

**A LIPID-PROTEIN FRACTION IN RHEUMATOID PLASMA  
PRECIPITABLE WITH CHONDROITIN SULFATE AFTER  
EUGLOBULIN REMOVAL**

Grace P. Kerby, ... , S. M. Taylor, Nancy M. Langley

*J Clin Invest.* 1961;40(10):1900-1905. <https://doi.org/10.1172/JCI104414>.

Research Article

**Find the latest version:**

<https://jci.me/104414/pdf>



# A LIPID-PROTEIN FRACTION IN RHEUMATOID PLASMA PRECIPITABLE WITH CHONDROITIN SULFATE AFTER EUGLOBULIN REMOVAL \*

By GRACE P. KERBY, S. M. TAYLOR AND NANCY M. LANGLEY

(From the Department of Medicine, Duke University School of Medicine, Durham, N. C.)

(Submitted for publication May 1, 1961; accepted June 15, 1961)

Acid mucopolysaccharides of human plasma have been observed (1) to vary as do acute phase reactants, i.e., to increase nonspecifically in any inflammatory state. The euglobulin fraction itself with which the acid mucopolysaccharides were associated increased also in such circumstances. On later comparing levels of acid mucopolysaccharides in samples of serum and plasma drawn simultaneously, it was noted that the acid mucopolysaccharides measurable in the euglobulin fraction were derived at least in part from the formed elements of blood during blood clotting (2). It seemed possible, therefore, that the amount of plasma protein precipitable as euglobulins by dilution at pH 5.8 might increase in inflammation as a result of the release of acid mucopolysaccharides into the blood, causing alteration of isoelectric point of plasma proteins already present. Precedent for such a possibility has been well established by the earlier observations of other investigators on the interactions of fibrinogen and  $\beta$ -lipoproteins and polyanions (3, 4). The possibility as it relates to rheumatoid plasma was mentioned in effect also by Badin, Schubert and Vouras (5).

To explore this possibility, studies were carried out on the interaction of chondroitin sulfate with the proteins remaining in plasma after isoelectric precipitation of spontaneously occurring euglobulins by dilution with mineral acid to pH 5.8. The additional protein available for precipitation under identical conditions of dilution and pH on addition of chondroitin sulfate (the fraction being called  $S_1$ ) was strikingly increased by prior dialysis of the plasma against balanced buffered salt solution. This increase was prevented if a heat-resistant, organic, dialyzable factor present in a pressure dialysate of plasma was restored

to the dialyzed plasma prior to use. The  $S_1$  fraction had a high cholesterol content. The amount available for precipitation from nondialyzed plasma of rheumatoid patients considerably exceeded that of patients without rheumatoid disease, with or without other inflammatory processes.

## METHODS

In dialysis experiments, oxalated plasma was dialyzed at 5° C for 21 to 27 hours in Visking 18/32-inch cellulose casing against 20 to 50 vol of balanced buffered salt solution.<sup>1</sup> Control plasma was held at 5° C for equal time and before use was made to volume (with balanced buffered salt solution) exactly equal to that of the dialyzed plasma (which increased in volume slightly during dialysis).

In surveying patients, oxalated blood for plasma was obtained from three groups of individuals, as previously defined in detail (1). Briefly, group I showed no evidence of inflammatory disease and had negative C-reactive protein tests; group II had significant inflammatory disease unrelated to rheumatoid or other primary connective tissue disease; and group III had clinically unequivocal active rheumatoid arthritis, in no instance being treated with hormones.

Euglobulins were precipitated from all plasma samples by the mineral acid dilution method used previously (1), and described briefly in Figure 1. Chondroitin sulfate (General Biochemicals, Inc.) was added, 2 mg per ml of physiological saline in volume one-half that of the original plasma, to the supernatant diluted plasma, to obtain the  $S_1$  fraction. The entire method of recovering fractions is summarized schematically in Figure 1. The protein content of euglobulin and  $S_1$  fractions was determined by biuret reaction (6), using human serum albumin as a standard and expressing content as milligram equivalence per 100 ml of original plasma. The cholesterol content of the fractions was determined after extraction with acid isopropyl alcohol and heptane (7), by the method of Zlatkis, Zak and Boyle (8). Results were expressed as milligrams per 100 ml of original plasma. A standard curve of 10, 25, 50, 100  $\mu$ g of puri-

<sup>1</sup> Ten ml of stock solution (7.5 per cent NaCl, 0.75 per cent KCl, 0.1 per cent  $\text{Na}_2\text{HPO}_4$ , 0.12 per cent  $\text{KH}_2\text{PO}_4$ , 0.05 per cent  $\text{K}_2\text{HPO}_4$ ) and 7 ml of 1 per cent  $\text{Na}_2\text{HPO}_4$  per 100 ml, aqueous (total phosphate concentration,  $6.7 \times 10^{-3}$  M).

\* This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, and the American Heart Association.

## SEPARATION OF PLASMA FRACTIONS

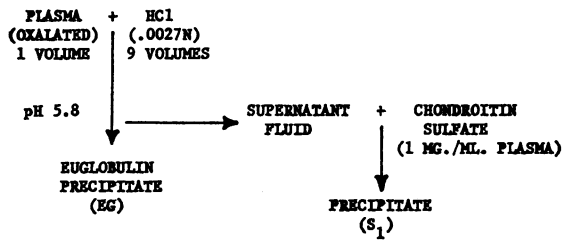


FIG. 1. SCHEMATIC SUMMARY OF PLASMA FRACTIONATION METHODS USED. Carried out at 5° C.

field cholesterol was run in duplicate with every set of determinations. All plasma fractions were derived and run in duplicate both for protein and cholesterol content, with close checks easily obtained.

## RESULTS

Table I illustrates the striking increase in protein available for precipitation in the S<sub>1</sub> fraction after dialysis of the original plasma. This was a consistent finding with all plasmas. The difference was gross, as shown in Figure 2 which compares the appearances of (a) S<sub>1</sub> fraction from nondialyzed plasma, (b) the same plasma after dialysis,

TABLE I  
Effect of dialysis of plasma on the protein (biuret) and cholesterol content of subsequent euglobulin and S<sub>1</sub> fractions

Noninflammatory plasma	Biuret	Cholesterol
mg/100 ml plasma		
Nondialyzed: Euglobulins	442	10.0
S <sub>1</sub> fraction	259	2.0
Dialyzed:*		
Euglobulins	423	22.0
S <sub>1</sub> fraction	742	45.0+

\* Dialyzed against 20 vol balanced buffered salt solution, pH 7.4, for 21.5 hours at 5° C.

and (c) dialyzed plasma to which a small amount of undiluted plasma dialysate (obtained by centrifuging plasma in Visking cellulose casing for 8 to 9 hours at 5° C at 1,000 G) was added before fractionation of the plasma. The factor (or factors) which restored the dialyzed plasma to its nondialyzed state in regard to S<sub>1</sub> precipitability was dialyzable, resisted heating to 100° C for 15 minutes, but was destroyed by ashing. Addition of certain known dialyzable plasma components and other substances to the buffered balanced

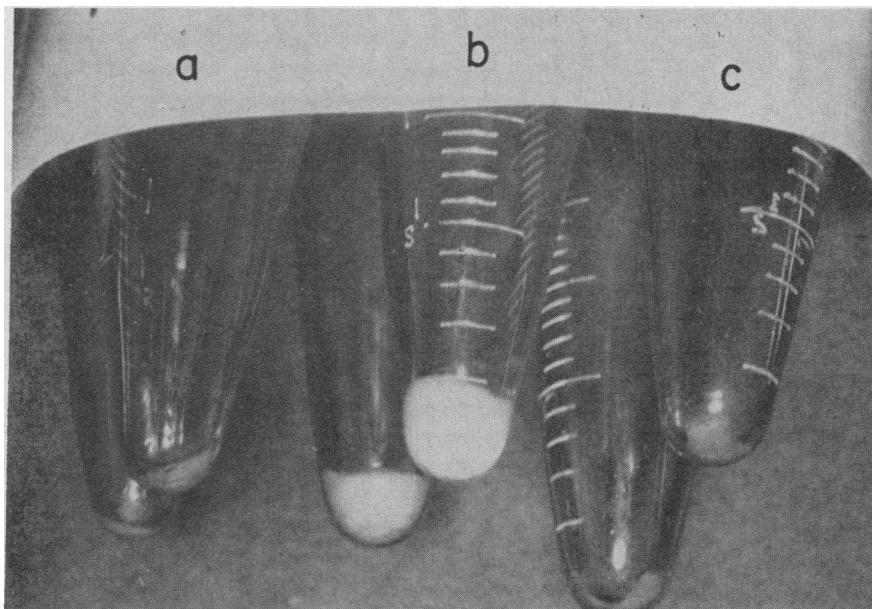


FIG. 2. GROSS EFFECT OF PLASMA DIALYSIS ON SUBSEQUENT S<sub>1</sub> FRACTION RECOVERED. S<sub>1</sub> fractions (in duplicate) centrifuged at 5° C and photographed after decantation of the supernatant fluid. (a) S<sub>1</sub> from original nondialyzed plasma. (b) S<sub>1</sub> from same plasma after dialysis. (c) S<sub>1</sub> from same plasma after dialysis and then addition of a plasma pressure dialysate before fractionation.

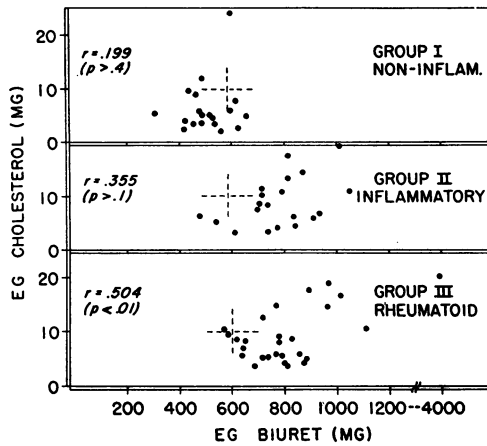


FIG. 3. CORRELATION DATA ON EUGLOBULIN FRACTIONS, CHOLESTEROL AND PROTEIN (BY BIURET) CONTENT (IN MG PER 100 ML ORIGINAL PLASMA).  $r$  = Correlation coefficient. The dotted crossed lines are arbitrarily and identically placed in the three boxes as visual reference aids.

salt solution before dialysis of plasma failed to prevent the subsequent increase in  $S_1$  fraction. These included  $(NH_4)_2SO_4$  ( $8.3 \times 10^{-4}M$ ),  $MgCl_2$  ( $2 \times 10^{-4}M$ ), glucose ( $7 \times 10^{-3}M$ ), urea ( $3.3 \times 10^{-3}M$ ), uric acid ( $2.4 \times 10^{-5}M$ ), glutamic acid ( $1.4 \times 10^{-4}M$ ), and sodium salicylate ( $2.5 \times 10^{-4}M$ ). Restoration of  $Ca^{++}$  to the system was without effect, and serum could be substituted for plasma without apparent alteration of results. Prevention of loss of oxalate during dialysis (by use of equal concentration outside the casing) did not prevent the subsequent increase in  $S_1$  fraction. On the chance that the dialysis effect simply reflected an alteration in labile lipoproteins, as noted on dialysis of plasma by Ray, Davisson and Crespi (9), the effect of  $Cu^{++}$  ( $1.5 \times 10^{-5}M$ ,  $4 \times$

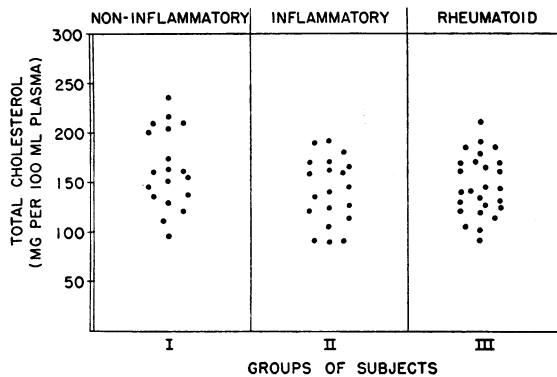


FIG. 4. TOTAL PLASMA CHOLESTEROL LEVELS.

$10^{-5}M$ ), of serine ( $4.8 \times 10^{-4}M$ ) and of versene (0.1 per cent disodium EDTA) was studied exhaustively in the system, with failure to demonstrate any change such as Ray and co-workers described as a cause of the increased  $S_1$  after dialysis of plasma. The amount of  $S_1$  obtained appeared, indeed, maximal when versene was added (0.1 per cent) to the balanced buffered salt solution used in dialysis. This was precisely the opposite effect from that expected if the  $S_1$  changes had been due to influences on lipoproteins of the type described by Ray and associates.

In surveying plasma of patients for evidence of possible biological significance in the above-described observations *in vitro*, the euglobulin frac-

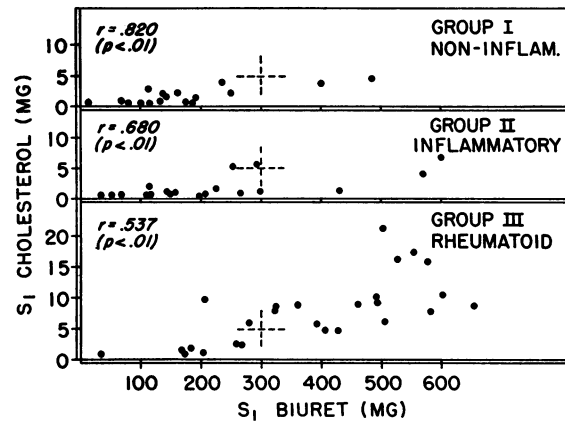


FIG. 5. CORRELATION DATA ON  $S_1$  FRACTIONS, CHOLESTEROL AND PROTEIN (BY BIURET) CONTENT (IN MG PER 100 ML ORIGINAL PLASMA). Dotted crossed lines and  $r$  as in Figure 3.

tions were found to be increased in the inflammatory groups II and III, as expected from previous studies (1). The euglobulin data are summarized in Figure 3, which plots protein content of the fraction against its cholesterol content. Only the rheumatoid group III showed significant correlation between protein and cholesterol content of the euglobulin fraction. Neither the cholesterol content nor the percentage of cholesterol in the euglobulin fraction correlated with the total plasma cholesterol in any of the three groups. Figure 4 shows the similarity in range of total plasma cholesterol in the three groups of patients studied.

Figures 5 and 6 summarize the  $S_1$  data. Here there was high correlation between biuret and cholesterol content of the fraction in all three

groups (Figure 5). The amount of lipid-protein fraction available for precipitation as  $S_1$  was considerably greater in rheumatoid group III than in either of the nonrheumatoid groups. In the nonrheumatoid groups I and II, those patients with the highest percentage of cholesterol in  $S_1$  fraction tended also to be those patients with highest total plasma cholesterol (Figure 6). This correlation did not exist in the rheumatoid group III.

The  $S_1$  fractions from the three groups of patients showed no qualitative differences between the groups, either by standard Ouchterlony plate techniques (rabbit antiserum to  $S_1$  fractions versus Cohn fractions of human plasma) or by electrophoretic techniques. However, the quantitative similarity between the  $S_1$  fraction from non-dialyzed rheumatoid plasma and that from dialyzed normal plasma was again emphasized on paper electrophoresis (Figure 7). Precipitation bands were obtained with  $S_1$  antiserum against both

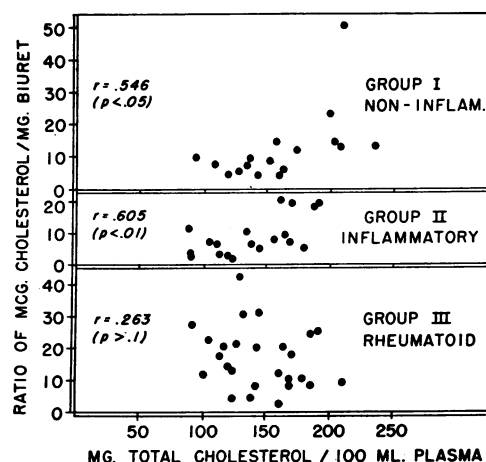


FIG. 6. CORRELATION BETWEEN PERCENTAGE CHOLESTEROL CONTENT OF  $S_1$  FRACTIONS AND TOTAL PLASMA CHOLESTEROLS.  $r$  as in Figure 3.

Cohn fractions I and III by Ouchterlony techniques, indicating that the  $S_1$  fraction included fibrinogen and  $\beta$ -lipoprotein among its components.

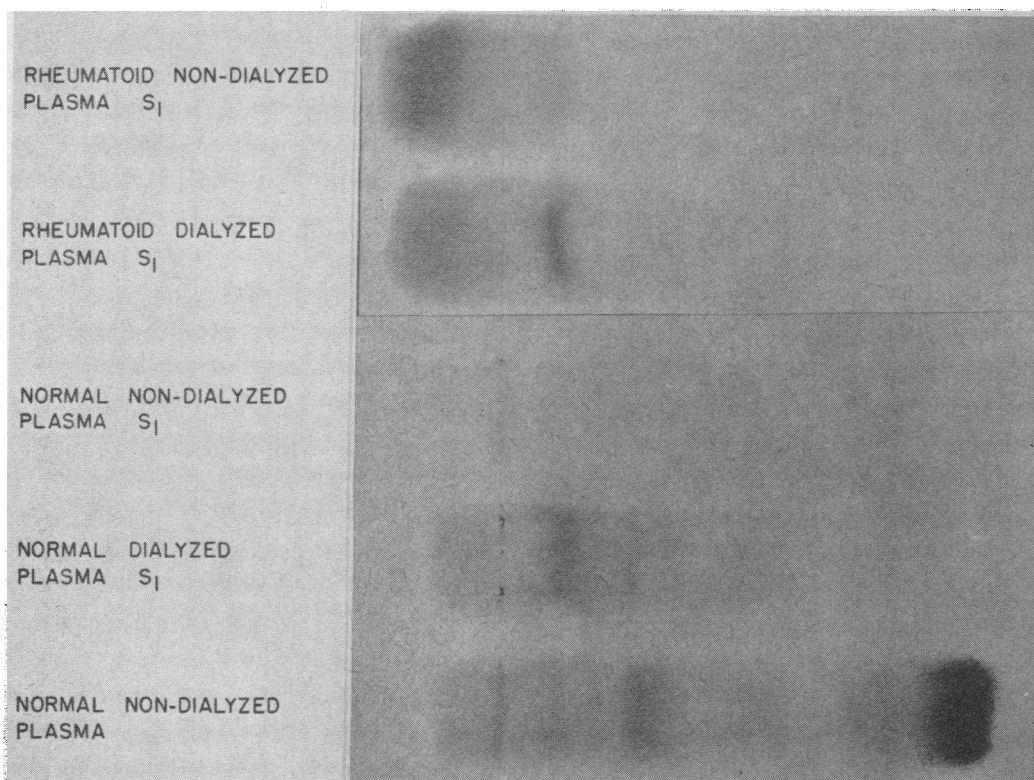


FIG. 7. PAPER ELECTROPHORESIS OF  $S_1$  FRACTIONS, AS INDICATED. All  $S_1$  fractions were concentrated twofold (taken up to one-half original plasma volume) for use here. The whole plasma was used at original concentration. Run at 10 ma for 7 hours on Whatman no. 3 MM paper in 0.12 M, pH 8.6, veronal buffer.

## DISCUSSION

Previous studies (10) have shown that predominant spontaneous occurrence of acid mucopolysaccharides of human plasma was in Cohn fractions III and IV-1.<sup>2</sup> In the present studies, the lipid-protein fraction precipitable from any human plasma with chondroitin sulfate after removal of spontaneously occurring euglobulins but under identical conditions of dilution and pH ( $S_1$  fraction) was found to be rather uniform and high in its cholesterol content. The spontaneously occurring euglobulin fraction of rheumatoid plasma, in contrast to that from nonrheumatoid patients with or without other inflammatory disease, showed uniformity and elevation of cholesterol content comparable with that of the  $S_1$  fraction produced artificially by appropriate addition of chondroitin sulfate. The possibility can be considered therefore that the increased euglobulins in rheumatoid plasma may represent in significant part plasma protein, the isoelectric point of which has been shifted by association with a polyanion such as an acid mucopolysaccharide so that it precipitates under the *in vitro* conditions of dilution and pH which define precipitability of euglobulins.

A further difference between rheumatoid and nonrheumatoid plasma lay in the presence of significantly increased amounts of demonstrable  $S_1$  fraction in rheumatoid plasma. Any plasma could be made to resemble rheumatoid plasma in this regard by first dialyzing out of it a heat-resistant, organic material which, in turn, could be re-added to dialyzed plasma before use to completely revert the plasma to its original nondialyzed state as far as subsequent  $S_1$  precipitability was concerned.<sup>3</sup> Because of the resemblance of dialyzed

<sup>2</sup> The validity of this observation would appear to be unchallenged by the later observation of Epstein and Block (11) who noted that only 0.5 per cent of total plasma hexosamine was found in lipoprotein fractions of human plasma. By the data from this laboratory (1) and by those of Bollet (12) on total acid mucopolysaccharides, and recognizing rough equivalence by weight of hexuronic acid and hexosamine content in acid mucopolysaccharides, less than 0.4 per cent of the total plasma hexosamine as measured by Epstein and Block and by Bollet could be expected to be found in total acid mucopolysaccharides of human plasma, regardless of where they occurred fractionally.

<sup>3</sup> It is of interest that the euglobulin fraction of dialyzed plasma regularly showed an increase in acid mucopoly-

plasma to rheumatoid plasma in this regard, an intensive effort is in progress to identify the dialyzable factor or factors involved. This effort has been furthered by finding that a similar if not identical factor can be demonstrated in both human urine and spinal fluid. Other quantitative studies in progress suggest that these findings may be of biological significance in rheumatoid disease. The presence of a dialyzable substance in biological materials capable of influencing the combination of acid mucopolysaccharides with proteins may offer a lead into the system of checks and balances which must certainly regulate this area of connective tissue physiology as similar systems regulate other biological processes such as blood coagulability.

## SUMMARY

1. The protein remaining in human plasma after isoelectric precipitation of euglobulins was studied for precipitability with chondroitin sulfate under the same conditions of dilution and pH which precipitated euglobulins. The protein so precipitated was called the  $S_1$  fraction. Cholesterol content of the lipid-protein  $S_1$  fraction showed a positive correlation with protein content.

2. This  $S_1$  fraction was significantly increased in the plasma of rheumatoid patients, in contrast to nonrheumatoids with or without other inflammatory disease.

3. Any plasma could be made to resemble rheumatoid plasma in this regard by first dialyzing it against balanced buffered salt solution.

saccharide content when measured by methods detailed elsewhere (1) as compared with the euglobulin fraction of the same plasma nondialyzed. This occurred regardless of whether the over-all protein content of the fraction increased, decreased, or remained unchanged after dialysis. Vertically paired data (below) show the acid mucopolysaccharide content (expressed as micrograms of hexuronic acid per 100 mg euglobulin protein) of nondialyzed (ND) and dialyzed (D) plasma in several experiments:

ND	75	55	76	89	50	58
D	161	156	139	159	120	95

This was assumed to reflect again the change in isoelectric point of proteins influenced by attached small groups which could be dialyzed off the protein. The euglobulin fraction after dialysis appeared to consist of proportionally more acid-mucopolysaccharide-associated protein than before dialysis.

4. Addition of plasma dialysate to dialyzed plasma prior to its use reversed the dialytic effect. The factor in the dialysate which accomplished the reversal was resistant to heating to 100° C for 15 minutes but not to ashing.

5. Further studies are intensively in progress to identify the dialyzable material which was shown to affect the combination of acid mucopolysaccharides with plasma protein.

## REFERENCES

1. Kerby, G. P. The effect of inflammation on the hexuronate-containing polysaccharides of human plasma. *J. clin. Invest.* 1958, **37**, 962.
2. Kerby, G. P., and Langley, N. M. Human thrombocytes and leukocytes as a source of some of the acid mucopolysaccharides of serum. *J. Lab. clin. Med.* 1959, **53**, 708.
3. Gordon, R. S., Jr. Interaction between oleate and the lipoproteins of human serum. *J. clin. Invest.* 1955, **34**, 477.
4. Bernfeld, P., Donahue, V. M., and Berkowitz, M. E. Interaction of human serum  $\beta$ -lipoglobulin with polyanions. *J. biol. Chem.* 1957, **226**, 51.
5. Badin, J., Schubert, M., and Vouras, M. Plasma polysaccharide fraction containing uronic acid, in normal subjects and in patients with rheumatoid arthritis. *J. clin. Invest.* 1955, **34**, 1317.
6. Jayle, M. F., Boussier, G., and Badin, J. Microdosage des protéines sériques par la méthode du biuret. *Bull. Soc. Chim. biol. (Paris)* 1951, **33**, 881.
7. Dole, V. P. A relationship between non-esterified fatty acids in plasma and the metabolism of glucose. *J. clin. Invest.* 1956, **35**, 150.
8. Zlatkis, A., Zak, B., and Boyle, A. J. A new method for the direct determination of serum cholesterol. *J. Lab. clin. Med.* 1953, **41**, 486.
9. Ray, B. R., Davisson, E. O., and Crespi, H. L. Experiments on the degradation of lipoproteins from serum. *J. phys. Chem.* 1954, **58**, 841.
10. Kerby, G. P. The distribution of acid mucopolysaccharides in Cohn fractions of human plasma proteins. *J. clin. Invest.* 1958, **37**, 678.
11. Epstein, F. H., and Block, W. D. Glycoprotein content of serum lipoproteins. *Proc. Soc. exp. Biol. (N. Y.)* 1959, **101**, 740.
12. Bollet, A. J. The hexosamine content of the serum globulins in normal and pathological sera. *J. clin. Invest.* 1957, **36**, 51.