Thomas, E. D., Rath, C. E., Haskins, D., Finch, S., and Fluharty, R. G., *Blood*, 1950, v5, 983.
6. Chase, M. S., Gubler, C. J., Cartwright, G. E., and Wintrobe, M. M., in press.

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Serum Lipids Studied by Electrophoresis on Paper. (19752)

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Several lines of investigation have indicated the lipids of human serum and plasma to be associated with proteins. Analysis of electrophoretically separated protein fractions of serum has revealed the presence of lipid in all(1), the highest concentration being in the β-globulin fraction(1-4). Nearly all the plasma cholesterol and phospholipid may be recovered in Cohn fractions III_0, IV, V, and VI(5) and in pooled plasma an α and a β lipoprotein have been described, the latter containing 75% of the total plasma cholesterol(6). A large number of lipoprotein classes has been disclosed by ultracentrifugation of plasma(7).

Microelectrophoresis of proteins on filter paper(8) has proved to be a useful method for the separation and study of serum proteins(8-11). In the present study histochemical and other procedures applicable to the detection and characterization of lipids in tissue sections have been applied to paper strips on which electrophoresis of human serum samples had been performed and the lipid and protein patterns were compared. A brief communication describing a similar approach to the study of blood lipoproteins has recently been published(12).

Methods. The procedure used for electrophoresis was, with minor modifications, that described by Durrum(8). The sera studied were obtained from 74 individuals and included both normal subjects and hospitalized patients suffering from various disorders. The serum sample (0.1-0.2 cc) was applied transversely as a band approximately 1 cm wide across a 42 x 11 cm piece of Whatman 3 MM paper and the paper adjacent to the band was then quickly moistened with sodium barbital buffer (0.025 M, pH 8.6). The paper was then placed in a lucite-covered glass aquarium so that the middle of the paper rested on a silicone-coated glass rod traversing the aquarium 20 cm above its floor and the ends (6 cm apart) dipped into buffer-containing electrode vessels in which were also immersed platinum electrodes connected, via mercury-filled glass tubes, to lead wires from the power supply. The paper on either side of the serum line was then drenched thoroughly with buffer and voltage applied. The line of application of the serum was generally 4.5 cm from the apex on the cathode side; in this position the γ globulins moved very little, their migration toward the anode being very nearly balanced by the electro-osmotic flow of liquid toward the cathode. If the line of origin was more toward the cathode there was a greater rate of movement of all bands toward the anode but without greater resolution, while if the placement were more toward the anode the components of least mobility moved toward the cathode. Three hundred volts, providing a current of 0.4 milliampere per cm of width, applied for 5 hours moved the front of the fastest band approximately 15 cm from the origin. The paper was then air-dried in a hood, heated for 10 minutes in an oven at 100°C, and, after removal of the outer 0.5 cm on each side, was cut into parallel longitudinal strips 1-2 cm wide to be stained separately. Proteins were stained according to Durrum's method(8) by immersion of a strip in alcoholic bromphenol blue solution saturated with mercuric chloride and subsequent washing in water. For demonstrating lipids another paper strip was immersed in a saturated solution of Sudan IV in 40% ethanol, 50% glycerol

and 10% water; this was prepared (except for the use of Sudan IV instead of III) according to the method of Rossman(13), by mixing equal volumes of glycerol and a saturated solution of the dye in 80% ethanol, and filtering. Papers were allowed to remain in this solution for approximately 16 hours at room temperature, and then were washed thoroughly in water and dried. The areas containing lipid were stained red, standing out against a faintly pink background; the latter was probably not caused by lipid originally in the paper since prolonged extraction of blank paper with petroleum ether prior to staining decreased it only slightly. The Sudan stain described gave better results than Sudan IV solution in 70% ethanol, 35% ethanol-50% acetone, and Sudan II in isopropanol (60%). Exposure of paper to osmium tetroxide vapors was also unsatisfactory as a method for fat demonstration, the method lacking sensitivity and specificity. The Schultz test(14), embodying the Lieberman-Burchard reaction, was modified for use under these conditions, as a means for demonstrating cholesterol in the paper. A paper strip was soaked in aqueous 20% ferric chloride solution for 15 minutes(15), washed thoroughly in water, rinsed in 2 N hydrochloric acid, and again in water, and dried in the oven. The paper was then laid on a glass plate over a white background, a solution of 1:1 glacial acetic-concentrated sulfuric acid applied with a pipette, and another glass plate was quickly superimposed; the presence of cholesterol was indicated by a greenish or black color developing after 10-20 minutes, well before the paper began to disintegrate in the strongly acid reagent (in about one hour). Pretreatment of the strip with the ferric chloride solution seemed to give better results than immersion in aqueous hydrogen peroxide or iodic acid. A spray of antimony pentachloride in chloroform(16) was also used as a cholesterol reagent in some instances, but the instability and non-specificity of this reagent made it less useful.

The relative quantities of protein and of sudanophilic lipid in different zones of the electrophoretograms were estimated by a densitometric procedure. The papers stained with bromphenol blue and Sudan IV, after drying, were rendered more translucent by applying Krylon Clarifier* with a cloth to one surface of the paper, and then a plastic coating was applied to both surfaces (Krylon Plastic Spray).* The strip thus prepared was passed through the horizontal slit in front of the photocell at the bottom of the cuvette well of the Coleman Junior Spectrophotometer. A metal plate contiguous with the paper and interposed between it and the light source, and provided with a 0.5 cm square aperture permitted the light beam to pass through an equal area of the paper strip, which was moved manually, in 0.5 cm steps, in a vertical direction, the optical density at each point being recorded. For the bromphenol blue papers a wave length of 630 μ was used, and for the Sudan-stained papers 540 μ. The photocell sensitivity was adequate at 630 μ to permit blank setting at O.D. of 0, but at 540 the zero setting was 0.200 or 0.300.

Results. The electrophoretic protein patterns revealed 4 bands which, judged both by comparison with published figures of paper electrophoretic protein patterns(8,10,11) and by relative mobility and apparent quantity, corresponded in order of increasing mobility to the γ, β, α_2 globulins and albumin of free electrophoresis. A discrete band corresponding to α_1 globulin was not observed. It is not clear whether this was attributable to poor resolution or to a failure to demonstrate small quantities of this protein by the dye technic used. Evidence in favor of the latter possibility was provided by radioautographs of paper strips on which electrophoresis had been performed of serum from a hyperthyroid patient who had received a therapeutic dose of I^{131} several days earlier and of normal serum to which labelled thyroxine had been added; in both instances the main radioactive zone was found between the α_2 globulin and albumin, confirming the observation of Gordon and associates(17), who identified this area as corresponding to α_1 globulin. On the other hand, the mobility on paper of a specimen of α_1 glycoprotein(18)† was the same as that of

* Manufactured by Krylon, Inc., 2601 N. Broad St., Philadelphia, Pa.

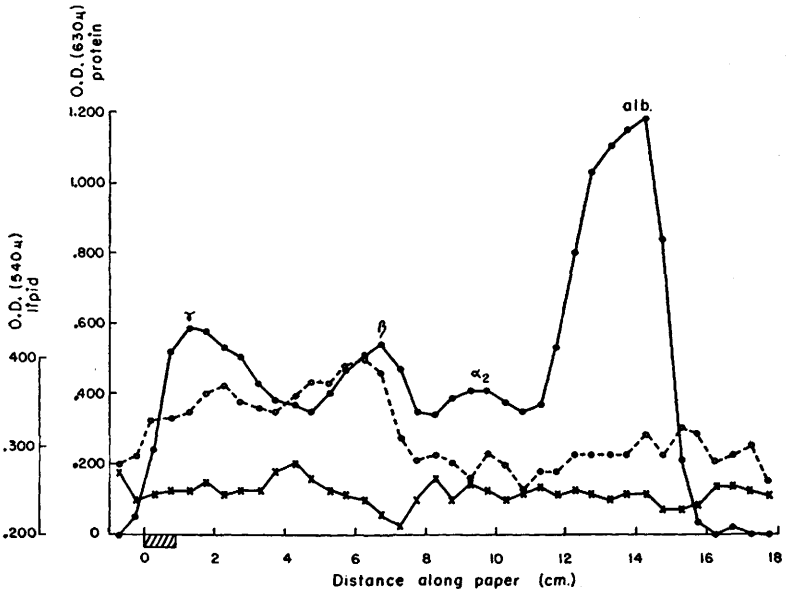

FIG. 1. Electrophoretic pattern of serum of fasting 30-year-old female subject. Optical density is plotted against distance along the paper. The cross-hatched area represents the line of application of the serum. In this and the other figures, solid circles, unbroken line represent protein (bromphenol blue); open circles, broken line, lipid (Sudan). The curve ×——× is the Sudan pattern of a parallel strip of paper extracted with alcohol-ether (3:1) after electrophoresis and prior to staining, and shows nearly complete removal of lipid.

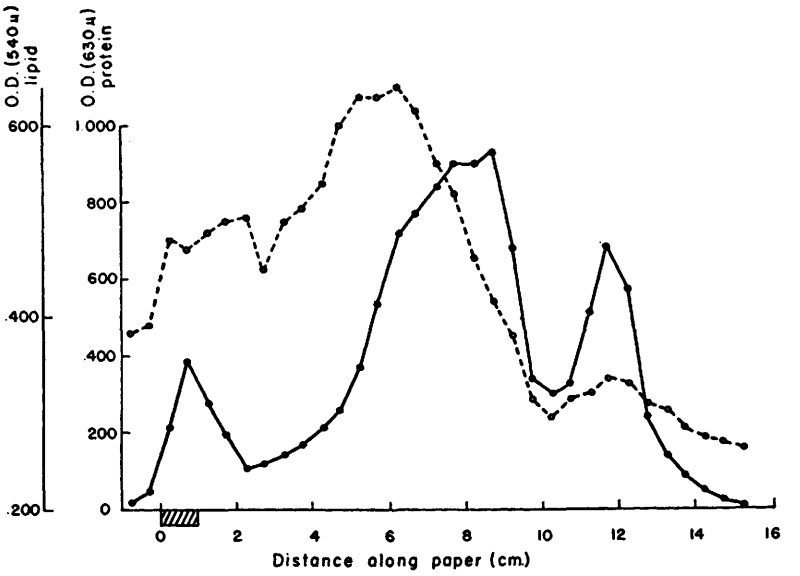

FIG. 2. Electrophoretic pattern of serum from patient with nephrosis and hypercholesterolemia. The figure illustrates the striking decrease in the albumin and γ globulin peaks and the large, fused α and β globulin zone, as well as the great increase in lipid as compared with the serum of Fig. 1.

albumin, and a mixture of these 2 proteins was not resolved, nor did addition of the glycoprotein to serum cause an increased quantity of dye to be retained between α_2 globulin and albumin.

† A specimen was generously supplied by Dr. D. M. Surgenor, Harvard Medical School.

The papers stained with the Sudan dye generally presented 2 distinct red zones, the more prominent corresponding to the β-globulin region and the less intense area to the albumin (Fig. 1). A faint pink trail from the line of application of the serum to the major peak was frequently noted. In some subjects with alimentary lipemia a distinct lipid band was present at the origin; this was not associated with the γ globulins which occupied nearly the same position, since placing the serum sample closer to the anode resulted in migration of the γ globulins toward the cathode while the lipid zone remained at the line of application. This would suggest that this lipid was unassociated with protein. In most serum specimens studied the peak of the main sudanophilic zone was at or close behind that of the β-globulin peak; in 5 subjects the pattern was different, the major Sudan-staining zone extending well beyond the β-globulin and almost reaching the α_2 globulin region.

The Schultz reaction on the papers paralleled, in general, the Sudan-stained papers, the major cholesterol band being in the β-globulin region, with a band occasionally visible at the line of application, and a light trail to the β-globulin zone. The intensity of the darkening produced by the acetic acid-sulfuric acid mixture paralleled the concentration of cholesterol in the serum, very dense black bands being noted in 3 nephrotic sera. In some instances a rather faint darkening in the Schultz test was observed at the albumin area corresponding to the more rapidly moving of the 2 sudanophilic zones, but in many cases it was difficult to be certain of a characteristic color change because of the persistence, even after washing, of a faint brownish color in this region after treatment with ferric chloride. It appeared that a greater proportion of the Schultz-reacting material on the electrophoretogram was in the β-globulin zone, as compared with Sudan-staining material.

No satisfactory histochemical procedure for the direct demonstration of phospholipids was devised. The Baker-Smith-Dietrich method (19) applied to the paper strips stained all 4 protein zones in proportion to the amount of protein in these areas. An indication of the position of phospholipid on the papers was obtained from the electrophoretogram of serum from a patient who had received a therapeutic dose of P^{32} 3 weeks previously.‡ Radioautographs were made of (a) the original strip, (b) a parallel strip after soaking in 5% trichloroacetic acid, and (c) a strip extracted with alcohol:ether (3:1). Radioactivity was present at the β-globulin and albumin areas and at the origin of (a) and of (b), but not (c). This result suggested the presence in these areas of phosphorus-containing substances precipitable with trichloroacetic acid and soluble in fat solvents. Experiments on other sera confirmed this finding. When, prior to Sudan staining, strips were boiled under a reflux for 2 hours in acetone saturated with magnesium chloride, in which phospholipids are relatively insoluble, the intensity of the stain in the β-globulin zone was considerably reduced as compared with unextracted strips, while there was little or no decrease in the sudanophilic lipid at the albumin region. Extraction of the strip with 3:1 alcohol:ether removed virtually all Sudan-staining lipid from both zones, and produced little change in the protein pattern. These findings, together with those in the Schultz test, suggest that there is relatively more cholesterol and less phospholipid in the β-globulin area as compared with the albumin zone, in agreement with chemically determined cholesterol-phospholipid ratios in the β and α lipoproteins (5,20).

The administration of small quantities of heparin is known to induce clearing of the plasma in alimentary lipemia (21) and marked changes in the ultracentrifugal lipoprotein patterns (22). It was of interest to observe that heparin also produced a change in the location and character of the lipid zones of the electrophoretogram, as shown by both the Sudan stain and Schultz test. The major lipid band presented a more diffuse appearance and extended further in the anodal direction, so that its peak, which in the pre-heparin specimen had been at, or slightly behind, the β-globulin peak, was nearly at the α_2 globulin. A similar shift toward the anode was sometimes seen in the smaller lipid band associated

‡ This serum specimen was kindly provided by Dr. J. F. Ross, Boston University School of Medicine.

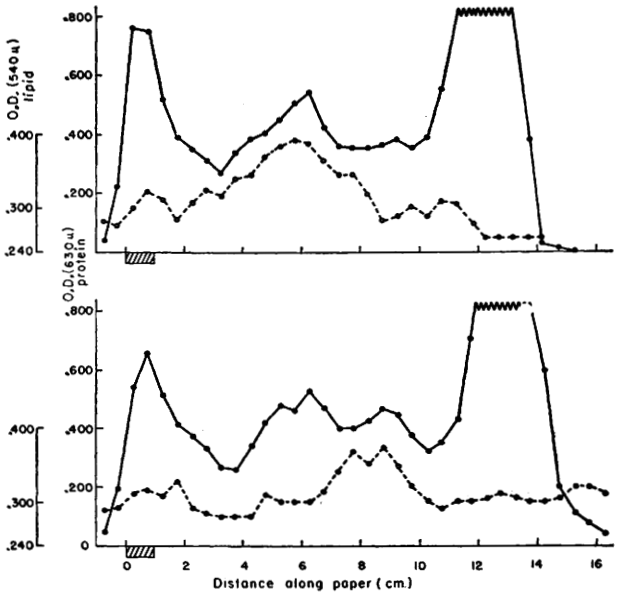

FIG. 3. Electrophoretic patterns of protein and sudanophilic lipid in serum of a male adult before (upper figure), and 35 minutes after (lower figure) the intravenous injection of 8 mg sodium heparin. The subject had ingested 250 cc of heavy cream 3 hr prior to the time the first blood sample was drawn.

with the albumin. The effect was apparent within 15-30 minutes after the intravenous administration of 8 mg sodium heparin, and was observed in the serum of 6 out of 8 fasting subjects and of all 6 of those with alimentary lipemia (Fig. 3). The addition of sodium heparin to serum *in vitro* to a final concentration of 0.1-0.2 mg/cc resulted in changes of a similar nature in the electrophoretic lipid pattern in 6 of the 7 sera so treated, although no clearing of lipemia occurred under these circumstances. In contrast to the marked effect of heparin on the lipid pattern, little change was noted in the protein pattern as demonstrated by bromphenol blue.

Summary. Serum was subjected to electrophoresis on filter paper and the distribution of lipid studied by application to the paper strips of Sudan stains, the Schultz test, and other histochemical procedures. Two principal lipid bands, probably corresponding to β and α lipoproteins, were recognized, the larger being in the β-globulin region, and the smaller in the albumin zone. In some cases lipid apparently associated with little protein was also found at the point of application of the serum. The administration of heparin to human subjects, or its addition to serum, rapidly produced a change in the distribution of sudanophilic and Schultz-reacting lipid.

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1. Blix, G., Tiselius, A., and Svensson, H., *J. Biol. Chem.*, 1941, v137, 485.

2. Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., *J. Exp. Med.*, 1945, v82, 411.

3. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, v70, 399.

4. Sterling, K., and Ricketts, W. E., *J. Clin. Invest.*, 1949, v28, 1469.

5. Russ, E. M., Eder, H. A., and Barr, D. P., *Am. J. Med.*, 1951, v11, 468.

6. Oncley, J. L., Gurd, F. R. N., and Melin, M., *J. Am. Chem. Soc.*, 1950, v72, 458.

7. Jones, H. B., Gofman, J. W., Lindgren, F. T., Lyon, T. P., Graham, D. M., Strisower, B., and Nichols, A. V., *Am. J. Med.*, 1951, v11, 358.

8. Durrum, E. L., *J. Am. Chem. Soc.*, 1950, v72, 2943.

9. Kunkel, H. G., and Tiselius, A., *J. Gen. Physiol.*, 1951, v35, 89.

10. Esser, H., Heinzler, F., Kazmeier, F., and

Schalton, W., *Arztliche Forsch.,* 1952, v4, 156.

11. Flynn, F. V., and DeMayo, P., *Lancet,* 1951, v261, 235.

12. Fasoli, A., *Lancet,* 1952, v262, 107.

13. Rossman, I., *Am. J. Anat.,* 1941, v69, 187.

14. Lee, B., *The Microtomist's Vade-Mecum,* 11th Edition, Blakiston Co., Philadelphia, 1950, p. 294.

15. Everett, J. W., *Am. J. Anat.,* 1945, v77, 293.

16. McMahon, J. M., Davis, R. B., and Kalnitzky, G., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 799.

17. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R., *Nature,* 1952, v169, 19.

18. Schmid, K., *J. Am. Chem. Soc.,* 1950, v72, 2816.

19. Baker, J. R., *Q. J. Micr. Sci.,* 1945, v85, 1.

20. Kunkel, H. G., and Slater, R. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 42.

21. Hahn, P. F., *Science,* 1943, v98, 19.

22. Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J., and White, S., *Circulation,* 1951, v4, 666.

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Localization of Proteolytic Activity in Muscle Protein Fractions. (19753)

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In the past most of the studies on muscle have been done in an attempt to learn more about the enzymes involved in the important carbohydrate energy cycle. Consequently, little is known about the proteolytic enzymes in muscle.

The following investigation was undertaken in an attempt to identify some of the proteolytic enzymes and localize them in muscle protein fractions.

Materials and methods. Muscles from the legs of 10 specimens of frog, *Rana pipiens,* were removed and placed in a mixture of ice and water (0 to 2°C) to inhibit bacterial growth and autodigestion. As much connective tissue and as many blood vessels as possible were excluded.

These muscles were then used as a source of muscle protein fractions. Myosin was extracted according to the method of Greenstein and Edsall(1). The procedure of Baranowski(2) was adapted for the extraction of myogen. The procedures outlined by Bate-Smith(3) and Meyer and Weber(4) yielded protein fractions rich in myoalbumin and globulin x, respectively.

A modified method of Anson(5) was employed to obtain some quantitative data concerning the proteolytic enzymes pepsin, trypsin, and cathepsin. Because absolute values could not be ascertained, the amount of proteolytic digestion by increasing quantities of muscle protein during a 10 minute period, was compared with that by standard solutions of pepsin, trypsin, and tyrosine. Standard tyrosine solutions were prepared for comparison with cathepsin because cathepsin is not available commercially.

Results. Very few, small crystals of myogen appeared. They were too small and few in number to separate from the myoalbumin. For that reason and because myogen cannot be extracted from myoalbumin in the native state(3), the myogen and myoalbumin fractions were analyzed together. When preliminary investigations revealed that no proteolytic activity was present in the globulin x fraction, analyses were made only on myosin and myosin-free fractions.

Cathepsin and pepsin were localized in the myosin fraction and trypsin in the myosin-free fraction. No enzyme appeared in both the myosin and myosin-free fractions.

Fig. 1 shows curves resulting from digestion of hemoglobin by increasing quantities of muscle protein fractions and of standard enzyme solutions during a 10 minute period. The curves of digestion by muscle proteins are almost parallel to each other.

Maximum digestion by trypsin in the myosin-free fraction occurred after 6 cc of myosin-

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