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Separation of dog serum lipoproteins by ultracentrifugation, dextran sulfate precipitation, and paper electrophoresis*

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SUMMARY

The separation of dog serum lipoproteins by ultracentrifugation at a density of 1.063 is hindered by the failure of the high density fraction to accumulate in the bottom portion of the centrifuge tube. This phenomenon interferes with the quantitative recovery of pure low density lipoproteins. By paper electrophoresis, a lipid-containing protein with the mobility of a β-globulin was detected in the lowest layer of the centrifuge tube. Comparison of the preparative ultracentrifuge technique with that of dextran sulfate precipitation of β-lipoproteins revealed the suitability of the latter procedure for the quantitative separation of β-lipoproteins. The dextran sulfate β-lipoprotein precipitate was shown to be free from α-lipoprotein by paper electrophoresis.

The separation of dog serum lipoproteins into an α- and a β-, or a high density and low density fraction, is complicated by the fact that in dog serum the amount of β- compared to α-lipoprotein is very small. It is of interest, therefore, to determine which one of several available procedures gives the most complete and specific separation of the lipoprotein fractions. If the newer precipitation procedures utilizing dextran sulfate could be shown to yield quantitative results, they would be superior to ultracentrifugation because of their simplicity and rapidity of performance.

Precipitation of β-lipoproteins with sulfated polysaccharide has been studied by Burstein and Samaille (1), Castaigne and Amselem (2), Bernfeld *et al.* (3), Oncley *et al.* (4), and Florsheim and Gonzales (5). Moreover, turbidimetric procedures based on the precipitation of β-lipoproteins with various polysaccharides have also been developed by Antoniades *et al.* (6) and Boyle and Moore (7). Although the principle of precipitating β-lipoproteins with sulfated polysaccharides is the same in all these procedures, the details of the technique vary considerably. Some of the authors utilized calcium ions in addition to a relatively low molecular weight dextran sulfate (1, 2, 5), whereas others used higher molecular weight polysaccharide sulfates without calcium (3, 4). Boyle and Moore (7), on the other hand, utilized an agar preparation. We examined a modification of the Burstein procedure suggested by Castaigne and Amselem (2), since the dextran sulfate they used is available from commercial sources.

EXPERIMENTAL

Blood from normal fasting male dogs was obtained carefully to avoid hemolysis, and was allowed to clot. The precipitation and isolation of the β-lipoprotein with dextran sulfate[1] was carried out as follows: To each milliliter of serum was added 0.04 ml of 5% dextran sulfate and 0.1 ml of 11% calcium chloride. After standing 2 hours at 4°, the mixture was centrifuged for 10 minutes at about 4,000 × *g* and the supernatant removed by decantation. For subsequent paper electrophoresis and lipid analysis, the precipitate was redissolved in 0.5 ml of 0.9% sodium chloride, and calcium ions were removed by the addition of 0.05 ml of a solution of 12.7% potassium oxalate and centrifugation (2).

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[1] Dextrarine, 2-ml ampoule containing 200 mg dextran sulfate. Obtained from l'Equilibre Biologique S. A., Commentry (Allier), France.

The ultracentrifugal separations were carried out in a Spinco Model L ultracentrifuge with a 40 rotor, essentially by the method of Havel *et al.* (8). In this procedure 10 ml of serum is adjusted to a density of 1.063 with NaCl–KBr solution, and centrifuged for 14 hours at 8° and an average acceleration of 105,000 × *g*. In later experiments the time of centrifugation was increased from 14 to 24 hours, and the amount of serum was decreased from 10 to 5 ml. To avoid the presence of radioactive potassium in preparations subsequently used for counting, sodium bromide was substituted for potassium bromide.

Paper electrophoresis was performed on a Spinco Model R unit with 10 to 50 lambda serum samples. In most instances electrophoresis was performed with a Spinco Model R unit, containing eight paper strips (S and S 2043 A g1), at room temperature for 16 hours with a current of 5 ma. Protein fractions, dissolved in concentrated salt solutions, exhibited electrophoretic mobilities lower than those obtained in whole serum. In one experiment we compared the mobility of crystalline bovine albumin dissolved in 0.9% or 5% sodium chloride In a parallel ultracentrifugation (d = 1.063) experiment seven layers obtained from the centrifuge tube were subjected to paper electrophoresis before and after dialysis against 0.9% sodium chloride containing 0.01% EDTA.[2] The mobilities of albumin and α-globulin in the higher salt concentration were decreased, even though only 50 lambda of the protein solutions were applied to the paper strip wetted with 0.075 M Na-barbital buffer pH 8.6. After dialysis the mobilities of these protein fractions were restored to normal.

At the termination of the electrophoresis, the strips were cut lengthwise into two portions: one portion was stained with bromophenol blue (9), and the other portion was stained with Sudan black B by the method of Moinat *et al.* (10). Lipids from serum and saline solutions after ultracentrifugation were extracted with 50 volumes of chloroform-methanol 2/1 (v/v) by an adaptation of the procedure of Folch *et al.* (11). After washing the chloroform-methanol extract with upper phase containing 0.02% $CaCl_2$, lipid phosphorus was estimated by a slightly modified method of Bartlett (12). The amount of phospholipid was obtained by multiplication of the phosphorus by 25. An aliquot of the extract was hydrolyzed with 2% KOH at 60° for 20 minutes, and extracted by low boiling petroleum ether (Skellysolve F, b.p. 35.5°–57°), according to the method of Abell *et al.* (13). The colorimetric determination of cholesterol was performed by the ferric chloride-sulfuric acid procedure of Zak *et al.* (14).

[2] EDTA = ethylenediamine-tetraacetate.

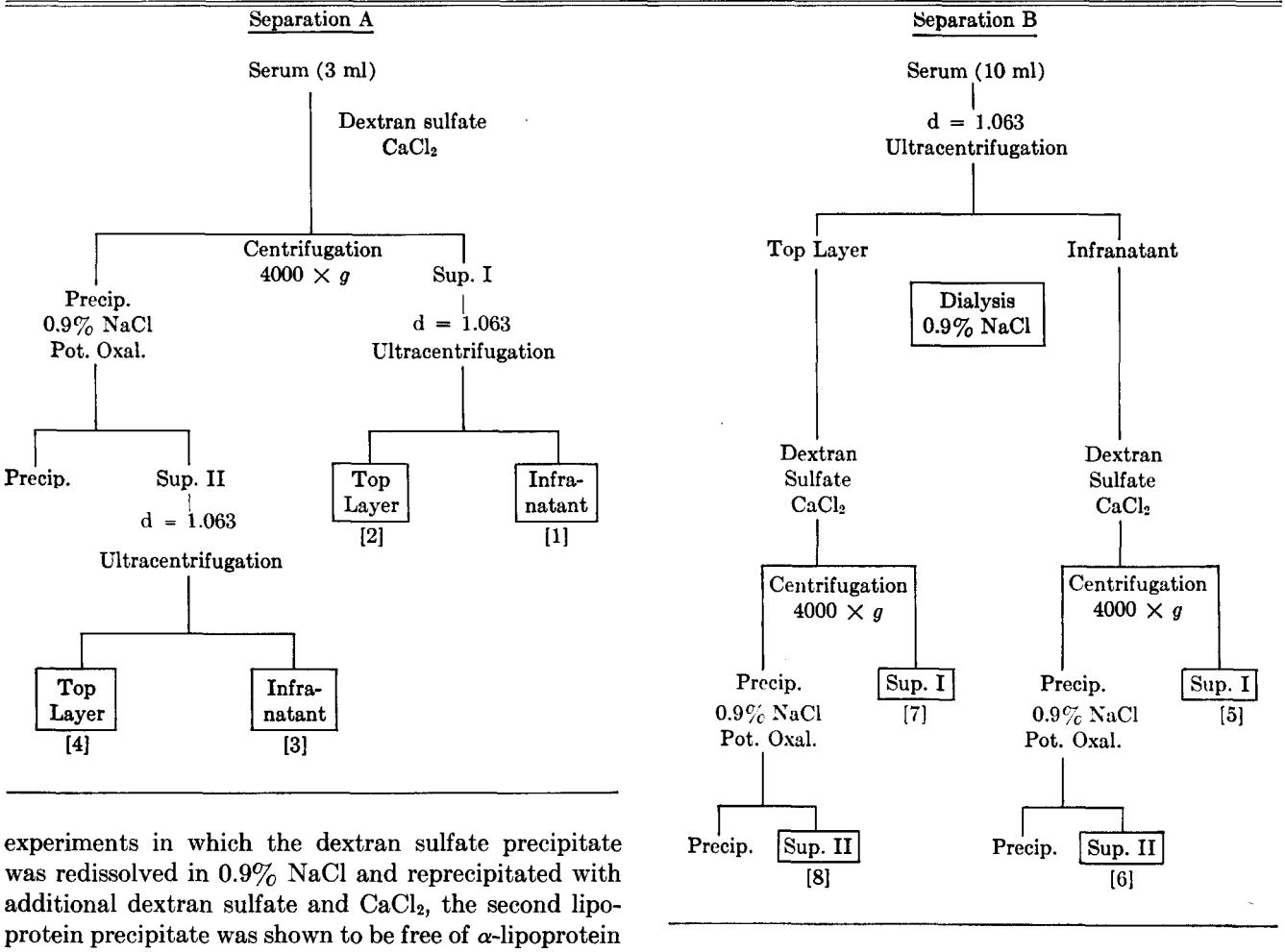
RESULTS

To compare the dextran sulfate precipitation with ultracentrifugation a single dog serum sample was divided into a 3- and a 10-ml portion. The 3-ml portion was precipitated first by dextran sulfate, as indicated by Table 1. After centrifugation the supernatant, Sup. I, was separated by ultracentrifugation (d = 1.063, 14 hours, 8°, 105,000 × *g*) into a high density lipoprotein, fraction [1], and a low density lipoprotein, fraction [2] by the use of a Spinco tube slicer. The dextran sulfate precipitate was redissolved in 0.9% NaCl, and Ca^{++} removed by the use of potassium oxalate. The saline solution, Sup. II, was ultracentrifuged to provide a high density lipoprotein [3] and a low density fraction [4].

The 10-ml serum aliquot was first subjected to ultracentrifugation at a density of 1.063 for 14 hours. Both the low density and the high density fractions, after being removed from the tube with the aid of a tube slicer, were dialyzed against 0.9% sodium chloride containing 0.01% EDTA for 24 hours. After adjusting the volumes to 25 ml, 5-ml aliquots were precipitated with dextran sulfate and calcium chloride, centrifuged, and precipitates were redissolved to furnish fractions [5], [6], [7], and [8] (Table 1). The results of cholesterol and phospholipid analyses of the 8 fractions listed in Table 1 are given in Table 2. In separation A, supernatant number I, obtained after dextran sulfate precipitation, should be α-lipoprotein. Accordingly, the high density lipoprotein fraction [1] in Table 2 contains more than 10 times the amount of cholesterol than fraction [2]. The small amount of cholesterol-containing material in fraction [2] might be either low density lipoprotein that had not been precipitated with dextran sulfate, or it might represent high density lipoprotein which had not centrifuged down after 14 hours at 105,000 × *g*. Apparently the latter is the case, since the cholesterol to phospholipid ratio of fraction [2] is very close to that for fraction [1] and is typical for the known cholesterol to phospholipid ratio of high density lipoprotein.

Fractions [3] and [4] are derived from the dextran sulfate precipitate and one would therefore expect the majority of the lipid to be in fraction [4], which represents the low density lipoprotein component. According to Table 2, more than 80% of the cholesterol is indeed present in fraction [4]. This lipoprotein has a cholesterol to phospholipid ratio of 0.84. The 20% of the cholesterol present in fraction [3], with a cholesterol to phospholipid ratio characteristic of the high density lipoprotein, actually represents a contamination of the dextran sulfate precipitate. In subsequent

TABLE 1. SEPARATION OF LIPOPROTEINS BY COMBINED DEXTRAN SULFATE PRECIPITATION AND ULTRACENTRIFUGATION



experiments in which the dextran sulfate precipitate was redissolved in 0.9% NaCl and reprecipitated with additional dextran sulfate and $CaCl_2$, the second lipoprotein precipitate was shown to be free of α-lipoprotein component as shown by paper electrophoresis (see Fig. 2, strip 4). Although the staining of lipoproteins with Sudan black B is not an extremely sensitive procedure, the application of a very concentrated β-lipoprotein solution to strip 4 and the absence of a visible alpha band would appear to eliminate appreciable contamination with α-lipoprotein.

In separation B, in which ultracentrifugation was carried out first, the low density and high density lipoprotein fractions had to be dialyzed, since dextran sulfate will not precipitate β-lipoproteins quantitatively in the presence of a high salt concentration. After dialysis, the high density lipoprotein fraction was precipitated with dextran sulfate. Very little cholesterol was found in the precipitate (fraction [6]), and the little lipoprotein that did precipitate exhibited a low cholesterol to phospholipid ratio, which indicates that it was high density lipoprotein.

The low density ultracentrifugal lipoprotein fraction was shown to contain an appreciable amount of high density fraction [7] that did not precipitate with dextran sulfate, and exhibited a low cholesterol to phospholipid ratio. This contamination is clearly the result of poor ultracentrifugal separation or cutting the centrifuge tube at too low a level.

In order to investigate the distribution of dog serum lipoproteins along the length of the centrifuge tube after ultracentrifugation, a 10-ml serum sample was centrifuged according to the method of Havel *et al.* (8).

TABLE 2. CHOLESTEROL AND PHOSPHOLIPID IN FRACTIONS OF TABLE 1

	Sup. I		Sup. II		HDL		LDL	
	[1] Infr.	[2] Top	[3] Infr.	[4] Top	[5] Sup. I	[6] Sup. II	[7] Sup. I	[8] Sup. II
Cholesterol	67.5	6.6	2.0	8.8	64	2.4	12.8	9
Phospholipid	185	16.6	5.9	10.4	150	5.6	29.5	10.5
Cholesterol/ phospholipid	0.36	0.40	0.34	0.85	0.43	0.43	0.43	0.86

All values are expressed as mg/100 ml of original serum.

TABLE 3. DISTRIBUTION OF DOG SERUM CHOLESTEROL AND PHOSPHOLIPID IN CENTRIFUGE TUBE

		10 ml Serum, 14 Hours Ultracentrifugation							Recovery
		Top Layer	Infranatant Fractions						
			1	2	3	4	5	6	
Cholesterol	*mg*	0.80	1.02	1.33	1.35	1.32	1.35	2.07	9.24
	*per cent**	9.1	11.5	15	15.1	14.7	15.1	23.2	103.9
Phospholipid	*mg*	1.35	1.89	2.75	3.36	2.80	3.23	4.72	20.1
	*per cent**	7	12.8	12.8	14.5	14.6	16.8	24.6	104.5
Cholesterol/ phospholipid		0.59	0.54	0.48	0.40	0.47	0.42	0.44	
		5 ml Serum, 24 Hours Ultracentrifugation							
Cholesterol	*mg*	0.50	0.21	0.34	0.48	0.77	1.10	0.98	4.38
	*per cent**	11.4	4.8	9.1	10.9	17.5	25	22.2	99.6
Phospholipid	*mg*	0.83	0.46	0.71	1.02	1.71	2.56	2.41	9.70
	*per cent**	8.6	4.8	7.4	10.6	17.8	26.6	25	100.5
Cholesterol/ phospholipid		0.60	0.45	0.48	0.47	0.44	0.43	0.40	

* Per cent of amount in original serum which contained: Cholesterol: 8.80 mg/10 ml; phospholipid: 19.2 mg/10 ml. Cholesterol/phospholipid ratio: 0.46.

Table 3 shows the concentration of cholesterol and phospholipid in various layers of the centrifuge tube after a 14-hour centrifugation at 105,000 × *g*. The "top layer," which contained about 1 ml of the uppermost layer in the centrifuge tube, was turbid and considered to contain predominantly low density lipoproteins. The rest of the centrifuge tube was divided as equally as possible into six divisions, containing approximately 2 ml each, numbered from 1 to 6, from the top to the bottom of the tube. It is apparent from the data in Table 3 that both cholesterol and phospholipids are distributed throughout the tube, although infranatant fraction 1 contains somewhat less lipids than the other fractions. Cholesterol to phospholipid ratios of the fractions near the top were higher than those in the bottom fractions. Apparently some contamination of the top layers with so-called high density lipoprotein existed. In Table 3 are also reported the results of a similar experiment, except that only 5 ml of the same serum was centrifuged, and the centrifugation was carried on for 24 hours. It is apparent that in the fractionation of 5 ml over a longer time period there is a better separation between the high density and low density layers. Infranatant fraction 1 contains only 4.8% of the total cholesterol, as compared to 11.5% in the previous experiment. Although Table 3 represents only two experiments, we have repeated these procedures each four times, and the results are consistent with one another.

The results of these analyses are confirmed by paper

TABLE 4. CHOLESTEROL PRECIPITATION AT VARIOUS TIMES AFTER ADDITION OF DEXTRAN SULFATE AND CALCIUM CHLORIDE TO 1 ML DOG SERUM

		10 Min	30 Min	60 Min	2 Hrs	5 Hrs	24 Hrs
Supernatant*	*mg*	0.837	0.827	0.784	0.779	0.801	0.797
	per cent‡	92.6	91.5	86.6	86	88.5	88
Precipitate†	*mg*	0.0787	0.0805	0.109	0.112	0.109	0.109
	per cent‡	0.6	08.9	12	12.4	12.1	12
Total recovery	*mg*	0.915	0.907	0.893	0.891	0.91	0.906
	per cent‡	101	100	98.6	98.4	101	100

* α-Lipoprotein.
† β-Lipoprotein.
‡ Per cent of amount in original serum which contained 0.905 mg cholesterol/ml.

electrophoresis. In both ultracentrifugal runs the top layers (Figs. 1*a* and 1*b*) contained β-lipoprotein. In the 10-ml sample a heavy alpha band was visible in infranatant layer 1 (Fig. 1*a*), and a little β-lipoprotein could be discerned. In the 5-ml sample neither alpha nor beta bands were visible in infranatant layers 1 and 2 (Fig. 1*b*). In lower layers of both experiments α-lipoprotein was the only band visible except in the very bottom layer (infranatant 6, Figs. 1*a* and 1*b*). Here a definite Sudan stain in the beta region was visible.

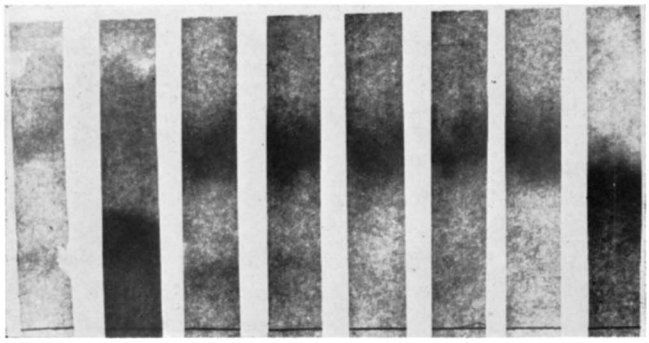


FIG. 1*a*. Paper electrophoretograms (Sudan black B stain) of whole serum (w.s.) and seven layers from top to bottom after ultracentrifugation of 10 ml of serum at density 1.063 for 14 hours in a 40 rotor. After dialysis all fractions were concentrated approximately threefold with the aid of Carbowax® (17).

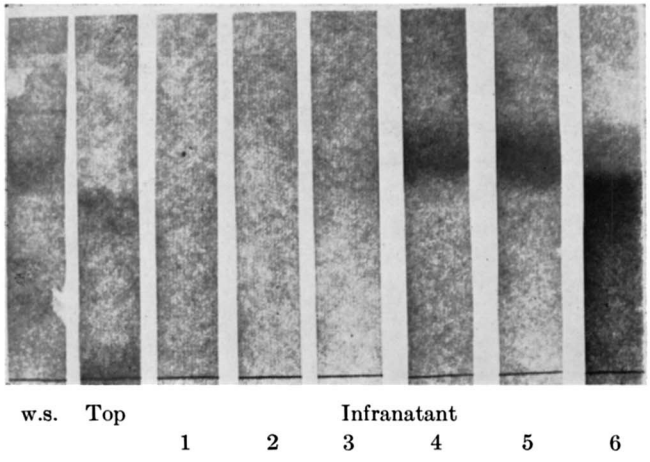


FIG. 1*b*. Same as Fig. 1*a*, except that 5 ml of serum was centrifuged for 24 hours. Photographs were made with transmitted light.

In order to circumscribe more precisely the conditions under which complete precipitation of β-lipoproteins in the presence of dextran sulfate and calcium occurs, six 1-ml serum samples from a fasting male dog were subjected to dextran sulfate precipitation, and the precipitate, as well as the supernatant, was analyzed for cholesterol after various periods of standing at 4°. Table 4 shows the results of one experiment with samples 10, 30, and 60 minutes, and 2, 5, and 24 hours after addition of dextran sulfate plus calcium. It is evident from Table 4 that most of the cholesterol precipitates within the first few minutes, and that the precipitation is essentially complete within 60 minutes. As a matter of safety, however, we have usually kept our samples at 4° for 2 hours prior to centrifugation.

It is interesting to note that in the experiment reported in Table 4, no further precipitation of cholesterol occurred even for a period of as long as 24 hours. However, when the supernatant was kept at 4° for a period of 7 days, an additional amount of precipitate occurred. Of the 4.47 mg of cholesterol present in 3-ml serum, 0.50 mg precipitated in 2 hours, and an additional 0.10 mg precipitated in the next 6 days. The additional precipitate exhibited a cholesterol to phospholipid ratio of 0.43, which is very close to the cholesterol to phospholipid ratio of 0.47 of the original, dextran sulfate-soluble, α-lipoprotein. Moreover, paper electrophoresis of the redissolved precipitate showed the presence of α-, and a small amount of β-lipoprotein (Fig. 2, strip 6), as well as other proteins including albumin. Three milliliters of serum diluted with 0.42 ml of 0.9% NaCl was kept at 4° for the same length of time

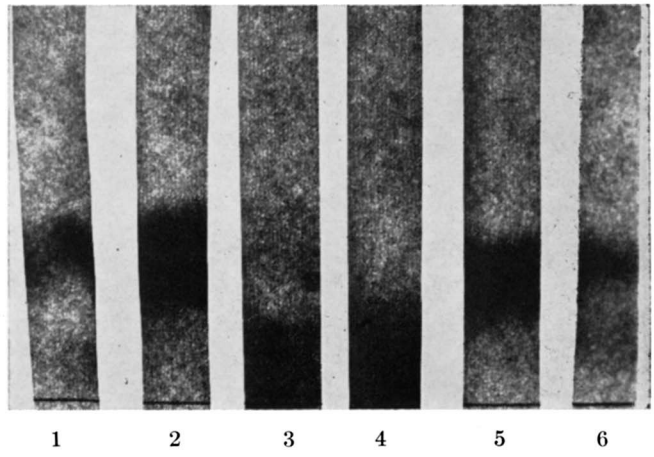


FIG. 2. Paper electrophoretogram of 50 λ of whole serum (strip 1), 50 λ of dextran sulfate supernatant from 3 ml of serum (strip 2), and 50 λ of dextran sulfate precipitate from 3 ml of serum redissolved in 1 ml of 0.9% NaCl and 0.1 ml K-oxalate (strip 3). The last strip shows the presence of a small amount of α-lipoprotein. Reprecipitation of this β-lipoprotein fraction with additional dextran sulfate and calcium chloride, and redissolution in 0.55 ml of NaCl, K-oxalate furnished a fraction essentially free of α-lipoprotein (strip 4). Strip 5 represents the supernatant 7 days after contact with dextran sulfate and $CaCl_2$ at 4°, and strip 6 the additional precipitate appearing between 2 hours and 7 days after dextran sulfate-$CaCl_2$ addition. Photographs were made with transmitted light.

as a control; this sample failed to show a precipitate. It appears, therefore, that after a prolonged period of time dextran sulfate, in the presence of calcium, will precipitate a small amount of proteins other than β-lipoproteins.

In order to obtain a more precise measure of the cholesterol to phospholipid ratio of β-lipoproteins of normal fasting male dogs, we precipitated the serum lipoproteins of eight additional dogs with dextran sulfate and calcium chloride. Afterward the precipitates were redissolved and subjected to ultracentrifugation at 105,000 × *g* at a density of 1.063 for 24 hours. By analyzing the top fraction in the centrifuge tubes, which corresponds to fraction [4] in Table 1, it was found that the cholesterol to phospholipid ratios of these beta, low density lipoprotein fractions were 0.85, 0.68, 0.55, 0.54, 0.66, 0.58, 0.78, and 0.55, with an average of 0.65 and a standard error of 0.013. This average value appears to be somewhat higher than the value of 0.53 reported by Havel *et al.* (8) for the fraction with a density between 1.019 and 1.063, but not quite as high as the value of 0.73 reported by Hillyard *et al.* (15) for the total low density fraction. As far as the relative proportions of α- or β-lipoprotein cholesterol are concerned, our eight male dogs showed an average content of 11% of β- and 89% of α-lipoprotein cholesterol. This proportion differs considerably from that reported by Castaigne and Amselem (2), who found 60% of the cholesterol in the β-lipoprotein fraction of dog serum by the dextran sulfate method. Hillyard *et al.* (15) reported 10%; Havel *et al.* (8), 9.5%; and Shafrir *et al.* (16), 11% of the cholesterol in the low density fraction.

DISCUSSION

Serum lipoproteins have been separated by ultracentrifugation, various forms of electrophoresis, and precipitation with sulfated polysaccharides. Although these procedures have been used by different investigators on human and animal sera, few comparative investigations of their respective values have been made. Particularly in animal sera in which the concentration of high density lipoproteins greatly exceeds that of the low density lipoproteins, it is useful to know which one of these procedures gives the best separation in the shortest possible time.

In our experiments it was shown that in dog serum, ultracentrifugation at a density of 1.063 failed to separate the high density and low density lipoproteins completely when such centrifugation was carried out according to the procedure of Havel *et al.* (8), which was designed for human serum. It was found that, in contrast to human serum, the high density lipoproteins of dog serum failed to settle in the bottom of the centrifuge tube, but instead exhibited a relatively uniform distribution throughout the tube. Only when centrifugation was carried out with smaller serum samples (5 ml), and for longer periods of time (24 hours), was a reasonably satisfactory separation of dog serum lipoproteins possible. Even then, however, one could judge only with difficulty the exact location of cutting the centrifuge tube for separation of high and low density fractions. This conclusion was reinforced by the centrifugation of dog serum prestained with Sudan black B, in which case an intense blue coloration was visible throughout the entire length of the centrifuge tube, although a slightly less colored region was found under the top layer in the tube containing 5 ml serum.

Among the variously available electrophoretic techniques, we chose that of paper electrophoresis. Although this technique does not lend itself easily to quantitative determination of α- and β-lipoproteins, it appears to be satisfactory for determining the purity of lipoprotein fractions obtained by other methods. The paper electrophoretic examination of the bottom layer of the ultracentrifuge tube after centrifugation for 24 hours at 105,000 × *g* disclosed the presence of two lipid-staining proteins, one with the mobility of α- and one with the mobility of β-lipoprotein. After dialysis of this solution for 24 hours against 0.9% NaCl, the two bands were still present and exhibited the normal mobilities of α- and β-globulins. The subnatant of density 1.063 was adjusted to a density of 1.21 and recentrifuged at 105,000 × *g* for 22 hours. The 7-ml bottom layer was dialyzed against 0.9% NaCl, concentrated to a small volume with Carbowax® (17), and an aliquot subjected to paper electrophoresis. This time lipid staining was observed only in a band corresponding to a mobility of β-globulin. Extraction of the lipids from the 1.21 bottom revealed the presence of phospholipid as the major lipid component. It appears, therefore, that in dog serum a lipid-containing β-globulin with a density greater than 1.21 is present.

The separation of lipoproteins with dextran sulfate appears to be the simplest procedure. However, this method too has its complications. It is not possible to obtain a really pure β-lipoprotein fraction. Although the second precipitate seems to be entirely free of α-lipoproteins, there is still present a little protein with the mobility of albumin. If one therefore is interested in the study of the protein portion of the lipoprotein, the dextran sulfate precipitated lipoprotein must be subjected to further purification.

REFERENCES

1. Burstein, M., and J. Samaille. *J. physiol.* (*Paris*) **49**: 83, 1957.
2. Castaigne, A., and A. Amselem. *Ann. biol. clin.* (*Paris*) **17**: 336, 1959.
3. Bernfeld, P., V. M. Donahue and M. E. Berkowitz. *J. Biol. Chem.* **226**: 51, 1957.
4. Oncley, J. L., K. W. Walton and D. G. Cornwell. *J. Am. Chem. Soc.* **79**: 4666, 1957.
5. Florsheim, W. H., and C. Gonzales. *Proc. Soc. Exptl. Biol. Med.* **104**: 618, 1960.
6. Antoniades, H. N., J. L. Tullis, L. H. Sargeant, R. B. Pennell, and J. L. Oncley. *J. Lab. Clin. Med.* **51**: 630, 1958.
7. Boyle, E., and R. V. Moore. *J. Lab. Clin. Med.* **53**: 272, 1959.
8. Havel, R. J., H. A. Eder and J. H. Bragdon. *J. Clin. Invest.* **34**: 1345, 1955.
9. Spinco Division: Beckman Instruments, Inc. Spinco Technical Bulletin TB 6050A, April, 1958.
10. Moinat, P., W. Appel and E. F. Tuller. *Clin. Chem.* **4**: 304, 1958.
11. Folch, J., M. Lees and G. H. Sloane-Stanley. *J. Biol. Chem.* **226**: 49, 1957.
12. Bartlett, G. R. *J. Biol. Chem.* **234**: 466, 1959.
13. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. *J. Biol. Chem.* **195**: 357, 1952.
14. Zak, B., N. Moss, A. J. Boyle, and A. Zlatkis. *Anal. Chem.* **26**: 776, 1954.
15. Hillyard, L. A., C. Entenman, H. Feinberg, and I. L. Chaikoff. *J. Biol. Chem.* **214**: 79, 1955.
16. Shafrir, E., K. E. Sussman and D. Steinberg. *J. Lipid Research* **1**: 109, 1959.
17. Kohn, J. *Nature* **183**: 1055, 1959.