# *CHARACTERIZATION OF CHOLESTEROL-BINDING GLOBULIN BY MODIFIED ZONE ELECTROPHORESIS AND O-(DIETHYLAMINOETHYL)CELLULOSE CHROMATOGRAPHY**

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Normal human serum has been shown[1] to exhibit cholesterol-binding properties upon immunoelectrophoresis on agar gel. The evidence clearly supports the concept of a specific type of binding of cholesterol by alpha-beta globulin. Accordingly, the name "transcholesterin" was proposed for the reactive component.

In order to evaluate the mechanism of *in vitro* interaction of cholesterol with transcholesterin, purified components are desirable. Sober and co-workers[2] introduced the use of *O*-(diethylaminoethyl) (DEAE)-cellulose chromatography for the fractionation and identification of serum proteins. However, DEAE-cellulose chromatography of serum proteins failed to resolve them into components homogeneous as judged by the criteria of paper electrophoresis.[3] Therefore, it has been necessary to combine electrophoretic separation with column chromatography in the fractionation of serum proteins. The present communication describes the initial findings in an attempt to isolate and characterize transcholesterin by the combination of zone electrophoresis and the modified chromatographic procedure and also by chemical analysis and immunodiffusion studies.

*Materials and Methods.—Human sera:* Pooled and individual fresh sera were obtained from healthy volunteers. The details of the procedure have been described elsewhere[1] for the following items: (*a*) agar-gel immunoelectrophoresis, (*b*) cholesterol solution in Teepol "L" (an alkyl sulfate detergent), and (*c*) transcholesterin assay involving immunoelectrophoresis.

*Lipoprotein and lipoprotein-free serum (LFF) preparations:* Lipoprotein fractions at densities 1.063, 1.10, and 1.21 were obtained in the Spinco preparative ultracentrifuge according to the method of Havel *et al.*[4] When total lipoproteins and LFF were desired, the preparative ultracentrifugation was carried out according to the procedure of Lewis *et al.*[5] at solvent density 1.21. All the fractions were adjusted to original serum volume with isotonic saline. Tests such as paper electrophoresis[6] and starch-gel electrophoresis[7, 8] were performed to determine the purity of the lipoproteins and LFF samples. Further characterizations of these samples were by the Ouchterlony double-diffusion technique.[9]

*Antisera against human serum lipoproteins:* Antisera against human alpha-1, alpha-2, and beta lipoproteins produced separately in rabbits were obtained from Behringwerke, A. G., Marburg-Lahn, West Germany.

*Total cholesterol determination:* A modified Liebermann-Burchard procedure[10] using the Bausch and Lomb spectronic 505 spectrophotometer to read the density of the color at wavelength 640 m$\mu$ was adopted for cholesterol quantitation. The reproducibility of the analytical procedure was good with a standard deviation of $\pm 5\%$.

*Vertical zone electrophoresis:* Buffer preparation was described elsewhere (Fig. 5),[1] except that the pH was adjusted to 8.6 instead of 8.1. Potato starch powder (Baker analyzed reagent) suspended in the buffer was slurried into a glass column (2.5 cm in diameter $\times$ 60 cm in length) stoppered at the bottom with a rubber stopper and center-

filled with a glass tubing which was filled with a glass-wool and Difco agar gel (2% buffered) to serve as a salt bridge. Excess buffer, which resulted after the starch had sedimented by gravity for 6 hr, was pipetted off. One piece of filter paper disk was put on the top surface of the packed starch column. Serum or serum protein preparation dissolved in the buffer was applied onto the starch column above the paper disk. Then starch powder was added to the liquid sample, with stirring, to adsorb the sample completely. Another piece of filter paper disk was layered on the starch medium containing the sample. More starch slurry was added so that the sample compartment was at least 10 cm below the additional starch medium. A layer of buffered agar gel which served as a reservoir for buffer covered the top surface. Electrophoretic technique and collection of fractions were described in an earlier communication.[1] Identification of serum protein fractions was performed by electrophoresis on cellulose acetate strips as described by Kohn[11] but with Hirschfeld[12] electrode buffer.

*DEAE chromatography:* DEAE-cellulose obtained from Carl Schleicher and Schuell Co. (standard no. 70; lot no. 1447; 0.89 meg/gm capacity) was prepared for chromatography by the method of Peterson and Sober.[13] A flow rate of 3.0 ml/min was maintained, and constant aliquots per tube were collected in the GME fraction collector. Serum sample, adjusted to original buffer condition, was passed through a DEAE column of the same buffer. The column was eluted with distilled water until the effluent absorbance at 280 mμ was less than 0.050, then with 0.05 *M* buffer (DEAE starting buffer), and finally with a linear gradient elution[14] of 1 *M* NaCl in a 0.05 *M* buffer. Different sets of pH and buffer were used in the separate experiments. The fractions were combined according to their respective peaks, lyophilized to small volumes, and dialyzed against distilled water for 4 hr and finally overnight in 0.154 *M* NaCl solution. These fractions were assayed for transcholesterin activity and serum proteins identified on cellulose acetate electrophoresis.

*Results.—DEAE column chromatography of a whole human serum at pH 4.75:* Figure 1 represents a chromatographic elution pattern of 20 ml of human serum (approximately 1.4 gm of protein) at pH 4.75. The transcholesterin activity is spread out across a wide section of the chromatogram. The last fraction eluted by 2 *M* NaCl contained only albumin and was negative in the transcholesterin test. All the other fractions, which have in them alpha-1 and alpha-2 globulins, exhibited positive transcholesterin activity. Even though the chromatographic procedure yields more than nine distinct peaks, the separation of serum proteins in terms of their electrophoretic identities on cellulose acetate was not clear-cut.

*DEAE column chromatography at pH 8.20 of lipoprotein-free serum or D 1.21 infranate:* In Figure 2 is shown the effluent diagram obtained with a lipoprotein-free serum chromatographed on the DEAE-cellulose column. A number of poorly resolved peaks are evident. The transcholesterin activity was found in the fractions containing either alpha-1 or alpha-2 globulin, or both. *No transcholesterin activity could be detected in either the isolated gamma globulin fractions or the purified albumin fractions.* It is quite interesting to note that all the transcholesterin activity is distributed over a fairly large NaCl gradient elution effluent from 0.13 to 0.48 *M* NaCl in 0.05 *M* Tris HCl, pH 8.2.

*Vertical zone electrophoresis of whole serum:* Figure 3*a* demonstrates that all the transcholesterin active proteins are found in a broad zone about 14.5 cm in length and including a well-defined yellow front band followed by a red diffused zone. The separation patterns of serum proteins for whole serum (Fig. 3*a*) were less well defined than for lipoprotein-free serum (Fig. 3*c*). Since

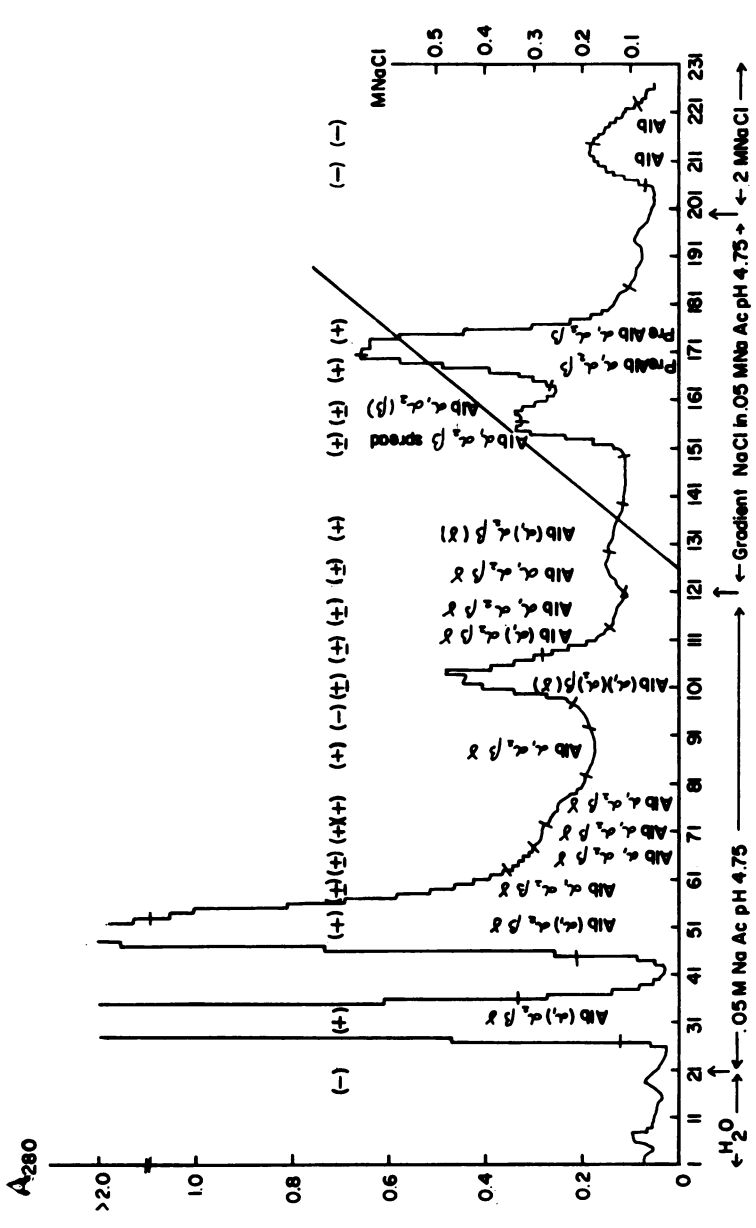


FIG. 1.—Chromatographic elution pattern of 20 ml of human serum on DEAE-cellulose column (47.5 × 3.4 cm) is described. Each electrophoretic category is listed (e.g., *Pre.Alb*, *Alb*, $\alpha$, $\alpha_2$, $\beta$, and $\gamma$ represent prealbumin, albumin, alpha-1, alpha-2, beta, and gamma globulins). (+), Positive transcholesterin activity; (±), weak transcholesterin activity. Arrows indicate the start of elution at the top of the column with the different eluents (NaAc is sodium acetate) at room temperature. Aliquots of 11.0 ml are collected. Short transverse lines on the graph indicate the pool cut regions.

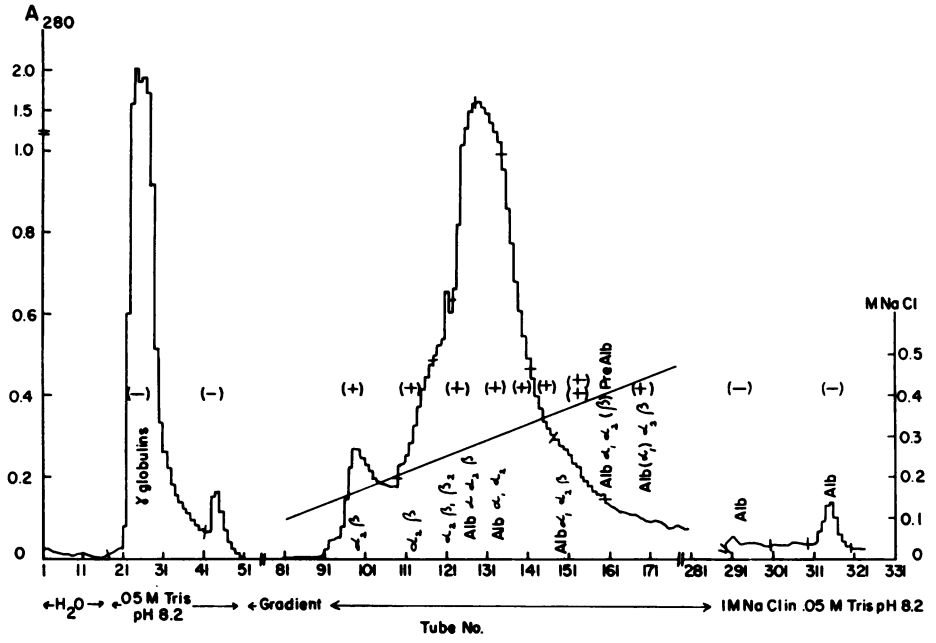


FIG. 2.—Elution diagram of about 180 mg of a pooled human lipoprotein-free serum on DEAE-cellulose column (42 × 2 cm). Aliquots of 3.6 ml are collected in a GME (Gilson Medical Electronics, Middleton, Wis.) fraction collector.

the active transcholesterin proteins were found to reside in this broad zone containing albumin, alpha-1, alpha-2, and possibly beta globulins, the active fractions from the zone electrophoresis were rerun on a newly prepared starch column and one of the peak fractions (fraction 9) of Fig. 3*a* was chosen. The electrophoretic run is shown in Figure 3*b*. Again, all the fractions which contained alpha globulins gave positive transcholesterin tests except for the one fraction of alpha-1 globulin in fraction 7. The positive tests with albumin fractions 1 and 2 were probably related to contamination of the albumin of whole serum by alpha-lipoprotein since the albumin of lipoprotein-free serum separated by zone electrophoresis (Fig. 3*c*) does not give transcholesterin activity.

An example of the vertical zone electrophoresis of the LFF is shown in Figure 3*c*. Alpha-1 globulin moved as a broad zone of approximately 12 cm between albumin and alpha-2 beta globulins; only two (fractions 4 and 5) of eight alpha-1 globulin fractions gave positive transcholesterin activity, whereas all fractions containing alpha-2 globulins were positive for transcholesterin activity. It appears that the transcholesterin active proteins were located in a certain fraction of alpha-1 globulin and in alpha-2 globulin regions of the electrophoresis.

During the course of the isolation and characterization of transcholesterin-active proteins by DEAE-cellulose chromatography and vertical zone electrophoresis, it became necessary to ascertain the identity of the fractionated samples.

*Further chemical and immunological characterization of serum fractions:* Further characterizations of the LFF serum and lipoprotein fractions by more specific and sensitive methods such as chemical and immunodiffusion techniques were carried out.

(1) *Analyses for total serum cholesterol on lipoprotein fractions and D 1.21 infranate:* The data showed that lipoprotein fraction (D 1.063) LDL contained 68.2 per cent of total serum cholesterol, which is in good agreement with the value given by Lindgren and Nichols[15] for lipoproteins obtained in this density range of up to 1.063. High-density lipoproteins such as (D 1.100) $HDL_2$ and (D 1.210) $HDL_3$ gave a combined total of 27.2 per cent for total serum cholesterol (i.e., 6.9% for $HDL_2$ and 20.3 for $HDL_3$). (D 1.210) $HDL_3$ contained as much as three times the amount of total serum cholesterol as (D 1.100) $HDL_2$. Despite the fact that (D 1.210) infranate was shown to contain no measurable total serum cholesterol, its unique character is interesting in that it is capable of binding free cholesterol in the transcholesterin test.

(2) *Transcholesterin test on partially delipidated and nontreated serum fractions:* Partial delipidation of serum fractions were conducted according to the method described by Levy and Fredrickson.[16] All nontreated lipoprotein fractions, (D 1.210) infranate, and whole serum yielded positive transcholesterin tests, whereas the partially delipidated samples of (D 1.063) LDL, (D 1.100) $HDL_2$, and (D 1.21) $HDL_3$ did not. Furthermore, (D 1.210) infranate and whole serum still exhibited positive transcholesterin activity even after they had been subjected to the delipidation treatment.

From the data presented, a general statement can be made concerning the stability of transcholesterin or transcholesterins. *The transcholesterin activity in all lipoprotein fractions is destroyed after partial delipidation.* In contrast, the activity in (D 1.210) infranate is stable after it is subjected to the same treatment.

(3) *Serological specificity of serum fractions with rabbit antisera in immunodiffusion:* The results of the characterization of whole serum, lipoprotein fractions, and density 1.210 infranate by immunodiffusion technique of Ouchterlony[9] with or without prior partial delipidation treatment are presented in Table 1. (D 1.210) $HDL_3$ reacted only with anti-alpha-1 lipoprotein to form a reaction line. Whole human serum was used as control and reacted with all three specific antisera. When the various serum fractions were subjected to

TABLE 1. *Characterization of whole human serum, lipoprotein fractions, and density 1.21 infranate by immunodiffusion technique of Ouchterlony[9] with or without prior partial delipidation treatment.*

| Serum Samples | | Immunodiffusion Test with Rabbit Antisera to: | | |
|---|---|---|---|---|
| Nontreated | Partially delipidated | Human $\alpha_1$-lipoprotein | Human $\alpha_2$-lipoprotein | Human $\beta$-lipoprotein |
| (D 1.210) $HDL_3$ | | + | − | − |
| | (D 1.210)$HDL_3$ | − | − | − |
| (D 1.210) infranate | | − | − | − |
| | (D 1.210) infranate | − | − | − |
| Whole serum | | + | + | + |
| | Whole serum | + | − | − |

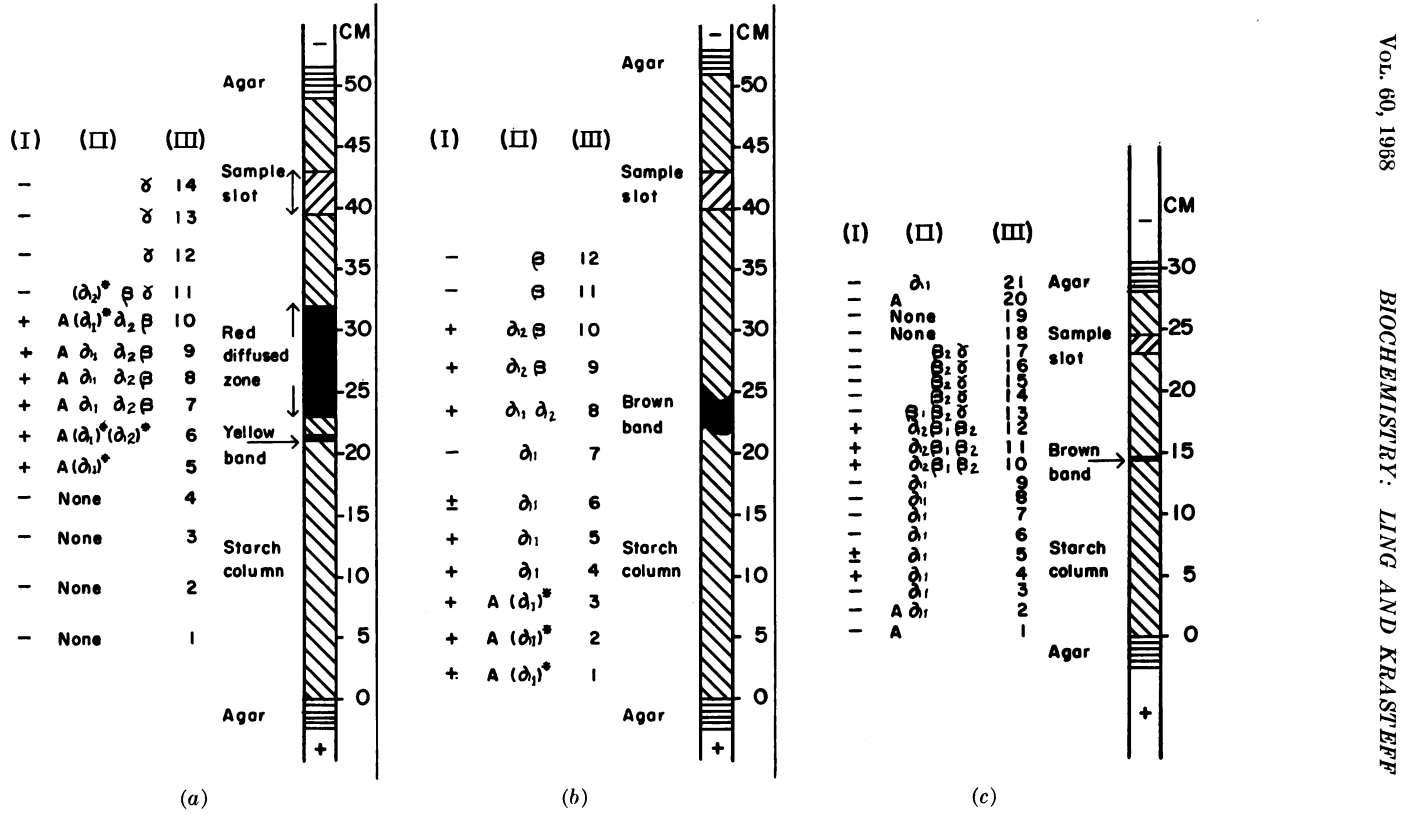


FIG. 3.—Electrophoretic patterns on vertical zone electrophoresis; pH 8.6 (Tris-barbital buffer); diameter of starch column, 2.5 cm. (I) Transcholesterin activity; (II) fractions identified by cellulose acetate electrophoresis; (III) fraction number.

(*a*) Normal human serum (10 ml); temperature, 18°C; duration of electrophoresis, 71 hr.

(*b*) Fraction 9 from (*a*); temperature, 18°C; duration of electrophoresis, 118 hr.

(*c*) 166 mg human LFF; temperature, 22–25°C; duration of electrophoresis, 67 hr. Each electrophoretic category is listed (e.g., $A$, $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, and $\gamma$ represent albumin, alpha-1, alpha-2, beta-1, beta-2, and gamma globulins). + and ±, Positive and weakly positive transcholesterin activity, respectively; −, negative transcholesterin activity.

prior partial delipidation, none of the treated samples gave a reaction line, but the delipidated whole serum reacted after immunodiffusion with anti-alpha-1 lipoprotein. (D 1.210) infranate was negative to each of the three specific antisera by the immunodiffusion test either before or after it was similarly treated, an indication that it did not contain lipoproteins of the nature discussed here. The (D 1.210) infranate preparations were thus generally free of alpha-1, alpha-2, and beta lipoproteins as determined by the Ouchterlony[9] double-diffusion procedure with commercial rabbit antisera.

*Discussion.*—Fractionation of the transcholesterin from lipoprotein-free serum by vertical zone electrophoresis on starch medium showed that one of the several separated alpha-1 globulins and all of the alpha-2 globulins yielded positive transcholesterin activity. Similar zone electrophoresis of the whole serum gave poorer resolution of the protein components. It seems that the lipoproteins present in the whole serum were smearing the electrophoretic pattern.

Sober and co-workers[2] applied DEAE-cellulose chromatography to the separation of serum proteins. They found that lipoproteins were eluted in fractions 10 and 11 of a total of 23 fractions.[17] This report and other papers[3, 18] have described the use of this technique for serum protein separation with minor modifications and have produced similar results. There has been an important need for suitable elution buffers at some pH values to reduce or increase the number of charges on the protein or exchanger. In the present study, two modified elution buffers (pH 8.2 and 4.75) used in developing the DEAE-cellulose chromatogram of serum proteins have been described.

The demonstration of transcholesterin activity in the several peak fractions of the DEAE-cellulose chromatograms containing alpha globulins suggests that this cholesterol-binding protein may actually comprise a system of several proteins rather than a single one. Hence, it is desirable to investigate further this class of alpha globulins exhibiting transcholesterin activity by immunological and by biological assay. For these purposes, adequate amounts of homogeneous purified materials must be obtained. Additional procedures are now under study in our laboratory.

Although the mechanism of *in vitro* interaction of free cholesterol with transcholesterin is not fully established, some experiments involving the technique of Avigan[19] to measure the relative uptake of $C^{14}$-cholesterol by serum fractions from radioactive cholesterol-coated celite 545 have produced evidence (which will be reported later) that the transcholesterin-positive protein or proteins can bind cholesterol without the use of the detergent Teepol "L". However, in an earlier communication,[1] the specificity of the precipitinlike reaction of transcholesterin was found to be limited to cholesterol and dihydrocholesterol dissolved in Teepol "L" solution. Close homologues of cholesterol such as desmosterol, zymosterol, and lanosterol dissolved in Teepol "L" have since been tested; they were unreactive. The evidence supports the idea that Teepol "L" serves only as a vehicle for cholesterol. The specific reaction would be dependent in part on the conformation of the protein, while the primary re-

quirement would be contributed by the steric configuration of the cholesterol molecule.

*Summary.*—The studies have presented evidence that the protein component(s) of lipoprotein-free serum which reacts with cholesterol in immunoelectrophoresis (termed transcholesterin) resides in the alpha globulins. It is apparently not a lipoprotein.

The albumin, alpha, and beta globulins were resolved into a number of subfractions of DEAE-cellulose column chromatography, but by this procedure were not completely separated from each other. Upon zone electrophoretic separation the lipoprotein-free serum yielded positive transcholesterin activity in one of the alpha-1 and all of the alpha-2 globulin regions. The electrophoretic patterns of whole serum were less distinct with considerable cross-contamination by lipids.

Further characterizations of the lipoproteins, the (D 1.210) infranate, and the LFF fractions were performed through Ouchterlony immunodiffusion technique and through chemical analyses for total serum cholesterol. The fractions of LFF with transcholesterin activity did not react with antilipoprotein sera.

The fact that transcholesterin activity is found in different eluted fractions of alpha-1 and alpha-2 globulins suggests that it may constitute a system of proteins actively combining with cholesterol.

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[1] Francis, T., Jr., N.-S. Ling, and T. Krasteff, these PROCEEDINGS, **53**, 1061 (1965).

[2] Sober, H. A., F. J. Gutler, M. D. Wyckoff, and E. A. Peterson, *J. Am. Chem. Soc.*, **78**, 756 (1956).

[3] Fahey, J. L., F. M. McCoy, and M. Goulian, *J. Clin. Invest.*, **37**, 272 (1958).

[4] Havel, R. J., H. A. Eder, and J. H. Bragdon, *J. Clin. Invest.*, **34**, 1345 (1955).

[5] Lewis, L. A., A. A. Green, and I. H. Page, *Am. J. Physiol.*, **171**, 391 (1952).

[6] Block, R. J., E. L. Durrum, and G. Zweig, in *A Manual of Paper Chromatography and Paper Electrophoresis* (New York: Academic Press, 1955), 2nd ed.

[7] Smithies, O., *Biochem. J.*, **61**, 629 (1955).

[8] Poulik, M. D., *Nature*, **180**, 1477 (1957).

[9] Ouchterlony, Ö., *Acta Pathol. Microbiol. Scand.*, **26**, 507 (1949).

[10] *Laboratory Manual of Biological Chemistry for Medical Students, Department of Biological Chemistry* (Boston: Harvard Medical School, 1948), 4th ed, p. 125.

[11] Kohn, J., *Clin. Chim. Acta*, **3**, 450 (1958).

[12] Hirschfeld, J., *Sci. Tools*, **7**, 18 (1960).

[13] Peterson, E. A., and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956).

[14] Bock, R. M., and N.-S. Ling, *Anal. Chem.*, **26**, 1543 (1954).

[15] Lindgren, F. T., and A. V. Nichols, in *The Plasma Proteins*, ed. F. W. Putnam (New York: Academic Press, 1960), vol. 2, p. 1.

[16] Levy, R. I., and D. S. Fredrickson, *J. Clin. Invest.*, **44**, 426 (1965).

[17] Sober, H. A., and E. A. Peterson, *Federation Proc.*, **17**, 1116 (1958).

[18] Tombs, M. P., K. B. Cooke, D. Burston, and N. F. Maclagan, *Biochem. J.*, **80**, 284 (1961).

[19] Avigan, J., *J. Biol. Chem.*, **234**, 787 (1959).