J. Lipid Research, January, 1962
Volume 3, Number 1

Dextran sulfate precipitation and ultracentrifugation of lipoproteins from hypercholesterolemic dog serum*

Toshio Sakagami† and D. B. Zilversmit‡

Department of Physiology, University of Tennessee, Memphis 3, Tennessee

[Received for publication September 8, 1961]

SUMMARY

Dogs fed a diet containing butter, cholesterol, and thiouracil develop severe hypercholesterolemia. Both α_2- and β-lipoprotein cholesterol and phospholipid increase whereas these lipids in the α_1 fraction appear to decrease. Comparison of the dextran sulfate-precipitable lipoproteins with lipoprotein fractions obtained by ultracentrifugation at densities 1.019, 1.063, and 1.21 and with paper electrophoretograms reveals that the addition of low molecular weight dextran sulfate and calcium chloride to diluted hypercholesterolemic dog serum quantitatively precipitates low-density lipoproteins. Paper electrophoresis revealed that the dextran sulfate-precipitable fraction contains both α_2- and β-lipoproteins.

The use of dextran sulfate for the precipitation of low-density or β-lipoproteins of human sera has been investigated extensively by Burstein (1, 2, 3, 4). Other workers have proposed the use of sulfated amylopectin (5), and agar-agar (6), high molecular weight dextran sulfate (7), heparin and synthetic heparinoids (1, 2, 3, 4, 8) for the precipitation of β-lipoprotein in human and animal sera. However, little or no data are available on the effectiveness of sulfated polysaccharides for the precipitation of low-density lipoproteins from hypercholesterolemic serum. The recent literature on the use and action of sulfated polysaccharides has been reviewed by Cornwell and Kruger (9).

Previous work from this laboratory has shown that dextran sulfate, used according to a slightly modified procedure of Burstein as proposed by Castaigne and Amselem (10), completely removed the low-density lipoprotein fraction from dog serum and made it possible to obtain this fraction in a form uncontaminated with other lipoproteins (11). Preliminary work with hypercholesterolemic serum showed, however, that the procedure, as described before, could not be applied without modification.

In the present paper, experiments are presented to demonstrate that for hypercholesterolemic dog serum a modified dextran sulfate precipitation procedure gives a satisfactory separation of high- and low-density lipoproteins.

EXPERIMENTAL METHOD

Blood samples were obtained from two dogs fed a diet containing butter, casein, sucrose, cholesterol, sodium cholate, and thiouracil according to a slightly modified diet of O'Neal *et al.* (12) as proposed by DiLuzio (13). Blood samples were allowed to clot and serum was obtained by centrifugation.

High- and low-density lipoproteins were separated at d = 1.019, 1.063, and 1.21 in the Spinco Model L centrifuge. Paper electrophoresis, cholesterol, and phospholipid phosphorus determinations on whole serum or lipoprotein fractions were performed as previously described (11).

After the addition of 0.04 ml of 5% dextran sulfate[1] and 0.1 ml of 11% calcium chloride per milliliter of serum, a flocculent precipitate appeared. The mixture was kept at 4° for 2 hours and the precipitate was separated by centrifugation at 4,000 × *g* for 10 minutes. The supernatant fraction, designated as dextran sulfate-

* Supported by Grants H-1238 and HTS-5279 from the National Institutes of Health, Bethesda 14, Maryland.

† Postdoctoral Research Fellow from Sapporo Medical College, Japan.

‡ Career Investigator of the American Heart Association.

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

TABLE 1. EFFECT OF DILUTION OF HYPERCHOLESTEROLEMIC SERUM ON PRECIPITATION OF LOW-DENSITY LIPOPROTEIN WITH DEXTRAN SULFATE

Material Fractionated	Fraction Analyzed	Phospholipid (mg/100 ml*)	Cholesterol (mg/100 ml*)	Cholesterol/ Phospholipid
Whole serum		600	719	1.20
Undiluted serum	DSL-1†	319	325	1.02
	DPL-1	254	375	1.48
	Total	573	700	
	DSL-2†	314	309	0.98
	DPL-2	242	363	1.50
	Total	556	672	
	DSL 1.063			
	Top	124	174	1.40
	Bottom	187	142	0.76
2-Times diluted	DSL	192	155	0.80
	DPL	348	490	1.41
	Total	540	645	
5-Times diluted	DSL	180	137	0.76
	DPL	410	566	1.38
	Total	590	703	

* All values expressed as milligrams per 100 ml undiluted serum.

† Numbers 1 and 2 refer to duplicate determinations.

TABLE 2. COMPARISON OF DEXTRAN SULFATE PRECIPITATION AND ULTRACENTRIFUGAL SEPARATION METHODS FOR ANALYSIS OF HYPERCHOLESTEROLEMIC DOG SERUM

Material Fractionated	Fraction Analyzed	Phospholipid (mg/100 ml*)	Cholesterol (mg/100 ml*)	Cholesterol/ Phospholipid
Whole serum		790	1212	1.53
Expt. 1				
Undiluted serum (2 ml)	DSL-a	203	291	1.43
	DSL-b	75	52	0.69
	DPL-b	128	239	1.87
	DPL-a	529	850	1.61
	DPL-(a + b)	657	1089	1.66
Expt. 2				
5-Times diluted serum (2 ml)	DSL	79	57	0.72
	DPL	713	1059	1.49
Expt. 3				
10 ml undiluted serum (ultracentrifuged at densities indicated)	1.019			
	Top	210	320	1.52
	Bottom	588	786	1.34
	1.063			
	Top	488	714	1.46
	Bottom	89	68	0.76
	1.21			
	Top	68	59	0.87
	Bottom	19.5	6.0	0.31

* All values expressed as milligrams per 100 ml undiluted serum.

soluble lipoproteins (DSL), was decanted; the precipitate, designated as dextran sulfate-precipitable lipoprotein (DPL), was redissolved in about 1 ml of 0.9% NaCl and treated with 0.1 ml of 12.7% potassium oxalate to remove the calcium. Potassium oxalate was not used if the DPL had to be reprecipitated with dextran sulfate and calcium.

RESULTS

In Table 1 are presented the results obtained on serum from a dog fed the high-cholesterol diet for 2 months. The total serum phospholipid concentration (lipid phosphorus × 25) was 600 mg per 100 ml of serum. The cholesterol content of total serum was 719 mg per 100 ml. The cholesterol-to-phospholipid ratio in whole serum was 1.20, which is considerably higher than the ratio of 0.46 found in serum from normal dogs (11). When whole serum was precipitated in duplicate (Table 1) with dextran sulfate according to the procedure described above, 50% to 52% of the total serum cholesterol appeared in the DPL fraction. After ultracentrifugation of the DSL fraction, it appeared that more than half still consisted of low-density lipoprotein cholesterol; i.e., cholesterol of lipoproteins with a density less than 1.063. Subsequent tests with serum diluted 2 and 5 times with 0.9% NaCl showed that the cholesterol of the DPL fraction increased to 68% and 79%, respectively. In the 5-times diluted serum, the DSL fraction contained 137 mg of cholesterol. This resembles closely the 142 mg of cholesterol contained in the bottom fraction of the DSL centrifuged at 1.063. Similarly, the cholesterol-to-phospholipid ratio of the DSL fraction in the 5-times diluted serum was 0.76, which is considerably lower than that found in the undiluted serum, and exactly the same as that found in the bottom fraction of the centrifuged sample. The cholesterol-to-phospholipid ratio of the DPL fraction of the 5-times diluted serum was the same as that of the 1.063 top after ultracentrifugation of the DSL fraction. It is evident, therefore, that precipitation of lipoproteins from this hypercholesterolemic serum was complete when the serum was diluted with 0.9% NaCl to one-fifth the original concentration.

In a second experiment, we tried to determine whether or not the addition of more dextran sulfate and calcium chloride would produce the same satisfactory results as diluting the original hypercholes-

terolemic serum. In Table 2 are presented the results of an experiment performed with serum from a hypercholesterolemic dog fed the high-cholesterol diet for 5 months. The total phospholipid concentration of this serum was 790 mg per 100 ml, the total cholesterol concentration was 1,212 mg per 100 ml, and the cholesterol-to-phospholipid ratio 1.53. In the first experiment of Table 2, 2 ml of serum was precipitated with the normal amounts of dextran sulfate and calcium chloride. The sample was centrifuged and the supernatant fraction decanted. Of the 1,212 mg of cholesterol per 100 ml serum, 850 mg was precipitated with dextran sulfate. As before, the dextran sulfate-soluble lipoprotein fraction still had a high cholesterol-to-phospholipid ratio of 1.43, showing appreciable contamination of this fraction (DSL-a) with dextran sulfate-precipitable lipoprotein. The further addition to the DSL fraction of the same amounts of dextran sulfate and calcium chloride as used before caused the precipitation of an additional 239 mg of cholesterol per 100 ml of serum (DPL-b) leaving only 52 mg of cholesterol per 100 ml of serum in the dextran sulfate-soluble fraction (DSL-b). This fraction exhibited a cholesterol-to-phospholipid ratio of 0.69.

A comparison of these results with those obtained on the same serum after 5-fold dilution (Expt. 2, Table 2) can be made by comparing the cholesterol and phospholipid in the total DPL fraction of Experiment 1, DPL-(a + b), with that obtained in Experiment 2. As may be seen, the cholesterol and phospholipid obtained under these two conditions were quite similar in amount and the cholesterol-to-phospholipid ratios of the DPL fraction in both instances were fairly close.

In Experiment 3, Table 2, we compare the above results with those obtained after centrifugation of 10 ml of serum successively at densities 1.019, 1.063, and 1.21. It may be seen that the total cholesterol in the combined 1.019 and 1.063 top fractions is 1,034 mg per 100 ml of serum. This value compares very well with the 1,089 and 1,059 values obtained with the two previous dextran sulfate precipitations. Similarly, the cholesterol-to-phospholipid ratios of the dextran sulfate-precipitable lipoproteins, namely 1.66 and 1.49, compare favorably with the cholesterol-to-phospholipid ratios in the 1.019 and the 1.063 top fractions. It would appear from the cholesterol-to-phospholipid ratio in the dextran sulfate-soluble fractions that these fractions might be less contaminated with low-density lipoproteins than the 1.063 bottom fraction obtained in the conventional manner.

Next we determined how much contamination of DPL with DSL exists, particularly when large amounts of serum lipoproteins are precipitated with dextran sulfate and when the precipitate becomes quite bulky. In Table 3 are presented the phospholipid and cholesterol data on DSL and DPL after one precipitation with dextran sulfate (DSL-1) and after redissolving the precipitate (DPL-1) and reprecipitating (DSL-2 and DPL-2). When 2 ml of 5-times diluted serum was used, the first DSL fraction contained 47 mg of cholesterol per 100 ml of serum. Redissolving of the precipitate and reprecipitation with additional dextran sulfate resulted in the loss of 9.2 mg of cholesterol per 100 ml of serum from the DPL fraction as determined directly on the supernatant fraction. Thus it appears that under these conditions the contamination of the first DPL fraction with DSL cholesterol was less than 1%. Since the phospholipid content of the DSL fraction is

TABLE 3. COMPARATIVE DATA OF DEXTRAN SULFATE PRECIPITATION AND ULTRACENTRIFUGAL SEPARATION METHODS ON HYPERCHOLESTEROLEMIC DOG SERUM

Material Fractionated	Fraction Analyzed	Phospholipid (mg/100 ml*)	Cholesterol (mg/100 ml*)	Cholesterol/Phospholipid
Whole serum		728	1100	1.51
5-Times diluted serum (2 ml)	DSL-1	76	47	0.62
	DPL-1			
	DSL-2	15	9.2	0.61
	DPL-2	622	978	1.59
5-Times diluted serum (15 ml)	DSL-1	77	46.5	0.60
	DPL-1			
	DSL-2	15	7.9†	0.53
	DPL-2	622	1001	1.61
Undiluted serum (10 ml)	1.019			
	Top	134	235	1.75
	Bottom	595	813	1.37
4.5 ml of 1.019 bottom per tube	1.063			
	Top	488	714	1.46
	Bottom	108	87	0.80
4.5 ml of 1.063 bottom per tube	1.21			
	Top	107	87	0.81
	Bottom	1.0	0	
DPL-2	1.019			
	Top	158	256	1.62
	Bottom	461	645	1.40
	1.063			
	Top	468	650	1.39
	Bottom	‡	‡	

* All values expressed as milligrams per 100 ml undiluted serum.

† This value is probably erroneously low.

‡ Non-detectable.

relatively greater than the cholesterol content, the contamination of the DPL fraction with phospholipid of DSL was a little larger than 2%. Apparently the DPL from 15 ml of 5-times diluted serum was no more contaminated than the DPL of the 2-ml aliquot (Table 3).

Comparison of the cholesterol content of the DPL fractions (987 and 1,001 mg per 100 ml) with the cholesterol found in the combined 1.019 and 1.063 top fractions (949 mg per 100 ml) shows satisfactory agreement. Again, the cholesterol-to-phospholipid ratio of the DSL fractions, namely 0.60 to 0.62, appeared to be significantly lower than the cholesterol-to-phospholipid ratio of 0.80 in the 1.063 bottom fraction, indicating less contamination of the DSL with low-density lipoproteins than in the high-density lipoprotein fraction obtained in the conventional manner.

A further check on the purity of the DPL fraction after one reprecipitation was made by subjecting this fraction to ultracentrifugation at densities of 1.019 and 1.063. As shown in Table 3, all the cholesterol and phospholipid was recovered in the 1.019 and 1.063 top fractions, as one would expect for a fraction uncontaminated with high-density lipoproteins.

Inasmuch as Burstein and others made comparisons between the selectivity in the precipitation of serum lipoproteins by dextran sulfate and the separation of these fractions by paper electrophoresis, we determined the paper electrophoretic pattern of normal and hypercholesterolemic dog serum as well as the patterns of various other centrifugal fractions of the latter. In Figure 1, all strips were stained with Sudan black B as previously described (11). Strip 1 shows the α_1- and β-lipoprotein fractions of normal dog serum. As has been reported previously, most of the lipids of normal dog serum occur in the α_1 fraction. The second strip was obtained from a dog fed the high-cholesterol diet for 4 months. It may be seen here that the β-lipoprotein fractions are increased but that the highest lipid concentration occurs in a region characteristic for the α_2-lipoprotein fraction. Paper electrophoresis showed the presence, in the 1.019 top fraction, of lipoprotein at the origin as well as a β-lipoprotein band; the 1.063 top fraction contained α_2- and β-lipoproteins. The amount of lipid in the 1.063 infranatant fraction was too small to show up by the usual staining procedure. For this reason, strips No. 7 (1.21 top) and No. 8 (1.21 bottom) were obtained with fractions that had previously been concentrated with carbowax (14). The presence of α_1-lipoprotein in the 1.21 top fraction may be easily seen. Even in the concentrated form, the amount of lipoprotein in the 1.21 infranatant fraction was too small to be detectable with certainty, although in the original strip a slight dark band with Sudan black B in the region of the globulins could be detected.

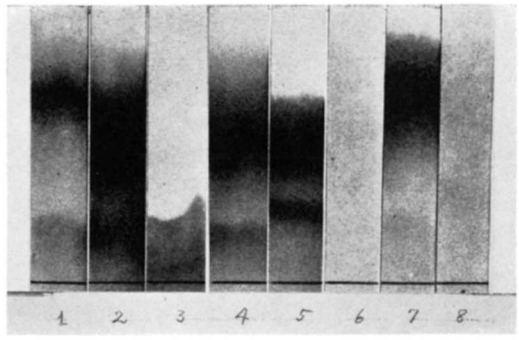

FIG. 1. Paper electrophoretograms stained with Sudan black B. (1) Normal whole dog serum, (2) hypercholesterolemic whole dog serum, (3) 1.019 top of (2), (4) 1.019 infranatant of (2), (5) 1.063 top of recentrifuged (4), (6) 1.063 infranatant of recentrifuged (4), (7) 1.21 top of recentrifuged (6) (concentrated with carbowax), (8) 1.21 infranatant of recentrifuged (6) (concentrated with carbowax).

When dextran sulfate-precipitable lipoprotein fractions were subjected to paper electrophoresis, it was at once evident that the DPL fraction contained not only β-lipoprotein but also a lipid-staining band with the mobility of α_2-lipoprotein (Fig. 2).

From these results, it appears that, in the dog maintained on the hypercholesterolemic regimen, the major portion of the lipid occurs in the α_2-lipoprotein fraction and somewhat less in the β-lipoprotein fraction. The absolute amount of lipid in the α_1-lipoprotein fraction appeared to be somewhat less than in the normal dog.

In the course of cholesterol feeding, the total cholesterol and phospholipid content of dog serum showed a steady increase over a 5-month period (Fig. 3). However, the different lipoprotein fractions, determined in 5-times diluted serum, did not change in the same manner. The DPL fraction changed its cholesterol content from an initial value of 10 mg per 100 ml of serum to values over 1,000 mg per 100 ml at the end of 5 months. During this time, the phospholipid content of this fraction also increased, but a little more slowly, so that the cholesterol-to-phospholipid ratio changed from 0.65 to 1.50 over the same period. The pattern for the DSL fraction is somewhat more complex. During the first month of cholesterol feeding, the cholesterol content rose from 80 mg per 100 ml to about 200 mg per 100 ml. The phospholipid content of this fraction changed very little during this time, so that the cholesterol-to-phospholipid ratio changed from 0.40 to 1.0. Subsequently, the cholesterol and

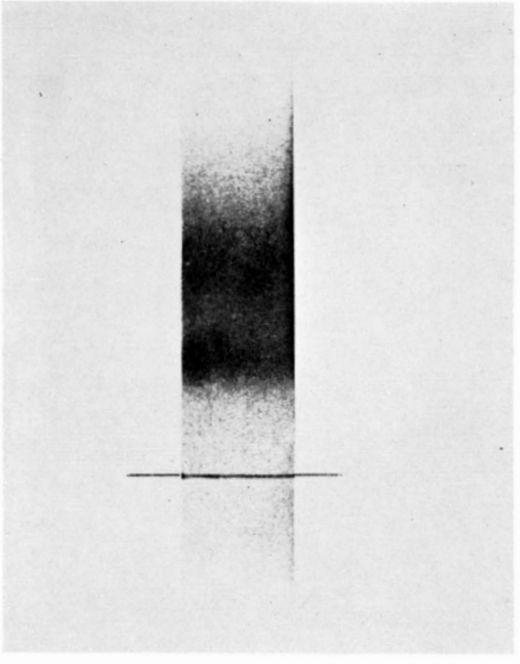

FIG. 2. Paper electrophoretogram of DPL fraction. Stained with Sudan black B.

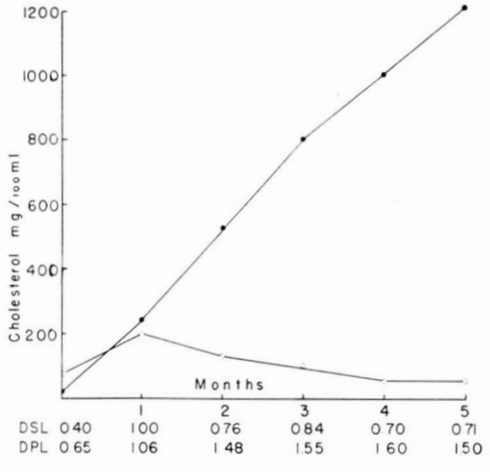

FIG. 3. Changes in serum lipoprotein cholesterol fractions in a dog fed a high-cholesterol diet. The numbers below the abscissa show the cholesterol-to-phospholipid ratios at 0 to 5 months after the high-cholesterol diet. ● = Dextran-precipitable lipoprotein. ○ = Dextran-soluble lipoprotein.

phospholipid content in the DSL fraction fell, the latter to below normal levels so that the cholesterol-to-phospholipid ratio, while decreasing to about 0.7, remained higher than normal. These results have been duplicated in another dog fed the same diet for 6 months.

DISCUSSION

Various investigators have examined the effectiveness of sulfated polysaccharides in the precipitation of low-density or β-lipoproteins. Most of the work, however, has been performed with so-called normal sera. The study of hypercholesterolemic sera offered an opportunity to examine whether dextran sulfate precipitates only β-lipoproteins or all the low-density lipoproteins. Inasmuch as the α_2-lipoprotein appears in the low-density fraction but is precipitated with dextran sulfate under the conditions of our experiment, it would seem more accurate to say that dextran sulfate precipitates all lipoproteins with a density less than 1.063.

One problem in the precipitation of low-density lipoproteins with dextran sulfate pertains to the relative amounts of dextran sulfate and lipoprotein that give optimal precipitation conditions. It was found in one experiment that from a serum with a total cholesterol content of 4.67 mg per ml, the addition of 0.04 ml of dextran sulfate and 0.1 ml calcium chloride precipitated only 1.23 mg of cholesterol. An additional 0.04 ml of dextran sulfate and 0.1 ml calcium chloride precipitated a further 1.17 mg of cholesterol. One might conclude from this that 0.04 ml of dextran sulfate per milliliter of serum could precipitate only 1.23 mg of cholesterol. However, when serum containing 7.9 mg of cholesterol per milliliter was tested, it was found that the addition of the first 0.04 ml of dextran sulfate precipitated 3.75 mg of cholesterol. It would seem, therefore, that the same amount of dextran sulfate is capable of precipitating more low-density lipoprotein when the latter is present in high concentrations. This might be explainable if one assumes the existence of a relatively high solubility product of the dextran sulfate-lipoprotein complex. On the other hand, it might mean that a certain amount of dextran sulfate combines with a given amount of lipoprotein-protein and that, in the more severe hypercholesterolemic animals, the low-density lipoproteins have more cholesterol per gram of protein.

To insure optimal conditions for the precipitation of low-density lipoproteins of hypercholesterolemic dog serum, it was found necessary to dilute the serum to one-fifth the original concentration with 0.9% NaCl. Although the addition of extra dextran sulfate and calcium chloride to the original serum also seemed to give satisfactory results in some cases, it did not consistently give quantitative precipitation. It would appear that the same problem might exist using sulfated polysaccharides in the precipitation of low-density serum lipoproteins of patients with hyper-

cholesterolemia or hyperlipemia. This subject has been reviewed extensively by Cornwell and Kruger (9).

The finding that the plasma lipoprotein pattern in hypercholesterolemic dogs differs from that in normal animals has been shown by other methods. Through the use of Cohn fractionation, Barr *et al.* (15) showed a decrease in the amount of cholesterol and phospholipid in their fraction A (α-lipoprotein). Milch *et al.* (16) showed by ultracentrifugal methods that 1 year after treatment with I^{131}, dogs showed hypercholesterolemia with an increase in high-density lipoproteins. Gonzalez *et al.* (17) studied I^{131}-treated dogs with and without cholesterol feeding. In six out of the nine cholesterol-fed animals, the high-density lipoprotein fraction fell while the low-density lipoprotein increased; this agrees with the results in our dogs on the high-cholesterol diet and made hypothyroid with thiouracil.

Our results relating to the paper electrophoresis of ultracentrifugal fractions of hypercholesterolemic dog serum appear to be at variance with results obtained for human serum using starch granules (18). Kunkel and Trautman (18) observed that the lipoprotein fraction of density less than 1.019 migrated by starch zone electrophoresis in the α_2 band. In our experiments with paper electrophoresis, this fraction fell in the β-lipoprotein region with a relatively high degree of sudanophilia near the origin. The fraction with a density between 1.019 and 1.063, which normally contains β-lipoproteins, appears to have two fractions in hypercholesterolemic dog serum; i.e., one with a mobility of β-lipoprotein and one with a mobility between that of the α_1- and β-lipoprotein, which we have provisionally labeled α_2-lipoprotein. This apparent difference between hyperlipemic human and dog serum deserves further investigation.

REFERENCES

1. Burstein, M. *Compt. rend.* **243:** 527, 1956.
2. Burnstein, M. *Compt. rend.* **245:** 586, 1957.
3. Burstein, M., and J. Samaille. *J. physiol. (Paris)* **49:** 83, 1957.
4. Burstein, M., and J. Samaille. *Clin. Chim. Acta* **3:** 320, 1958.
5. Bernfeld, P., V. M. Donahue, and M. E. Berkowitz. *J. Biol. Chem.* **226:** 51, 1957.
6. Boyle, E., and R. V. Moore. *J. Lab. Clin. Med.* **53:** 272, 1959.
7. Oncley, J. L., K. W. Walton, and D. G. Cornwell. *J. Am. Chem. Soc.* **79:** 4666, 1957.
8. Florsheim, W. H., and C. Gonzales. *Proc. Soc. Exptl. Biol. Med.* **104:** 618, 1960.
9. Cornwell, D. G., and F. A. Kruger. *J. Lipid Research* **2:** 110, 1961.
10. Castaigne, A., and A. Amselem. *Ann. biol. clin. (Paris)* **17:** 336, 1959.
11. Sakagami, T., and D. B. Zilversmit. *J. Lipid Research* **2:** 271, 1956.
12. O'Neal, R. M., W. A. Thomas, and W. S. Harroftt. *Am. J. Cardicl.* **3:** 94, 1959.
13. DiLuzio, N. R. *Nature* **185:** 616, 1960.
14. Kohn, J. *Nature* **183:** 1055, 1959.
15. Barr, D. P., E. M. Russ, H. A. Eder, F. E. Kendall, and L. L. Abell. *Circulation Research* **3:** 199, 1955.
16. Milch, L. J., A. A. Renzi, N. Weiner, L. G. Robinson, and S. S. Wilson. *Proc. Soc. Exptl. Biol. Med.* **97:** 56, 1958.
17. Gonzalez, I. E., L. N. Norcia, M. R. Shetlar, C. W. Robinson, L. L. Conrad, and R. H. Furman. *Am. J. Physiol.* **197:** 413, 1959.
18. Kunkel, H. G., and R. Trautman. *J. Clin. Invest.* **35:** 641, 1956.