemistry.* **1:** 521.
22. Greenberg, D. M. 1955. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **2:** 54–68.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193:** 265.
24. Dole, V. P. 1956. *J. Clin. Invest.* **35:** 150.
25. Nelson, G. J., and N. K. Freeman. 1959. *J. Biol. Chem.* **234:** 1375.
26. Parker, F., and N. F. Peterson. 1965. *J. Lipid Res.* **6:** 455.
27. Rhodes, D. N., and C. H. Lea. 1957. *Biochem. J.* **65:** 526.
28. Hanahan, D. J., J. C. Dittmer, and E. Warashina. 1957. *J. Biol. Chem.* **228:** 685.
29. Marinetti, G. V. 1961. *Biochim. Biophys. Acta.* **46:** 468.
30. Condrea, E., A. De Vries, and J. Mager. 1962. *Biochim. Biophys. Acta.* **58:** 389.
31. Ansell, G. B., and J. N. Hawthorne. 1964. Phospholipids. Elsevier Publishing Co., New York. 153–159.
32. Switzer, S., and H. A. Eder. 1965. *J. Lipid Res.* **6:** 506.
33. Goodman, D. S., and E. Shafrir. 1959. *J. Am. Chem. Soc.* **81:** 364.