TRITON HYPERLIPEMIA IN DOGS

I. In Vitro Effects of the Detergent on Serum Lipoproteins and Chylomicrons*, ‡

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In 1951, Kellner, Correll, and Ladd reported that injection of a non-ionic detergent (tween 80, triton A-20) induces in animals sustained hyperlipemia (1). The mechanism was not determined. Later studies by Friedman and Byers (2, 3) and Hirsch and Kellner (4, 5) provided support for the hypothesis that the hyperlipemia may be caused by primary physical or chemical changes induced by the detergent in plasma lipoproteins; as a consequence of these changes lipid would remain trapped or locked in circulating plasma. Consistent with this hypothesis are the findings by Brown, Boyle, and Anfinsen (6), that non-ionic detergents *in vitro* cause irreversible changes in serum lipoproteins.

Since studies on the nature of the interaction between triton and lipoproteins may help in understanding the role of plasma lipoproteins in lipid transport, we have further studied the problem. The *in vitro* studies are reported here.

Methods and Procedures

Sera from normal, fasting donor dogs were used. Low density lipoproteins (D 1.006 to 1.063) were separated by ultracentrifugation according to Gofman *et al.* (7). After removal of this fraction, the residue was spun at D 1.21 according to Lewis, Green, and Page (8). Both D 1.063 and D 1.21 lipoproteins were purified as described previously (9). Lipoproteins were dialyzed against 0.15 M NaCl before use.

α_1-lipoprotein protein (α-LP) was separated from serum, and α-P prepared according to the delipidation procedure of Scanu, Lewis, and Bumpus (10).[1] Pooled lipemic sera obtained from blood of dogs fed 500 ml. of 18 per cent cream, were used as a source of chylomicrons. They were separated and purified by the ultracentrifugal procedure described by Scanu and Page (11).

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[1] The abbreviations used in the text are: D 1.006 to 1.063 lipoproteins or β-lipoproteins, β-LP; D 1.063 to 1.21 lipoproteins or α_1-lipoprotein, α-LP. α-P is the protein from α-LP after removing the lipid.

Total lipids were determined gravimetrically by the method of Sperry (12), and triglycerides according to Van Handel and Zilversmit (13). Cholesterol determinations were performed by the procedure of Abell *et al.* (14). Phospholipids were calculated as 25 times the lipid phosphorus values obtained by the method of Fiske and SubbaRow (15). Protein concentration was determined by the Lowry method (16). In samples containing triton, protein content was calculated by multiplying by the factor 6.25 the nitrogen values obtained by the micro Kjeldahl procedure of Lange (17).

I^{131} labeling of the lipoproteins was performed according to McFarlane (18). Free iodine was removed by passing the radioiodinated protein 3 times through an anion exchange resin, ioresin® (Abbott Laboratories, North Chicago). The labeled protein was estimated to have about one atom of iodine per molecule. The labeling with I^{131} of the protein moiety of chylomicrons was performed as previously described (9, 18), based on the interaction between chylomicron protein and α-P–I^{131}.

Lipomul®,[2] a cottonseed oil emulsion, was also used in our experiments. Each milliliter of the emulsion contained approximately 150 mg. of total lipids. The complexes lipomul–α-LP and lipomul–α-LP–I^{131} were prepared as described by Scanu and Page (19).

Triton WR-1339®,[3] a polyoxyethylene ether of an alkyl phenol, was employed as the nonionic detergent. When radioiodinated triton was needed, the following iodination procedure was performed: aliquots of a 12.5 per cent solution of triton in 0.15 M NaCl were brought to pH 8.6 by gradual addition of a glycine-NaOH buffer (18). From 0.1 to 1 ml. of a solution of NaI^{131} containing 25 mc. of I^{131} per ml., was mixed with 5 ml. of a 1 mM solution of ICl and this mixture rapidly added to the buffered triton in the ratio of 0.1 ml. of ICl per ml. of triton. The free radioiodine was removed through ioresin. The labeled triton thus obtained had the same detergent action as unlabeled triton. When the specific activity was too high for our purposes, unlabeled triton was used to dilute the radioiodinated product.

The ***in vitro*** studies on the interaction between triton and whole serum or between triton and purified lipoproteins were carried out in Spinco lusteroid tubes ½ inch × 3½ inches. During incubation at 37°C. the tubes were gently shaken at 10 minute intervals. In order to carry on all the reactions at a constant pH, the reactants were dissolved in phosphate buffer, pH 7.2, $\Gamma/2$, 0.1. Concentrations of detergent and lipoproteins and times of incubation are reported in the next section. Following incubation, the mixtures were analyzed by electrophoresis and by preparative and analytical ultracentrifugation. Starch gel electrophoretic analyses were performed at 4°C., following the technique described by Smithies (20) with the discontinuous system of buffers (tris–boric acid, pH 8.2) proposed by Poulik (21); amido Schwartz 10 B Bayer was used for protein staining and oil red O Spinco for lipid staining. When labeled samples were used, the curve of radioactivity of the starch pattern was obtained as reported previously (9). Elution of proteins from unstained starch blocks was performed by displacing the protein, electrophoretically, from the starch into cotton wool, which had been interposed in the starch block according to Jarrige and Lafoscade (22). The proteins were squeezed from the cotton wool by suction through a Büchner filter.

Moving boundary electrophoresis was determined by Longworth's modification of the Tiselius method employing barbital buffer, pH 8.6, $\Gamma/2$, 0.1 and paper electrophoresis using a Durrum (23) cell with barbital buffer of pH 8.6, $\Gamma/2$, 0.05. Brom-phenol blue was used for protein staining, and Sudan black for lipid staining. Scanning of the strips was performed by the Spinco analytrol, model RA. When labeled material was used, radioactivity was determined by a windowless paper chromatogram scanner (Atomic Accessories, Bellerose, New York).

For ultracentrifugal analysis, the mixtures of detergent and serum or purified lipoproteins

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[2] Upjohn Company, Kalamazoo, Michigan.

[3] Winthrop Laboratories, New York.

were dialyzed for 48 hours against the desired salt solutions at D 1.006 (NaCl), 1.063 (NaCl), and 1.21 (NaCl-KBr). Before ultracentrifugal analysis, the density of the mixtures containing triton was determined by pycnometry, using pycnometer bottles of 1 ml. capacity. A Spinco ultracentrifuge model L preparative, with a 30.2 rotor and a Spinco model D analytical ultracentrifuge were used in these studies.

Post-heparin plasma from donor dogs was the source of lipoprotein lipase. The animals were injected intravenously with heparin (liquaemin, sodium, organon) in doses of 25 u/kg. and blood was withdrawn 10 minutes later. Enzymatic assays were carried out at pH 7.2, 37°C., using lipomul as a substrate. The reactions were followed by readings of optical density in a Coleman spectrophotometer at 660 mμ., and by production of fatty acids according to Dole's method (24).

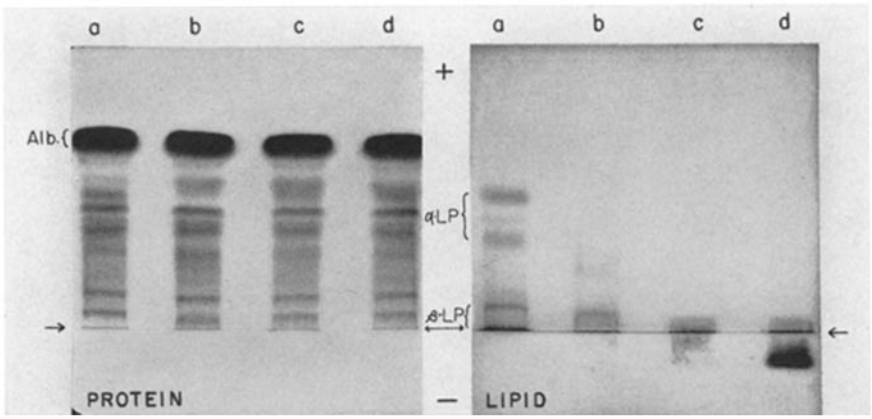

FIG. 1. Starch gel electrophoretic patterns of normal serum alone (*a*) and with triton. For each milliliter of serum either 4 mg. (*b*), 20 mg. (*c*), or 40 mg. (*d*) of triton was added. Volumes were adjusted so that each 0.1 ml. aliquot contained the same amount of serum. The starch block was cut into two slices along the plane of migration. The slice on the left was stained for protein (amido Schwartz) and the one on the right, for lipid (oil red O). The arrows indicate points of application of the samples.

RESULTS

Effects of Triton on Whole Serum.—Various amounts of triton (2 to 40 mg.) were added to 1 ml. aliquots of serum, each aliquot containing 72 mg. of total protein of which 2.4 mg. was lipoprotein protein. The mixtures were incubated for 2 hours at 37°C., pH 7.2, and then analyzed by starch gel electrophoresis. Some of the results are reported in Fig. 1, where *a* is the pattern of a normal serum and *b*, *c*, and *d* are mixtures of 1 ml. of serum and 4, 20, and 40 mg. of triton, respectively. As indicated in the protein-stained pattern, addition of detergent increased the mobility of the α-LP protein boundary in the post-albumin zone; this effect is more marked in *c* and *d* where larger concentrations of triton were present. The other protein boundaries do not appear to be affected by triton. The lipid-stained pattern, *a*, shows the three boundaries of

α-LP, previously described (19), and one broad boundary of β-LP. After addition of 4 mg. of triton, *b*, β-LP lipid remained unchanged, whereas the lipid boundaries of α-LP were not seen in the starch pattern. When amounts of triton

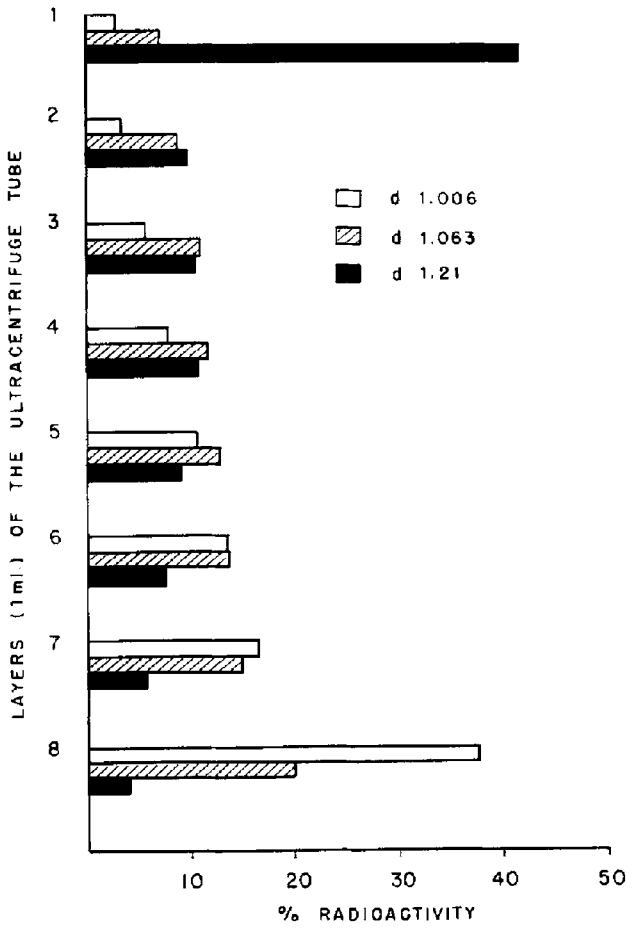

FIG. 2. Distribution of radioactivity in ultracentrifuge tubes after centrifugation of triton-I^{131} at D 1.006, D 1.063, and D 1.21. No. 1 is the top layer.

up to 40 mg. were used, most of the lipids moved cathodically from the point of application.

Experiments were performed in which serum and triton were incubated for 2 hours at 4°, 24°, and 37°C. At 24°C. the results were essentially identical to the ones observed at 37°C. At 4°C., the effects of triton on serum were less marked; the lipid of α-LP showed a 50 per cent reduction in electrophoretic

mobility; the β-LP boundary showed very minor changes. Various times of incubation were also studied (10, 60, 120, and 180 minutes). The effect of triton in changing electrophoretic mobility of serum lipids was favored by increasing the length of incubation. However, if the incubation was too prolonged (over 2 hours), the results were difficult to interpret because of the denaturing effect of heat on the lipoproteins. Unless otherwise stated, in all the experiments reported below mixtures of triton and serum or triton and purified lipoproteins, were incubated for 2 hours at 37°C.

Lipase inhibitors, tri-o-cresyl phosphate and diisopropyl fluorophosphate

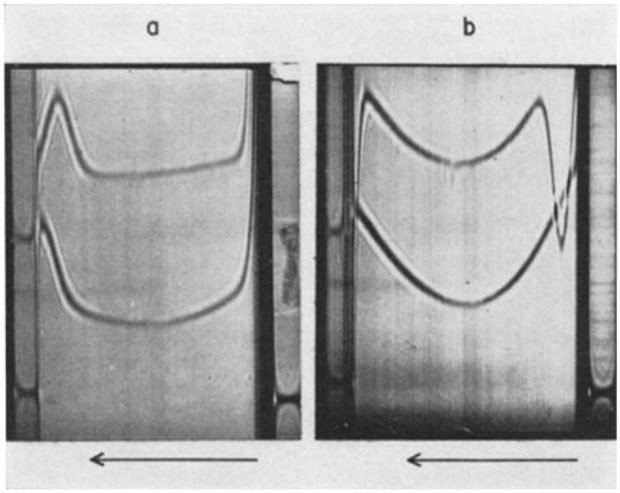

FIG. 3. Ultracentrifugal patterns of triton (10 mg.) at D 1.006, D 1.063, and D 1.21: (*a*) upper pattern, D 1.006; lower pattern, D 1.063; (*b*) upper pattern, D 1.21; lower pattern, salt blank of D 1.21. 52,640 R.P.M., 50 minutes, 26°C.

(paraoxon),[4] were mixed with 1 ml. of serum in concentrations from 10^{-2} to 10^{-4}M, prior to addition of 20 mg. of triton. These inhibitors did not prevent the action of the detergent on serum. Further, triton remained effective when added to serum which had been preheated for 5 minutes at 60°C.

Since these preliminary studies indicated that triton induces marked changes in the lipoproteins of whole serum, further experiments were performed on lipoproteins separated and purified from canine serum by ultracentrifugation. First, however, it was necessary to determine certain physical characteristics of triton itself.

Electrophoretic and Ultracentrifugal Properties of Triton.—10 mg. aliquots of

[4] Kindly provided by Dr. G. Schrader, Farbenfabriken Bayer A. G., Wuppertal-Elberfeld, West Germany.

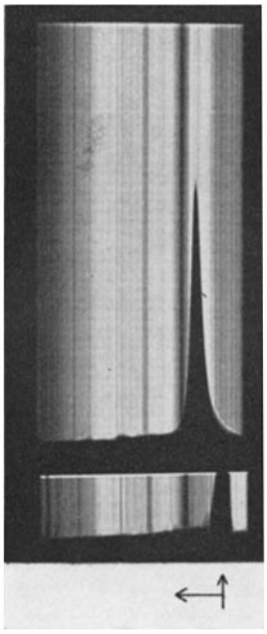

FIG. 4. Tiselius pattern of triton (10 mg.), descending boundary. Barbital buffer, pH 8.6, $\Gamma/2$ 0.05, 82 minutes, 4°C.

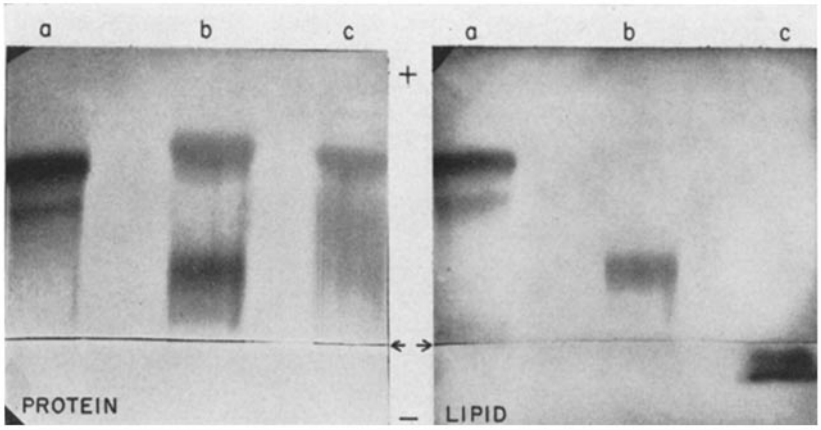

FIG. 5. Starch gel electrophoretic patterns of α-LP alone (*a*) and after incubation with triton. Triton was added to the α-LP protein in a weight ratio of 2:1 (*b*) and 20:1 (*c*). In each instance, the aliquots applied to the starch contained 2 mg. of α-LP protein. Left, protein staining; right, lipid staining.

triton-I^{131} were dissolved in salt solutions of D1.006, D 1.063, and D 1.21 and spun at 79,420*g* for 18 hours at 16°C. The distribution of radioactivity in the various layers of the ultracentrifuge tubes (Fig. 2) indicates that triton sediments at D 1.006 and D 1.063 and floats at D 1.21. These results were cor-

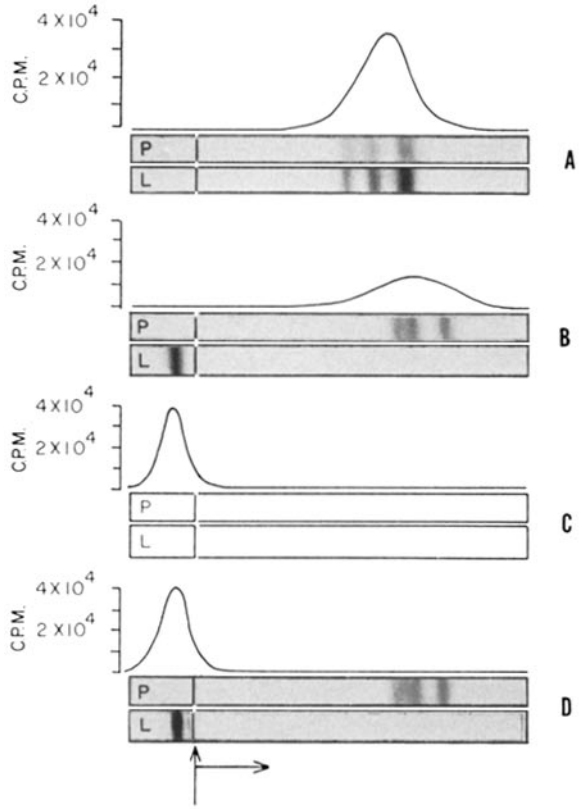

FIG. 6. Starch gel electrophoretic patterns and curves of radioactivity of labeled mixtures of triton and α-LP. A: α-LP–I^{131}, 2 mg., specific activity 0.5 μc./mg.; B: mixture of α-LP–I^{131}, 2 mg. and triton, 40 mg.; C: triton–I^{131}, specific activity 0.2 μc./mg.; D: mixture of α-LP (2 mg.) and 40 mg. of triton-I^{131}. P, protein stained pattern; L, lipid stained pattern.

roborated by the flotation diagrams in the analytical ultracentrifuge. As indicated in Fig. 3, triton floated at D 1.21 with a peak of flotation of −S 2.2. The detergent sedimented at the lower densities.

In starch gel electrophoresis, triton could not be detected by either protein or lipid staining. When triton-I^{131} was used, the curve of radioactivity gave a peak in the zone just behind the point of application. By moving boundary

electrophoresis (Fig. 4), triton gave a single boundary with a mobility of -1.4×10^{-5} cm.^{2}/volt sec.

Effect of Triton on α-LP.—The effect was found dependent on the amount of triton used. Some of the results by starch gel electrophoresis are reported in Fig. 5, where *a* represents native α-LP. After addition of 2 mg. of triton per mg. of protein, *b*, the protein of α-LP shows two boundaries moving respectively faster and slower than the major protein boundary in *a*. The faster moving fraction appears lipid-free as indicated by the lipid staining technique. With a higher concentration of detergent (20 mg. of triton per mg. of α-LP protein), a complete splitting of the protein and the lipid moieties occurred, these moieties moving in opposite directions in the electric field, *c*. The protein, apparently free of lipids as determined by staining, when eluted from starch contained 3 to 6 mg. of lipid per 100 mg. of protein. This protein exhibited the property of recombining with lipids as tested by the method of Scanu and Hughes (9) and gave an immunoprecipitin reaction in gel media with its homologous antibody.[5] In parallel experiments it was found that α-P, obtained from α-LP by delipidation with ethanol-ether (10), incubated with triton and dialyzed to remove unbound detergent, retained its capacity to recombine with lipids.

Mixtures of α-LP–I^{131} and unlabeled triton, and α-LP and labeled triton were studied by starch gel electrophoresis. These results (Fig. 6) show that removal of lipids from α-LP–I^{131} by unlabeled triton, had no effect on the peak of radioactivity of the α-LP protein. When triton-I^{131} was used, the lipids, removed from α-LP, were in the zone occupied by the labeled detergent. As shown in Fig. 6 *C*, triton did not stain either for protein or lipid.

By paper electrophoresis (Fig. 7), 2 mg. of detergent per mg. of α-LP protein, reduced the mobility of α-LP–I^{131}. With a higher concentration of triton (20 mg. per mg. of α-LP protein), the lipid moiety remained close to the origin, in a position where triton-I^{131} was found to move. The resulting protein, greatly impoverished of lipids, had a mobility lower than intact α-LP.

The analysis of mixtures of triton and α-LP by free boundary electrophoresis is shown in Fig. 8. Purified α-LP gave a single boundary with a mobility of -6×10^{-5} cm.^{2}/volt sec. Addition of 2 mg. of detergent reduced the electrophoretic mobility of α-LP (-3.2×10^{-5}) and gave appearance to a small boundary (probably triton) on the shoulder of α-LP. With a larger amount of triton (20 mg.), two boundaries were clearly visible, although incompletely dissociated; the major one (probably triton) had a lower mobility (-0.8×10^{-5}) than the minor one (-2.4×10^{-5}), which probably represented α-LP. These two boundaries could not be further separated by prolonging the time of electrophoresis or by using an acid buffer (acetate buffer, pH 4.5, $\Gamma/2$, 0.1).

In analytical ultracentrifuge, native α-LP floated with a coefficient of $-S$

[5] A more detailed report on these experiments will be presented elsewhere.

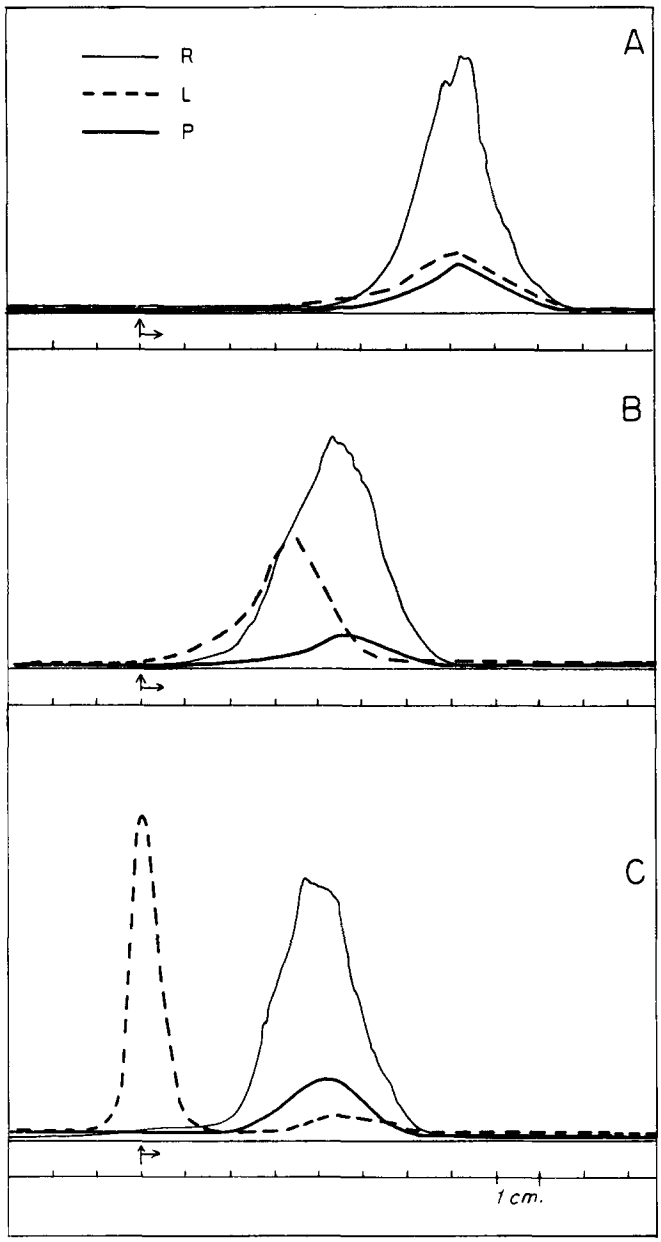

FIG. 7. Paper electrophoretic pattern of α-LP–I^{131} alone and with triton. A: α-LP–I^{131}, specific activity 0.5 μc./mg.; B: mixture 2:1 (by weight) of triton and α-LP–I^{131}; C: mixture 20:1 of triton and α-LP–I^{131}. In each case 200 μg. of labeled α-LP was applied to the paper strip. R, radioactivity; L, lipid; P, protein.

5.0 (Fig. 9 *a*, lower pattern). The effects of 2 mg. and 20 mg. of triton are indicated in Fig. 9 *b* (upper and lower patterns). The upper pattern shows two peaks of floatation with coefficients of −S 5.2 and 1.0; the lower one, a large, single peak with a very low flotation coefficient (−S 0 to 1). Ultracentrifugal studies of 20:1 mixtures of triton and α-LP were performed at D 1.21. In absence of triton, approximately 75 per cent of the protein, cholesterol, and

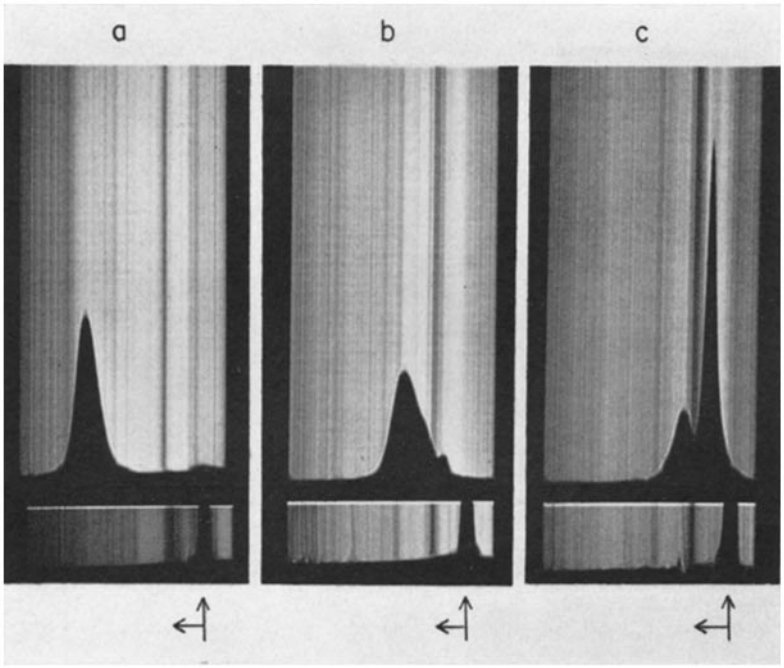

FIG. 8. Tiselius patterns (descending boundary) of α-LP alone (*a*), 2:1 mixture of triton and α-LP (*b*), and 20:1 mixture of triton and α-LP (*c*). In *a* and *b*, 2 mg. of α-LP protein were analyzed; in *c* the sample was diluted to contain only 1 mg. of α-LP protein, to permit complete visualization of the triton boundary.

phospholipid of α-LP were found in the top layer (Table I). In presence of triton, only 37 per cent of the lipoprotein protein floated, the per cent of cholesterol and phospholipid in the top layer being respectively 69 and 71. Thus, the amount of lipid in the top fraction, relative to the amount of protein, was greater than with native α-LP.

Effect of Triton on β-LP.—β-LP, like α-LP, showed changes which were dependent on the dose of triton used. Electrophoretic analyses in starch gel (Fig. 10) showed that triton, which in small concentrations reduced the electrophoretic mobility of the lipoprotein, at higher levels (40 mg.) displaced part of its lipid so that the protein moved in the same zone as triton. However,

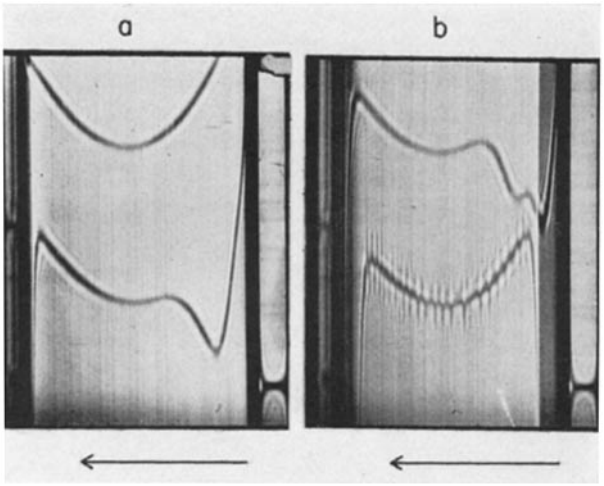

FIG. 9. Ultracentrifugal patterns of α-LP alone and with triton. (*a*) upper diagram, salt blank of D 1.21; lower pattern, α-LP; (*b*) upper pattern, 2:1 mixture of triton and α-LP; lower pattern, 20:1 mixture of triton and α-LP. Each sample contains 2 mg. of α-LP protein. 52,640 R.P.M., 36 minutes, 26°C.

TABLE I

Distribution of Protein and Lipids after Ultracentrifugation at D 1.21 of α-LP Alone or with Triton

Layers (1 ml.)		α-LP						α-LP + triton					
		P		TC		PL		P		TC		PL	
		mg.	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
Top	1.....	10.50	74.95	2.59	78.25	5.60	75.06	5.28	37.02	2.27	69.42	5.65	70.80
	2.....	0.91	6.50	0.17	5.13	0.57	7.64	2.91	20.40	0.37	11.32	0.86	10.78
	3.....	0.87	6.21	0.17	5.13	0.47	6.30	2.30	16.13	0.26	7.95	0.58	7.27
	4.....	0.44	3.14	0.08	2.42	0.21	2.82	1.67	11.72	0.18	5.51	0.30	3.76
	5.....	0.37	2.64	0.08	2.42	0.16	2.14	0.75	5.26	0.07	2.14	0.18	2.26
	6.....	0.30	2.14	0.08	2.42	0.14	1.88	0.60	4.21	0.04	1.22	0.17	2.13
	7.....	0.32	2.28	0.08	2.42	0.14	1.88	0.45	3.15	0.04	1.22	0.12	1.50
Bottom	8.....	0.30	2.14	0.06	1.81	0.17	2.28	0.30	2.10	0.04	1.22	0.12	1.50

P, protein; TC, total cholesterol; PL, phospholipids.

The sample of α-LP contained 15 mg. of α-LP protein; prior to centrifugation it was incubated with 300 mg. of triton for 2 hours at 37°C. As a control, a sample of α-LP was kept for 2 hours at 37°C., in absence of the detergent.

the displacement of lipid from β-LP was incomplete even with concentrations of detergent up to 80 mg. per mg. of β-LP protein. Similar results were obtained with paper electrophoresis.

β-LP, analyzed by moving boundary electrophoresis (Fig. 11), gave two

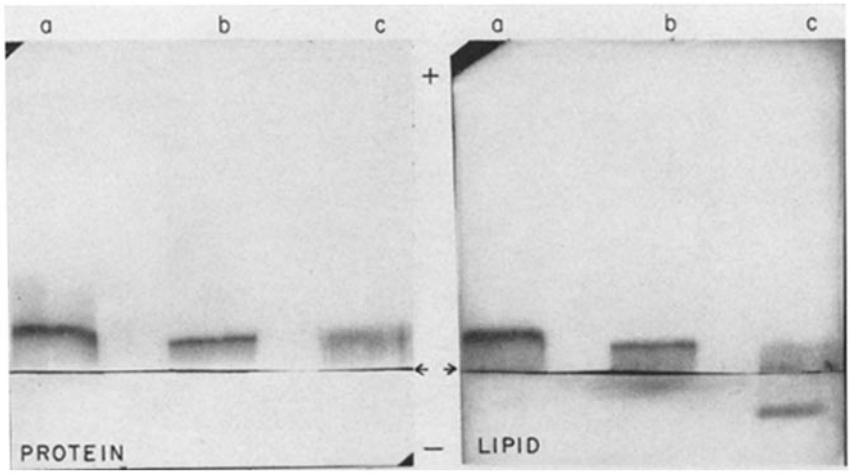

FIG. 10. Starch gel electrophoretic patterns of β-LP alone (*a*) and after incubation with triton. Triton was added to the β-LP protein in a weight ratio of 2:1 (*b*) and 40:1 (*c*). In each instance, the aliquots applied to the starch contained 1 mg. of β-LP protein. Left, protein staining; right, lipid staining.

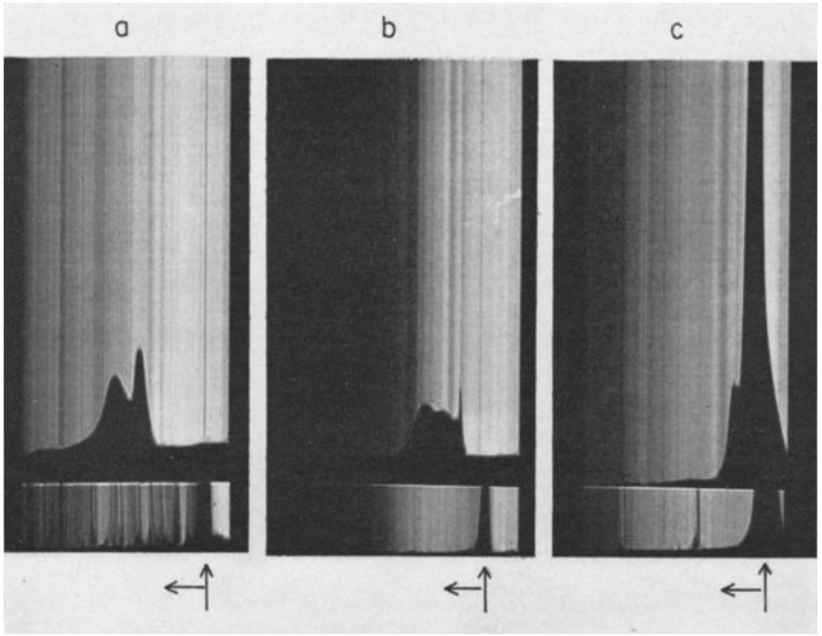

FIG. 11. Tiselius patterns (descending boundary) of β-LP alone (*a*), 2:1 mixture of triton and β-LP (*b*), and 40:1 mixture of triton and β-LP (*c*). In each instance, the sample analyzed contained 1 mg. of β-LP protein.

boundaries with mobilities of -4.6 and -3.0×10^{-5} cm.^{2}/volt sec. Addition of triton reduced the electrophoretic mobility of β-LP. This is particularly evident in *c* where a 40:1 mixture of triton and β-LP was analyzed. A large boundary (very likely triton) with a mobility of -0.8×10^{-5} cm.^{2}/volt sec. was closely associated with a smaller boundary (β-LP or β-P?) of a slightly higher mobility (-1.6×10^{-5} cm.^{2}/volt sec.).

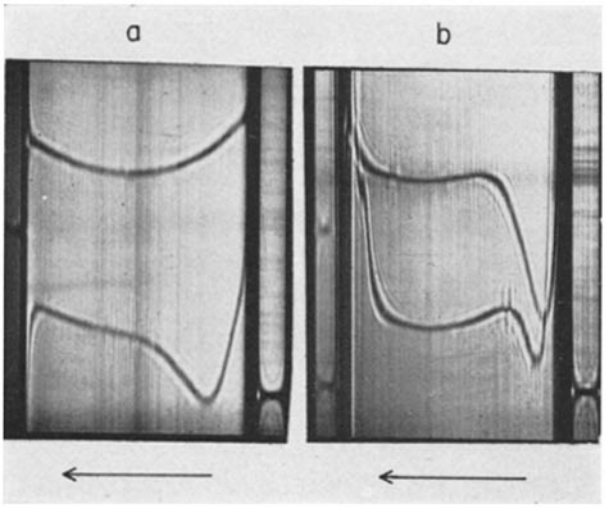

FIG. 12. Ultracentrifugal patterns of β-LP alone and with triton. (*a*) upper pattern, salt blank, D 1.063; lower pattern, β-LP; (*b*) upper pattern, 2:1 mixture of triton and β-LP; lower pattern, 40:1 mixture of triton and β-LP. Each sample contains 1 mg. of β-LP protein. 52,640 R.P.M., 36 minutes, 26°C.

Ultracentrifugal flotation at D 1.063 of β-LP, gave a peak with an S_f value of 5 (Fig. 12 *a*, lower curve). Addition of 2 mg. of triton per mg. of β-LP protein changed the flotation rate of the lipoprotein (s_f 2). At higher concentrations (40 mg. per mg. of protein) of triton, only a small amount of β-LP floated, the remainder sedimenting at the bottom of the analytical cell probably in combination with triton (Fig. 12 *b*, lower curve).

Mixtures of triton and β-LP, 40:1, were fractionated in a preparative ultracentrifuge at D 1.063, and various layers were analyzed for protein, cholesterol, and phospholipid. Samples of β-LP were run comparatively. In absence of triton, about 74 per cent of the protein, cholesterol, and phospholipid of β-LP appeared in the top layer. In the presence of triton, only 20 per cent of the lipoprotein constituents was found in the top; 22 per cent was recovered in the bottom layer and the remainder was distributed in intermediate fractions. In all the experiments it was observed that β-LP, ordinarily opalescent, was cleared by addition of triton.

Effect of Triton on the Extraction of Cholesterol and Phospholipids from Lipoproteins by Ethyl Ether.–

1 ml samples of α-LP and β-LP, containing 2 mg. of protein, were incubated for 2 hours at 37°C. with various amounts of triton (2, 20, and 40 mg.). As a control, samples of lipoproteins were incubated for the same period in the absence of triton. After incubation, each sample was transferred to a 25 ml. volumetric flask containing 20 ml. of redistilled ethyl ether. The mixtures were shaken for 3 minutes, brought up to 25 ml. mark with ether and left at 4°C. for 48 hours. 5 ml. aliquots were then separated for determination of cholesterol and phospholipid. In parallel samples, extraction was performed by a 24:1 (*v/v*) mixture of ethyl ether–ethanol.

TABLE II

Effect of Triton on Extractibility of Lipids from α-LP and β-LP

Mixtures analyzed	Per cent extracted lipid by ether alone		Per cent lipid extracted by ether-ethanol 24:1, *v/v*	
	Cholesterol	P lipid	Cholesterol	P lipid
	A	B	C	D
β-LP + saline	93.4	17.4	98.9	70.0
" + triton, 2 mg.	28.4	12.2	99.0	69.0
" + " 20 "	7.2	4.3	97.6	65.4
" + " 40 "	7.3	5.1	98.6	57.0
α-LP + saline	94.2	17.2	97.2	96.0
" + triton, 2 mg.	24.2	10.5	98.9	91.2
" + " 20 "	11.6	5.2	99.0	83.2
" + " 40 "	11.2	5.4	99.0	81.4

For experimental conditions, see text. α-LP and β-LP contained 2 mg. of protein.

The results are reported in Table II. In the absence of triton, ether alone removed 93 to 95 per cent of cholesterol from β-LP and α-LP, and 17 per cent of the phospholipids. When triton was present less cholesterol and phospholipids were extracted by ether (Table II, columns *A* and *B*). Ethanol, when added to the ether, partially opposed the effect of triton; it increased the extraction of cholesterol and phospholipids (columns *C* and *D*).

Effect of Triton on the Complexes Chylomicron–α-P–I^{131}, (Lipomul–α-LP)–α-P–I^{131}, and Lipomul–α-LP–I^{131}.—As previously described (9), these complexes are formed by mixing chylomicrons with α-P–I^{131} (A), lipomul–α-LP with α-P–I^{131} (B), and lipomul with α-LP–I^{131} (C). In these experiments samples of 1 mg. of either α-P–I^{131} or α-LP–I^{131} of a specific activity of 100,000 C.P.M./mg. were mixed with aliquots of chylomicrons or lipomul containing 20 mg. of triglycerides. A, B, and C mixtures, either alone or after addition of 20 mg. of triton, were centrifuged for 18 hours at D 1.006 and the various layers were analyzed for protein content and radioactivity. All of the triglycerides

were found in the top layer. In the absence of triton, 75 to 78 per cent of the radioiodinated protein also appeared in the top layer. In samples A and B the specific radioactivity was of the order of 20,000 C.P.M./mg., approximately 1/5 the activity of the original radioiodinated lipoprotein protein. The drop of specific radioactivity probably reflected the mixing of labeled protein with unlabeled protein attached to the triglycerides in the chylomicrons and the lipomul–α-LP complex. After addition of triton, the labeled protein partially

TABLE III

Effect of Triton on the Flotation of α-P–I^{131} or α-LP–I^{131} in Medium of Density 1.006

Layer	A Chylomicron–α-P–I^{131}				B (Lipomul–α-LP)–α-P–I^{131}				C Lipomul–α-LP–I^{131}			
	Protein				Protein				Protein			
	Alone		Plus triton		Alone		Plus triton		Alone		Plus triton	
	per cent	*C.P.M./mg.*	*per cent*	*C.P.M./mg.*	*per cent*	*C.P.M./mg.*	*per cent*	*C.P.M./mg.*	*per cent*	*C.P.M./mg.*	*per cent*	*C.P.M./mg.*
Top.....	78.2	20,000	42.7	1,600	75.2	26,200	*	—	76.4	99,600	*	—
Bottom...	*	—	34.6	19,500	*	—	78.1	25,400	*	—	72.6	99,000

The original samples of α-LP–I^{131} and α-P–I^{131} used for the interaction with lipomul and chylomicrons, respectively, had a specific activity of 100,000 C.P.M./mg. All the samples contained 20 mg. of triglycerides. In the samples with triton, the detergent was added in order of 1 mg. for each mg. of triglycerides. 9,600 *g*, 16°C., 18 hours.

* Too low to be accurately determined.

(sample A) or totally (samples B and C) was sedimented by ultracentrifugation of the mixtures for 18 hours at D 1.006 (Table III).

Samples of chylomicrons or lipomul, containing 20 mg. of triglycerides, when incubated with 20 mg. of triton for 2 hours at 37°C. and then equilibrated with 1 mg. of α-P–I^{131} or α-LP–I^{131}, failed to combine with the radioiodinated lipoprotein protein, as indicated by the absence of a labeled top layer after ultracentrifugation of the mixtures at D 1.006.

A, B, and C, either alone or after mixing with triton, were also analyzed by starch gel electrophoresis. In all instances, the bulk of lipids remained at the origin. In the absence of triton, the peak of radioactivity was also at the origin. With triton, the peak of radioactivity was in the area of α-P. The results obtained with the complex chylomicron–α-P–I^{131} are presented in Fig. 13.

Unlabeled chylomicrons or the labeled A, B, and C complexes were used, either alone or after mixing with triton, as substrates for lipoprotein lipase from post-heparin plasma. In each case, triton exhibited an inhibitory effect on the release of free fatty acids. This effect was proportional to the concentration of triton present in the medium. The results obtained with unlabeled chylomicrons are shown in Table IV.

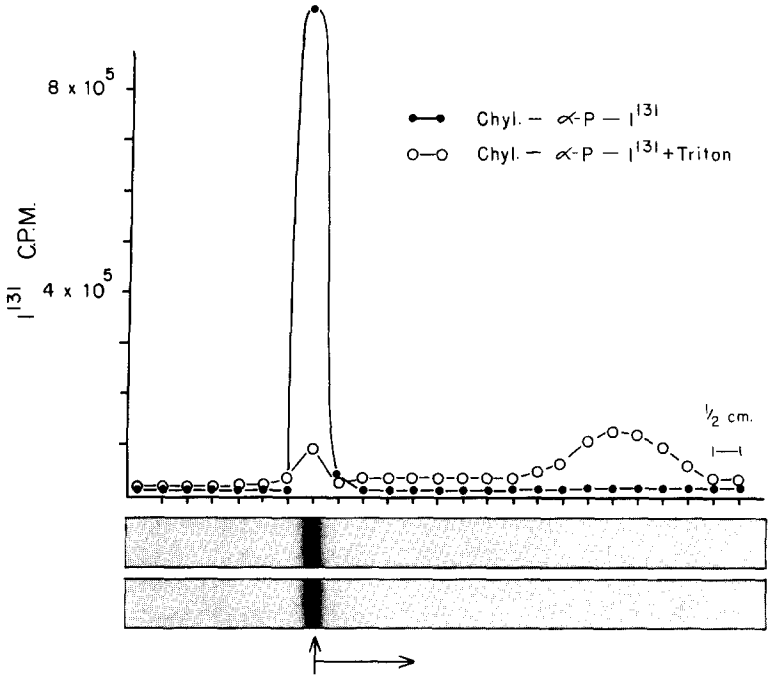

FIG. 13. Starch gel electrophoretic patterns and curves of radioactivity of a chylomicron–α-P–I^{131} complex alone and after incubation with Triton. Lipid staining shown by the dark bands in the two strips at the bottom.

TABLE IV

Effect of Triton on Lipoprotein Lipase from Post-Heparin Plasma

Substrate	Fatty acids produced in 60 min.
	m.eq./liter ± S.D.
Chylomicrons + saline	0.70 ± 0.08
" + triton, 0.5 mg.	0.52 ± 0.05
" + " 1.0 "	0.21 ± 0.02
" + " 2.0 "	0.11 ± 0.001
" + " 4.0 "	0.05 ± 0.005

Chylomicrons contained 2 mg. of triglycerides and were incubated with the various amounts of triton reported in the Table, for 2 hours at 37°C. To each chylomicron-triton mixture, (0.2 ml.), 0.5 ml. of 3 per cent bovine serum albumin and 0.1 ml. of canine post-heparin plasma were added. Fatty acid production was measured after 60 minutes' incubation at 37°C.

Samples of lipomul containing 20 mg. of triglycerides were incubated for 2 hours at 37°C. with various amounts of triton-I^{131} (1, 5, 10, 20, and 100 mg.). The mixtures were spun at D 1.006 for 18 hours at 9,600*g* and the radioactivity in the eight 1 ml. layers of the ultracentrifuge tube determined. About 78 per

cent of the triglycerides of the original sample of lipomul, was recovered in the top layer; milkiness did not appear affected by the detergent. From the amount of radioactivity present in the top layer and on the basis of the values of specific activity of the labeled detergent, it was calculated that a maximum of about 10 mg. of triton combines and floats with 10 mg. of lipomul (as triglycerides). As we mentioned before, triton alone sediments at D 1.006.

DISCUSSION

Our data indicate that triton, a non-ionic detergent, combines with serum lipoproteins and induces changes which are dependent upon the concentration of the detergent and the class of lipoprotein. Triton had the greatest affinity for the high density lipoprotein class (α-LP). Changes in this lipoprotein were first to appear when the detergent was added to fasting serum. At relatively low concentrations of triton (ratio by weight of detergent to α-LP, 2:1) α-LP showed a reduced mobility in Tiselius, paper, and starch gel electrophoresis, and small changes in the ultracentrifugal flotation pattern. When more triton was added to α-LP (ratio triton: α-LP, 20:1), the mixture analyzed by starch gel and paper electrophoresis exhibited a nearly lipid-free protein moving anodically and a triton-lipid complex moving slightly toward the cathode (Figs. 1 and 7). A possible explanation of this phenomenon is that triton exerts a solubilizing effect on the lipid moiety of α-LP because of its property of lowering interfacial tension. By this process, lipids pass into the micelles of detergent, where they remain enclosed, probably sandwiched between the hydrocarbon chains of the detergent, without apparently disturbing water solubility and electrophoretic mobility of the detergent. According to our interpretation, the triton-lipid complex replaces the lipid of α-LP, becoming associated with the protein moiety by forces weaker than the ordinary lipoprotein bonds; under these conditions, an added force, like paper or starch gel electrophoresis, separates the triton-lipid complex from the protein moiety of α-LP. Whether this phenomenon occurred in free boundary electrophoresis cannot be said since the composition of the resulting two boundaries was not determined.

Triton, being a non-ionic detergent, would not be expected to move in an electric field. The small degree of migration, which was observed in our studies, could be related to the general property of colloidally dispersed material to become charged in respect to the major bulk of the solvent (25).

The interpretation of the results obtained by ultracentrifugation of mixtures of triton and α-LP at D 1.21 has to take into account that triton alone floats at this density (Fig. 3), although at a lower rate than α-LP. The patterns shown in Fig. 8 indicate that α-LP and triton form a complex which floats at a rate lower than native α-LP. Chemical analysis of the various layers of a 20:1 mixture of triton and α-LP after ultracentrifugation at D 1.21 (Table I) also indicates that lipids and protein of α-LP float at different rates. These findings

suggest that protein and lipid of α-LP have split, corroborating the electrophoretic findings.

From the above results it appears that the site at which triton combines with α-LP is its lipid moiety. We may assume that cholesterol, being the most non-polar lipid, is primarily involved in the combination. This would be consistent with the findings of Pethica and Schulman (26) showing that non-ionic detergents can displace cholesterol from lipoproteins of cell membranes. That lipids are the site of attack of triton on lipoproteins, is also indicated by the apparent lack of interaction between it and the lipoprotein protein as shown by: (*a*) the failure of triton-I^{131} to form a radioactive complex with α-P, the delipidated product of α-LP, (*b*) the identical electrophoretic mobility of α-P alone and mixed with triton, (*c*) the capacity of α-P, after incubation with triton and prolonged dialysis (to remove unbound triton), to recombine with lipids and to give a precipitin reaction when put in contact with its specific antibody. The ability to recombine with lipids and to react with its homologous antibody was also exhibited by the α-P obtained by electrophoresis in starch gel of a 20:1 mixture of triton and α-LP. The observation that the protein moiety of α-LP is not affected by triton is consistent with the concept that non-ionic detergents, in general, do not alter proteins (27) as do ionic detergents, especially the anionic (27).

Low density lipoproteins (β-LP), were also greatly affected by triton. The observed differences between α-LP and β-LP were that (*a*) the affinity of triton for β-LP was less than for α-LP; twice as much triton was needed to induce electrophoretic and ultracentrifugal changes in β-LP as in α-LP, and (*b*) the amount of lipid displaced from β-LP by triton was only partial. Obviously, it should be borne in mind that, per milligram of protein, there is more bound lipid in β-LP than in α-LP, the ratio lipid:protein being 2:1 and 1:1 respectively (28). There is, however, the possibility that the action of triton on β-LP and α-LP is dependent on the properties of their protein moieties, which have been shown to be structurally (29, 30) and immunochemically (31–33) different. A dependence of the activity of anionic detergents on the characteristics of the protein moiety of lipoproteins, has been already suggested by Macheboeuf and Tayeau (34).

Concerning anionic detergents, it has been shown, in analogy with triton, that their action on lipoproteins varies with the dose of detergent. At high concentrations, they favor ether extraction of lipids from lipoproteins (34) whereas, at lower concentrations, they simply affect electrophoretic mobility (34–36). In regard to the favoring effect of anionic detergents on the ether removal of lipids from lipoproteins, it is interesting to point out that triton exhibited just the opposite effect; namely, it prevented ether extraction of lipids from α-LP and β-LP. This difference in results may be due to the different solubility of ether of anionic and non-ionic detergents. Triton is insoluble in

ethyl ether; therefore, since we assume that lipids remain enclosed within the micelles of the detergent, they will fail to partition into the ether phase. In fact, in the presence of ethanol, which precipitates triton, ether extraction can be restored, the lipid being free to pass into the ether phase.

Recently, Tsaltas *et al.* (37, 38) reported that tween 40 and 80, non-ionic detergents, when added in small amounts to serum, reduce the electrophoretic mobility of α-LP and cause ultracentrifugal changes, characterized by accumulation of most of the lipoproteins in the top of the ultracentrifuge tube at D 1.063. Because incubation at 37°C. was essential to eliciting these changes, the mechanism of production was considered enzymatic, related to the lipolytic activity of lipoprotein lipase. In view of the different experimental conditions, these results cannot be compared with ours. That an enzymatic mechanism could be responsible for the changes in lipoprotein observed with triton is unlikely since the results were not affected by potent enzyme inhibitors, or by preheating the lipoproteins at 60°C. for 10 minutes. Further, it has been shown, and our data are in agreement, that triton inhibits lipoprotein lipase activity (6, 39). The results by Schotz *et al.* (39) had shown that inhibition of lipoprotein lipase activity by triton is due to the action of the detergent not on the enzyme, but on the substrate. It has been already shown that either chylomicrons, which contain α-LP (11, 40) or an artificial fat emulsion, which had been incubated with plasma lipoproteins (41), more precisely with α-LP (11), represent an optimal substrate for lipoprotein lipase. It would appear, therefore, that α-LP plays the role of an "activator" in either case. Since triton either prevents the triglyceride–α-LP complex from forming or causes its disruption when the detergent is added to an already formed complex, we may infer that the inhibitory action of triton on lipoprotein lipase activity is prevention of α-LP from combining with triglycerides to form a suitable substrate. How triton prevents the interaction between an artificial triglyceride emulsion and α-LP, cannot be established on the basis of our experiments. It is conceivable that since the physical properties (degree of turbidity, flotation characteristic) of either synthetic or natural triglycerides are not affected by triton, this detergent may simply coat the fat particles with consequent covering of the sites involved in the interaction between triglycerides and α-LP.

If the changes that triton induces when mixed *in vitro* with lipoproteins and chylomicrons of serum also occur in the plasma of tritonized animals, they may well be the cause of a sustained hyperlipemia. The enclosing of lipids in the micelles of detergent could interfere, for instance, with the physiologic exchange of lipids, which occurs among lipoproteins (42–44) and also in their rate of removal from plasma and utilization in tissues. As we mentioned before, activation of triglycerides by α-LP is an indispensable requisite for their hydrolysis by lipoprotein lipase; this hydrolytic process may play a role in the removal of triglycerides from circulation. Triton, by interfering with lipoprotein

lipase activity may totally prevent or delay the egress of triglycerides from plasma, thus causing their accumulation in the blood stream.

SUMMARY

Triton WR-1339, a non-ionic detergent, added to canine serum or to ultracentrifugally separated lipoproteins, induced changes in the lipoproteins which were dependent upon concentration of detergent and class of lipoproteins.

D 1.063 to 1.21 lipoprotein (α-LP) was especially sensitive to the action of triton. Addition of 2 mg. of triton to 1 mg. of α-LP (based on protein content), induced only slight changes in the electrophoretic and flotation characteristics of the lipoprotein. With a tenfold increase of the detergent (triton:α-LP, 20:1), the mixture, analyzed by starch gel and paper electrophoresis, yielded a triton-lipid complex which remained close to the origin, and a nearly lipid-free protein with electrophoretic mobility higher (starch gel) or lower (paper) than native α-LP. The splitting of the lipid and protein moieties of α-LP could not be clearly shown when the same mixture was analyzed by free boundary electrophoresis. Triton alone moved only slightly in an electrical field (paper, starch gel, Tiselius); it sedimented during ultracentrifugation at D 1.006 and D 1.063 and floated at D 1.21.

D 1.006 to 1.063 lipoproteins (β-LP), required larger amounts of triton to show changes. These were evident in 40 to 80:1 mixtures of triton and β-LP. In starch gel and paper electrophoresis triton retained, in a position close to the origin, part of the lipids of β-LP; the remaining β-LP fraction, impoverished of lipids, had electrophoretic mobility similar to native β-LP. The triton-lipid complex sedimented at D 1.063.

After addition of triton to complexes [chylomicron–α-P–I^{131}] or [lipomul-α-LP-I^{131}], the electrophoretic and ultracentrifugal analyses of these mixtures revealed that the labeled protein was removed from the triglyceride component. Triton also prevented the occurrence of the interaction between lipomul and α-LP and the hydrolysis of both chylomicrons and lipomul–α-LP by lipoprotein lipase.

It is postulated that, if the changes in lipoproteins and chylomicrons observed *in vitro* occur *in vivo*, they could account, at least in part, for the hyperlipemia which develops in animals following administration of triton.

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