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A STUDY OF THE AMINO ACID COMPOSITION OF SOME OF THE SERUM LIPOPROTEINS INVOLVED IN THE DEVELOPMENT OF ATHEROSCLEROSIS

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May 29, 1952

Berkeley, California

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ABSTRACT

Gofman and co-workers have presented evidence for the existence of a series or "spectrum" of human serum lipoproteins and have shown a definite correlation between certain of these lipoproteins and the development of atherosclerosis. An obvious way to extend our understanding of lipid metabolism and atherogenicity was to study the protein moieties of serum lipoproteins.

This investigation was concerned with the development of analytical procedures adequate for the characterization of these protein moieties and with their preliminary application. It was felt that, once the analytical procedures had been developed, valuable information might eventually be secured regarding the nature of these newly isolated lipoproteins and consequently the relationship of the protein moieties of this series (spectrum) of lipoproteins to each other and to the development of atherosclerosis.

The significant achievements of this investigation may be listed as follows: (1) Development of a satisfactory small-scale procedure

for denaturing and delipidizing homologous sets of serum lipoproteins. (2) Development of a technique for the chromatography of whole native lipoproteins and other proteins. (3) Development of a new quantitative ultramicro procedure for the determination of protein after hydrolysis. (4) Development of satisfactory qualitative, semi-quantitative and quantitative chromatographic techniques for analyzing the protein hydrolysates of homologous sets of serum lipoproteins. (5) Preliminary application of these techniques to the analysis of a limited number of homologous sets of human serum lipoproteins.

A STUDY OF THE AMINO ACID COMPOSITION OF SOME OF THE SERUM LIPOPROTEINS INVOLVED IN THE DEVELOPMENT OF ATHEROSCLEROSIS

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STATEMENT OF THE RESEARCH PROBLEM

Gofman, et al.[1,2,3] have presented evidence for the existence of a series or "spectrum" of human serum lipoproteins and have shown a definite correlation between certain of these lipoproteins and the development of atherosclerosis.[4,5,6] For a better knowledge and understanding of lipid metabolism and atherogenicity, it seemed desirable to study the protein moieties of these lipoproteins.

This investigation was concerned with the development of analytical procedures adequate for the characterization of these protein moieties and with their preliminary application. It was felt that, once the analytical procedure had been developed, valuable information might eventually be secured regarding the nature of these newly isolated lipoproteins and consequently the relationship of the protein moieties of this series (spectrum) of lipoproteins to each other and to the development of atherosclerosis.

CHOICE OF EXPERIMENTAL APPROACH

Considerable study was devoted to the choice of the most desirable experimental approach for the adequate characterization of protein moieties of various lipoproteins. The difficulties here lay principally in the fact that the analytical procedure must be capable of characterizing proteins available in amounts as small as 0.1 milligram; that is, the procedure must be sensitive enough to handle the series (spectrum) of lipoproteins isolated from a single donor (homologous sets of components).

In general, proteins can be characterized by studies on the intact protein and/or on the hydrolyzed protein. The application of physical or physico-chemical* methods to the study of the intact protein did not seem too encouraging especially in view of repeated failure to prepare a delipidized protein without drastically altering the physical or physico-chemical properties of the "native" protein. The application of purely analytical chemical techniques to the characterization of the intact delipidized proteins seemed more encouraging; but such analyses were of limited scope and of dubious accuracy;** and are used with confidence only for proteins which have been subjected to previous study of their corresponding hydrolysates.

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* For example, infra-red spectrophotometry, viscometry, osmometry, and electrophoresis.

** More recent work may force eventual reconsideration of studies on the intact protein (cf. especially the unpublished studies on determination of arginine in the intact protein molecule, by Singer[7]).

Characterization of new proteins by study of their hydrolysates seemed to be more promising than studies on the intact protein. Except for those functional groups chemically unavailable in the intact molecule, chemical analyses on the total hyrolysate offered little advantage over those on the intact protein; but biological methods, particularly microbiological assay, suggested real possibilities for characterizing these protein hyrolysates. Microbiological assay methods,[8] however, are best adapted for routine analyses of well-defined proteins; they could not be used with confidence unless supported or corroborated by detailed chemical study of the composition of these new protein hydrolysates. For the limitations imposed by this investigation, there seemed to be no alternative but to subject the protein hydrolysates to some satisfactory isolative procedure and to characterization of the isolated compounds.

Furthermore, another experimental requirement became obvious during the preliminary study of the first protein hydrolysates: the necessity for an ultramicro procedure for the direct determination of the amount of protein (or some property directly related to the protein) present as contrasted with the micro-Kjeldahl procedure by which the final protein-nitrogen was determined <u>after</u> substraction of the lipid-nitrogen. It was hoped that such a protein assay method might be adapted from the method(s) used to determine the amino acids isolated from those same protein hydrolysates whose protein contents are to be assayed prior to extensive study.

BRIEF DESCRIPTION OF THE EXPERIMENTAL PROCEDURE

It is desired to present here a brief description of the overall experimental procedure as it developed during the course of this investigation -- an outline that might serve as a rough guide for the reader while he is going through the mass of experimental details with which this investigation was of necessity concerned.

The various lipoprotein components are prepared by preparative and differential ultracentrifugation in this laboratory by Frank T. Lindgren.

The samples are aliquoted immediately after preparation and stored in previously calibrated centrifuge-hydrolysis tubes at - 1° C.

Denaturation of the lipoprotein is effected by alternate heating (80° C) and chilling (-20° C) in the presence of 75-80 percent ethanol.

Delipidization of the lipoprotein is effected by a series of simple batch extractions using 75 percent ethanol and then absolute ethanol.

Hydrolysis of the delipidized protein is effected by heating with highly purified constant-boiling hydrochloric acid on a steam bath for twenty-four hours. Hydrolysates are stored at -20° C. when not being used. It is to be noted that all manipulations of the lipoprotein are effected in the original centrifuge-hydrolysis tubes in order to avoid material transfer losses.

The protein concentration of each hydrolysate is determined by the use of the quantitative Moore-Stein ninhydrin reagent and a suitable "protein equivalent" calibration curve which has been prepared from an *ad hoc* mixture of amino acids simulating a *gamma*-or *beta*-globulin hydrolysate.

The hydrolysates are studied qualitatively by small-and large-scale two-dimensional paper chromatography. The amino acids are tentatively identified by their relative positions. They are conclusively identified by repeated chromatography with any of the compounds that are suspected of being identical; if further proof is indicated, spot tests for specific functional groups and other standard organic qualitative ultramicro tests are performed as needed.

The hydrolysates are studied semi-quantitatively by simple visual comparison of small-scale (8 in. x 8 in.) two-dimensional paper chromatograms run in triplicate at the 20 and 100 microgram protein levels, or more accurately by the marker-dilution technique in which those spots containing known amounts of amino acids added to smaller volumes of hydrolysate are compared to those spots derived from larger volumes of hydrolysate containing no added amino acids.

Finally, the protein hydrolysates may be studies quantitatively. The hydrolysates are subjected to large-scale two-dimensional paper chromatography; the amino acids are located by heating at 100-150° C. for fifteen minutes and observing the fluorescence under an ultraviolet light.

The individual amino acid spots are cut out and the amino acid removed by elution chromatography. The eluates are evaporated to dryness, and the amino acid determined by means of the quantitative Moore-Stein ninhydrin reagent and the standard curve for the corresponding amino acid.

PRELIMINARY EXPERIMENTAL STUDIES

Study of the Whole Lipoprotein.

It was hoped that the whole lipoprotein (except for the removal of any free amino acids) might be hydrolyzed and the hydrolysate chromatographed without further treatment.

The lipoproteins isolated* for this preliminary study were obtained by Frank T. Lindgren and Alexander Nichols by means of repeated differential ultracentrifugation[9] similar to that procedure used for the final quantitative study. Seven human and one rabbit sample were obtained and stored at 1° C.

In view of the extremely small quantities of protein available, it seemed desirable to design an apparatus suitable for carrying out all the steps prior to paper chromatography without any necessity for transfer of the protein samples. A regular 15 - ml graduated centrifuge tube was cut off at the 11 - ml. mark; the female (outer) part of a 19/22 - joint (standard taper) was cut off 1/4 inch from the end-bulge and fused to the centrifuge tube section. The final tube was made short enough to fit into a regular clinical centrifuge trunion and head. The male (inner) part of the 19/22 joint was used to make

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* The general isolative procedure used for the lipoproteins in this investigation was as follows: The lipoproteins were first removed from the other serum proteins including the high density (alpha) lipoproteins by a preparative ultracentrifuge (Spinco Model L). The lipoproteins were further fractionated into their particular components and purified by sucessive differential ultracentrifugation till the desired "homogeneity" was obtained--the fractionation and purification processes being followed by the analytical ultracentrifuge (Spinco Model E).

a condenser for the "centrifuge-hydrolysis" tube. The top of the male joint was cut off at 4.5 cm from the ground glass section; a piece of 12 mm (O.D.) tubing was fused to the bottom of the ground glass section and sealed off leaving a small but pointed tip. This section, equipped with two pieces of 4 mm (O.D.) glass tubing, 5 inch and 6 inch long, inserted in a two-holed #00 or #0 rubber stopper would serve as a condenser for the centrifuge-hydrolysis tube during the hydrolytic procedure. The entire assembly is pictured in Fig. 1. An ordinary steam bath was used for the hydrolysis; the tubes were placed in a 5 - hole heavy brass plate (Fig. 2) which rested on top of the steam bath.

The following simple procedure was outlined for the first lipoproteins: The sample was to be placed in a centrifuge-hydrolysis tube, and absolute ethyl alcohol added until the liquid was 75 percent ethyl alcohol by volume. The tube was heated to complete the precipitation (denaturation-coagulation). The precipitate washed with aliquots of 75 percent ethyl alcohol until washings (extracts) shown to be free of amino acids by the "ninhydrin-paper" test. Hydrochloric acid (6 N) was added to the air-dried precipitate and hydrolysis effected by heating on a steam bath. The progress of the hydrolysis was to be followed by paper-chromatographing small aliquots of the hydrolysate removed at various time intervals.

The first sample studied was a 5 S_f* lipoprotein containing

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* One S_f unit represents a flotation rate of 1×10^{-13} cm/sec/dyne/gm in a sodium chloride solution of density 1.063 gm/cc at 26° C.

10 mg component and about 2 mg protein in 0.7 cc. A 0.50 cc sample was pipetted into a centrifuge-hydrolysis tube. Absolute ethyl alcohol was added dropwise until the final volume was 2.0 cc. A yellowish flocoulent (voluminious) coagulum formed after a few tenths of a milliliter of alcohol had been added. By the time the rest of the alcohol had been added the coagulum had settled down as large particles. The liquid was stirred with a glass rod. The tube was sealed with a piece of "Parafilm" and centrifuged for 5 minutes at 3000 rpm. the supernatant was clear; a yellow, resinous, compact precipitate was at the bottom. The supernatant was checked for the presence of free amino acids by placing an aliquot on a piece of filter paper (Whatman #1), air-drying, spraying with a ninhydrin-butanol solution (0.25 percent ninhydrin), and heating at 80° C. in an air-oven. The resulting color was compared with valine standards prepared simultaneously in the same way. The supernatant gave a color reaction equivalent to 0.5 mg valine per ml. Removed supernatant without stirring; added 0.5 percent cc 75 percent ethyl alcohol. The ninhydrin-paper test indicated about 0.1 mg/ml or less. This wash was removed by careful pipetting*. Hydrocholoric acid (6 N) 1.0 cc, was added to the moist lipoprotein mass; the tube was heated on a steam bath for 24 hours. Samples (200 microliters) were removed at end of 10, 22, and 24 hours for chromatographic assay.

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* It will be noted that no effort was made to check completeness of precipitation nor to remove any more lipid than might have been removed by this mild procedure.

At end of the 10 hour period a small drop of light brown oily material was floating on the surface of the hydrolyzing liquid; this droplet remained without apparent change at the end of the hydrolysis. The centrifuge-hydrolysis tube was very satisfactory; no appreciable loss of hydrochloric acid occurred. Aliquots of the hydrolysate at the various stages indicated were studied by the ninhydrin-paper spot test and showed that in so far as total amino acid color was concerned, the hydrolysis may be almost as complete after 10 hours as after 24 hours; paper chromatography of these 10-hour aliquots , indicated that appreciable quantities of ninhydrin-reacting material remained at the point of application of the sample to the paper. These preliminary chromatrograms presented a rather confusing picture probably due to the presence of a large brown spot where several of the amino acids and/or peptides should have been (R_f 0.2 - 0.3). Further chromatographic study of the final hydrolysate indicated that this brown spot was tying up several of the amino acids; distinct fluorescing spots corresponding to only 5 amino acids were obtained on extensive heating (105°C) of the papers in addition to the brown spot which had given an intense diffuse brownish-blue flourescence with ultraviolet light and a reddish-blue background with ninhydrin. There seemed to be no alternative but to remove the interfering brownish* material. Two possibilities for the removal of the interfering material were briefly studied:

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* No special attempt was made to study the nature of this interference beyond the attempts made to remove it as discussed above.

1. Removal of the material from the final acid hydrolysate by extraction or some other means, and

2. A pre-hydrolytic extraction of the coagulated lipoprotein. Removal of the interference from the final acid hydrolysate was attempted first. The hydrolysate was extracted with three times its volume of diethyl ether by vigorous prolonged shaking. The two phases were chromatographed (one-dimensional paper) the diethyl ether fraction contained none of the interfering brownish material; the aqueous fraction gave essentially the same chromatogram as it did prior to extraction. Surprisingly enough, chromatography of the original 75 percent ethyl alcohol supernatant obtained from the original precipitation of the lipoprotein (removal of free amino acids) showed appreciable amounts of this brownish interference. It was rather obvious that the better approach would be the removal of the brownish interference during the pre-hydrolytic treatment of the lipoprotein.

It seemed reasonable to assume that the brown interference was a more or less complex mixture of lipid and lipid-like materials derived from the precipitation and subsequent hydrolysis of the lipoprotein. The behavior of this substance was reminiscent of some unpublished studies[10,11] with lecithin and other lipids such as cholesterol and triglycerides -- especially those lecithin-containing mixtures that were old (paritally oxidized). In view of these observations, it seemed best to repeat this preliminary study using a 75 percent ethyl alcohol solution. to extract the free amino acids and any of the lipid that

might accompany this alcohol-water solvent, and then absolute ethyl alcohol to remove as much of the interfering lipid as possible.

A $7S_f$ lipoprotein was selected for the study of the removal of the brownish lipid interference. A 0.05 cc sample (approximately 5 mg. lipoprotein) was placed in a centrifuge-hydrolysis tube. Absolute ethyl alcohol was added dropwise; precipitation began when 0.1 ml had been added. The addition of the alcohol continued until the final volume was 2.0 ml. The mixture was stirred and centrifuged. The supernatant was removed and the extraction repeated with 1.5 ml of 75 percent ethyl alcohol. Both supernatants were found to be low in amino acid content (ninhydrin paper test). The residue was dried <u>in vacuo</u> at room temperature. Absolute ethanol, 2.5 ml, was added to the yellow, plastic mass. The mixture was vigorously stirred for several minutes and left standing for 30 minutes: no noticeable dissolution or other change was apparent. It was thought that heating on a steam bath might accelerate any changes that might be occurring. Accordingly, the centrifuge-digestion tube containing this sample was heated 70 - 75°C for 2 hours. The only change seemed to be that of some darkening of the yellow mass. The mixture was contrifuged, the supernatant removed and the lipoprotein dried <u>in vacuo</u> as before.

Obviously, the bulk of the lipid remained attached in some manner or other, to the protein. It seemed interesting to try a more effective fat solvent such as a chlorinated hydrocarbon for removal of the lipid. Chloroform seemed a good choice; so 3 ml were added to the

brownish mass and refluxed for 30 minutes on the steam bath. An appreciable fraction of the brownish solid seemed to go into solution. Suspension was centrifuged for 5 minutes, the solid particles floated to the top and had to be separated by pouring the entire suspension onto a pad of filter papers (S and S Analytical, #576, 18 1/2 cm). The paper was air-dried and the particles returned to their original centrifuge-hydrolysis tube. The partially delipidized protein was hydrolyzed by adding 1.0 cc 6 N HCl and heating on the steam bath for 24 hours. The hydrolysate looked quite clear and light brown with small specks of dark brown particles at the bottom; the particles constituted about 10 percent (by volume) of the pre-hydrolytic lipid-protein. The hydrolysate was then checked by one and two-dimensional paper chromatography. The "brown" interference had disappeared to the extent that it was no longer visible except by its fluorescence with an ultra-violet light source; nevertheless enough of the interference remained so that several amino acid spots merged together. It was obvious that even more of this interfering material would have to be removed.

These preliminary runs were repeated with the same results. In view of the failure to eliminate the interference material by these preliminary trials, it was thought worthwhile to pause and study the whold lipoprotein itself in the hope of securing some clues as to the nature of this strange, unstable lipoprotein and some better techniques for handling it before and after hydrolysis.

Chromatography of the Native Lipoprotein.

Among the many approaches that occurred to this investigator at this stage was that of devising some better micro-procedure for the removal of the lipid before the liproprotein complex underwent extensive modification as was obviously the case from the above preliminary experiments.

A considerable amount of work had been done (1948-1949) in this laboratory in an attempt to chromatograph whole native proteins. Egg albumin, whole serum, hemoglobin, trypsin, thyroglobulin and other proteins were studied principally on filter paper in aqueous media. Moderate success had been achieved in separation of various mixtures in one-dimensional chromatograms but the work was limited by the lack of sufficiently sensitive specific reagents for the detection of the micro amounts of the proteins on paper and by a lack of knowledge and skill in preparing and handling the easily denatured proteins. This work was never published, but along with the recent work of other investigators[12,13,14,15,16] it confirmed the feeling that successful chromatography or adsorption analysis of many if not most of the proteins would be a logical development of the next decade.

The unusual properties and the availability of these new and rather unstable lipoportein components seemed to indicate that paper chromatography might yield some interesting information regarding not only the lipoprotein components themselves but also some ideas as to improving the technique for separating proteins by chromatography.

Fortunately Drs. Richard Lemmon and William Stepka of Prof. Melvin Calvin's photosynthetic group, having had considerable success in detecting a few micrograms of tyrosine and other compounds containing diazotizable groups by means of the modified Pauly[17] test, suggested that the Pauly test might effectively be applied to the detection of small amounts of protein on paper.

Pauly Test: The following solutions are made up.

Soln. 1. 1% aq. sulfanilic acid plus 8 cc conc. Hcl for each 100 cc of soln.

Soln. 2. 0.69% of $NaNO_2$

Soln. 3. 10% soln. of Na_2CO_3.

Mix equal volumes of solns. 1 and 2. Make up fresh every day.* Then spray on paper chromatogram using a fine spray (cf. Fig. 3) and avoiding a great excess of reagent. Dry** at room temperature with a fan; then spray with solution 3***.

The Pauly test was checked with egg albumin; 50 micrograms per sq. cm of Whatman #1 filter paper seemed to be about the lower

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* It was found that this initial spray reagent was stable for at least 3 or 4 days if stored in a refrigerator at 0° degrees C.

** Drying was necessary for obtaining uniform application of the Na_2CO_3 solution and for preventing the reagents from running all over the paper.

*** Use a gas mask (full-face type covering the eyes) for all spray operations.

limit of this procedure. It appeared, however, that the test might be made more sensitive thus eliminating the necessity for overloading the paper chromatogram (protein overloading decreases greatly any possible sharp resolution of the different proteins). It was soon found that it was difficult to secure complete reaction by a single spraying of each solution without greatly over-wetting the paper, making the paper slmost impossible to handle. Te effect complete reaction, the sulfanilic-nitrite solution was sprayed on three times with drying at room temperature after each application; this was followed by spraying the paper with carbonate solution three times and again drying after each application. After this careful spraying, the papers usually had a yellow and/or orange background that sometimes obscured the color of the diazotized protein; this color background could be completely eliminated by dipping the papers in a beaker of cold running tap water to remove most of the color and then hanging for about 5 min. in a cylinder of distilled water. The paper was then dried in an air stream at room temperature. It was usually sufficient to examine the developed papers in visible light; if, however, there was any doubt about the presence of a spot, examination over a good ultra-violet light source was usually effective in doubling or tripling the sensitivity. By using this multiple spray modification and ultra-violet examination it was found possible to detect 10 micrograms (per sq. cm of Whatman #1 filter paper) of several proteins. This sensitivity was thought sufficient to detect chromatographable quantities of at least some of the lipoproteins, especially those with $S_f = 6$ (~25 percent protein) and $S_f = 13$ (~12 1/2 percent protein).

The initial chromatographic experiments were performed with a series of components having S_f values of 6, 13, and higher. Aliquots of the samples were placed 10 cm from the ends of strips of Whatman #1 filter paper, 18 in. x 2 in.; the samples were air-dried and developed chromatographically in sets of four by suspending them from the cover of the top of a glass cylinder (18 in. high, 6 in. diameter) containing 250 cc. distilled water (Fig. 4). All chromatographic development was performed in a constant temperature room. The development was complete in eight hours. The strips were immediately air-dried. Examination under an ultraviolet light source revealed a faintly absorbing spot at the starting position and an intensely fluorescing round or slightly elliptical spot about halfway up the paper. The Pauly test revealed that all the protein was at the starting position; apparently the intensely fluorescing spot was either part or all of the lipid or, very improbably, some non-lipid associated with the lipid moiety of the lipoprotein.

The thoroughness of the denaturation-coagulation of the protein moiety was quite unexpected; previous studies with impure and purified globulin preparations had always revealed some movement or at least extensive tailing in the direction of the solvent. Even more surprising was the consistent, extensive movement in distilled water of the lipid material as a sharp spot.

It was soon discovered that the denaturation-coagulation of the protein moiety could be completely eliminated by preventing the sample from drying on the paper prior to inception of chromatographic

development. The procedure was modified to prevent evaporation. The sample was applied quickly and the paper strip immediately suspended in a duplicate chromatographic cylinder or chamber containing a wet sponge (Fig. 4); when four strips had been prepared, they were quickly plunged into a chamber containing distilled water and developed as before. Examination of the dried papers with an ultraviolet light revealed only one spot, a spot similar to the lipid spots obtained with the dried samples; there was practically no ultraviolet absorbing region at the starting position. The Pauly test revealed that the protein had moved as a very sharp, elliptically shaped spot near the middle of the more diffuse fluorescing lipid-like material. This movement of the protein was evidenced by all of the lipoproteins components studied with S_f values ranging from 4 to 100. In view of the large amount (about half) of lipid material overlapping and hence not directly associated with the protein, it seems reasonable to conclude that, during chromatography at least, the "native" protein is associated with only a part of the lipid. The question of whether such partial association of the protein with lipid exists in the native freshly prepared lipoprotein or whether it represents a partial dissociation induced by the mild surface forces of paper chromatography cannot be answered at this time although this work does suggest that chromatography might be an interesting technique for evaluating the nature and extent of the association of lipid and protein in the native lipoprotein. It can be concluded, however, that the lipid-protein "association"

is not as strong as had been anticipated and therefore any attempts to remove the lipid fraction should be restricted to mild extraction procedures.

The results with "dry" and "wet" samples of lipoproteins was confirmed by repeating this work with a P^{32} -labelled lipoprotein prepared in this Laboratory by Mr. Dean Graham; all of the P^{32} was organically bound and presumably associated with the lipid fraction. In case of chromatographically developed dry samples, all of the radioactivity was associated with the lipid fluorescing spot ($R_f = 0.5$); with wet samples all the radioactivity as well as all of the protein was associated with this lipid-like fluorescing spot. These P^{32} -tagged experiments increased the feeling that most if not all of the lipid was to be found in these intensely fluorescing spots of $R_f = 0.5 - 0.6$.

The lipoproteins demonstrated another chromatographic property in common with other proteins: If a paper strip is loaded with an amount of protein greater than 50 micrograms per square centimeter a phenomenon that might be characterized as "overloading" occurs in which the movement of the solvent front is slowed down and the protein R_f value is increased at the same time. Overloading with the various lipoproteins usually began when more than 200 micrograms of lipoprotein sample had been added to each square centimeter of paper at the starting position; in the 250 to 350 microgram per square centimeter range, the rate of movement of the solvent is slowed down about 30 percent and the R_f values are increased to about 0.8, the lipoprotein now

moving as a band across the full width of the paper instead of as a compact spot. Overloading the paper with dry samples, thus removing the protein, results in essentially the same phenomenon of "overloading" as given by the undenatured lipoprotein. We may have here some clue as to the nature of protein overloading: If it can be assumed that the deproteinized lipid is present in a state of colloidal or micellular aggregation similar to proteins and lipoproteins, then the phenomenon of protein overloading is characteristic not necessarily of proteins but rather of large molecules or particles. Hence, it might be expected that the phenomenon of overloading in chromatography might be expected for any colloidal substance. In the final analysis, overloading probably represents a breakdown in the chromatographic processes with the resulting movement of the substance by mere mechanical forces.

Finally, some attempt was made to separate mixtures of two or more lipoprotein components using only distilled water as the developing medium. No distinct separations were achieved. This investigation was too limited to permit of extensive speculation regarding the feasibility of such separations by chromatographic techniques; much more information would have to be secured regarding the chromatographic properties of single homogeneous lipoprotein components before any intelligent attempt could be made to separate mixtures of these components.

In conclusion it might be pointed out that these investigations suggest interesting possibilities not only for the characterization of the native lipoproteins but also for the isolation and further study of the lipid moiety as well. And, in so far as this investigation is concerned, these chromatographic studies of the whole lipoprotein suggest that the lipoproteins can be readily delipidized by comparatively mild extracting conditions without resorting to extensive pre-treatment of the lipoprotein.

Further Attempts to Remove Lipid Interference.

The work with whole native lipoprotein components in paper chromatography simply supported the feeling of this investigator that the lipid interference could best be removed by using a series of ethanol-water and ethanol extractions under mild conditions thus avioding any extensive oxidative and/or polymeric changes which would decrease greatly the solubility of the lipid moiety of the molecule.

A freshly prepared 6 S_f -lipoprotein (slightly inhomogeneous, Code #22r-10T_{o-2}), 0.5 cc containing 5 mg liproprotein, was placed in a centrifuge-hydrolysis tube. Absolute ethanol, 1.5 ml, was added with vigorous stirring. A yellowish white fibrous coagulum formed; the supernatant was removed by decantation after centrifugation. The mass was extracted three times with 2 cc. 75 percent ethanol, stirring centrifuging, and decanting each time. This was followed by three extract ons with absolute ethanol. The absolute alcohol removed

more of the lipid and all of the yellow color, leaving a whitish, gel-like mass. The gel was air-dried to give a white solid. Hydrochloric acid, 1 cc of 6 N, was added to the solid; the tube was placed on a steam bath for 24 hrs. Aliquots of the hydrolysate were chromatographed by small scale* one- and two-dimensional paper chromatography. Apparently the brownish lipid interference had been completely removed; if any remained, it was simply another ultraviolet fluorescing spot that did not interfere with any individual amino acids or their distribution pattern.

The modified procedure for delipidizing was next tried on three freshly prepared lipoprotein components that were more homogeneous than the sample studied above:

1. 41R, 2-10 T_4 - containing highly purified 6 S_f lipoprotein.
2. 37R, 9-15 T_1 - containing component with $S_f = 13$.
3. 41R-T_1 - containing components with $S_f = 17$ to 40.

When these samples were subjected to the precipitation and extraction procedure described above, hydrolyzed, and paper-chromatographed, it was found that the lipid interference had been satisfactorily eliminated. In each case, a white, gelatinous protein was obtained after the ethanol extractions. Hence, as far as this investigation was concerned, enough of the lipid had been removed to permit a rapid qualitative study of the amino acid composition of the protein moiety of any of the human

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* To be described in next Section.

serum lipoprotein components with ultracentrifugal flotation rates (S_f) from 4 to 40, and, as subsequent studies were to demonstrate, up to an S_f value of 300 and eventually from 400 and up to about 100,000 (chylomicrons).

Large-Scale Descending and Ascending Paper Chromatography

Descending Paper Chromatography One- and Two- Dimensional

Although large-scale descending paper chromatography had not as yet been employed in this investigation, it seems appropriate to describe the technique at this time: not only was large-scale paper chromatography used extensively throughout the remainder of this investigation, but the newer small-scale, ascending paper chromatography is only a slight modification of the older large-scale technique.

Following the lead of the English investigators Gordon, Consden, Martin, Synge[18], considerable effort in this Laboratory from 1947 to 1950 was expended in designing suitable equipment for developing and handling large-scale one- and two-dimensional descending paper chromatograms.

In so far as its operational principles are concerned paper chromatography is simple enough: It consiste of applying a sample of the substance to be analyzed to a small area of a piece of filter paper, the point of application being away from the edge of the paper.

The edge near the sample spot is placed in a suitable solvent trough in such a way as to hang freely. The chromatogram is "developed" by adding an appropriate solvent to the trough and then permitting it to move down the paper by capillary action until it approaches or reaches the end of the paper. The entire operation is carried out in a gas-tight chamber in order to maintain a saturated atmosphere and to prevent drastic alteration of the solvent system by evaporation from the extensive surface of the paper. At the end of development the paper chromatogram is removed from the chamber and dried at room temperatures or in an air oven. This gives a so-called "one-dimensional" chromatogram. If successfully performed, the components of the mixture will be satisfactorily separated or resolved along a straight line and the chromatogram is now ready for further study. If the resolution is inadequate, as is frequently the case for complex mixtures of the similar compounds that are usually separated by chromatographic techniques, then the one-dimensional chromatogram is repeated using a large square sheet of paper in the following manner: The sample is placed near one corner away from both edges of the paper. One edge is held in a suitable solvent trough and the first solvent is added as in case of the one-dimensional runs. When development is complete, the paper is dried; the edge next to the line of partially resolved compounds is then dipped into a clean solvent trough and as second solvent different from the first is added. The paper is developed in the "second-dimension", air- or oven-dried, and is ready

for further study. Once the spots have been located with the help of various physical and/or chemical agents, some qualitative information can be secured from 1) the distance the spot moves relative to that of the solvent front (R_f value), and 2) the relative distribution pattern of all the spots. The procurement of further qualitative and/or quantitative data will be described in the remainder of the dissertation; in fact, it has been the central technical problem of this investigation.

Two kinds of apparati were found to be effectively convenient for one-dimensional descending paper chromatography. For narrow strips (1 in. to 1 1/2 in.) it is good to use a doughnut-shaped solvent trough as the solvent reservoir to help maintain a vapor-saturated atmosphere; the entire assembly is enclosed in a large glass cylinder fitted with ground glass end-plates. Twelve to sixteen strips can be developed at one time (Fig. 5). For broad strips (1 3/4 in. to 2 1/2 in.) it is sufficient to use grooved bacteriological staining dishes resting on a modified wooden funnel support; three of these staining dishes with their support can be placed in a glass jar (18 in. height, 12 in. diameter) fitted with a ground glass cover. Three four-inch cluture dishes are used as the solvent reservoirs. Six strips can be developed at one time in this apparatus (Fig. 6; cf. also Fig. 7 for a smaller unit).

Somewhat more elaborate apparatus is required for large-scale two-dimensional descending paper chromatography than for the

one-dimensional. The following apparatus has been used with consistent success for all two-dimensional paper chromatography in this Laboratory for over four years. The solvent trough is made by cutting lengthwise a 27-inch piece of glass tubing (1 3/8 in. diameter) and sealing the ends by fusing with semicircular glass plates. The solvent trough is suspended in angle slots at the top ends of a paraffined chamber made of two window sashes and heavy wooden sides. A glass frame (27 in. long, made of 1 cm glass rod and 1/2 in. wider than the solvent trough) is placed on top of the solvent trough; this frame serves to hold the paper up and away from the trough edge. The filter paper to be chromatographed is folded 3/4 in. from the edge to be dipped into the solvent; a special glass rod (24 in. long, prepared by bending up and back the 6 in. ends of a 36 in. long glass rod of 1 cm diameter--the ends for extra weight and for convenience in adjustment and removal) is placed in the paper fold, and the rod and paper* placed in the solvent trough; one end of the glass rod is held upright by means of a slotted wooden block. The solvent is added slowly through a long stem funnel that is kept pressed tightly down on the middle of the glass rod until the solvent has crept at least to the top of the glass frame**. The chamber is now leveled to the point where the solvent

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* Two papers can be run just as conveniently as one; the two creases oppose each other under the rod and hang over the opposite edges of the glass frame.

** Otherwise the movement of the paper as it wets and folds over the glass frame is vigorous enough to "float" the glass rod and loosen the paper.

surface is the same distance from the top of the glass rod along its entire length. The chamber, the bottom of which has been covered with open shallow vessles serving as a solvent reservoir, is now sealed against evaporation and opened only when the development is complete (Fig. 8). The papers can be removed individually, but the large size of these filter papers, their wet, weakened condition, and the highly objectionable harmful odors of the solvents usually used in paper chromatography combine to make individual removal at best sloppy technique. The following procedure was devised to avoid handling the individual chromatograms until they were dry: When development is complete, the excess solvent in the trough is siphoned off. Stainless steel frames, designed to hold the solvent trough and glass frame firmly together and having a hole in the middle for a rod and a hooked end for hanging, are slipped over the solvent trough and glass frame at each end, and a heavy metal rod is slipped through the holes in the steel frames and the ends bolted (Fig. 9); the entire assembly is now quickly removed by means of the long rod, and transferred to an air-dryer. At the end of about an hour, the glass rod used to hold the papers in place is removed to permit the paper edges in the trough to dry more rapidly. When dry, the paper is chromatographed similarly in the second dimension.

Ascending Paper Chromatography One- and Two- Dimensional

Ascending chromatography differs fundamentally from descending chromatography only in that the solvent moves up against gravity. In

practice this results both in slower solvent movement and in definite limits as to how far the solvent can move irrespective of time of development*. The limitations of ascending solvent flow place definite restrictions on the possible resolving power of any solvent system. However, the solvent movement is quite rapid during the first eight hours and the resolution for that time and for the distance covered is much greater than that for any corresponding descending chromatogram; these facts were the basis for the development and extensive use in this investigation of small-scale ascending one- and two-dimensional chromatography. Furthermore, the equipment required was simple, cheap, and easy to construct; all that was required was a test tube, jar, or

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* For the solvents used for amino acid resolution, the rate of movement was as follows:

Time of Run Hours	Distance Inches
1	3
2	4
8	8
16	12
24	15
48	15

As yet no explanation for this stopping of the solvent front has been put forward. Since the movement is by "capillarity" against the force of gravity, it may be plausible to argue for a situation somewhat analogous to that of the classical movement of liquid up a capillary tube. However, some recent unpublished experiments by Karler and Hoeger[19] indicate that this is at best only part of the explanation. These investigators carried out a series of ascending chromatograms under conditions in which there was no evaporation of solvent from the surface of the paper (Fig. 10). It was found that, after the first day's movement of six inches under the especial conditions of these experiments, the solvent front progressed steadily until the end of the experiment (14 days later) at the rate of 1 1/4 inches per day.

other chamber equipped with a sealable top from which the papers could be suspended. Since small-scale ascending chromatography was so vital to the qualitative and semi-quantitative aspects of this investigation, the subject seems worthy of detailed discussion and is presented in the next and some of the succeeding sections.

As implied above, large-scale ascending chromatography proved to be of limited value in view of solvent movement being restricted to a maximum of about 15 inches. Nevertheless, it was sometimes found efficacious to develop large two-dimensional chromatograms by ascension in one-dimensional and descension in the other. A simple apparatus was designed for this purpose. It consisted of a large pyrex glass jar (12 in. x 24 in.) with "handles" made by pushing in the glass wall and a gas-tight ground glass cover; the paper holder is prepared from a circular piece of wood attached to another piece of wood which rests on the handles (Fig. 11).

It was also found that separation often analogous to large-scale (20 in. x 20 in.) descending two-dimensional chromatography could be obtained by medium-scale (11 1/2 in. x 11 1/2 in.) two-dimensional ascending chromatography. The apparatus consisted of a pyrex glass jar (12 in. height, 12 in. diameter) fitted with a wooden cover (rubber weather-stripping used as sealer), to which was attached a circular piece of wood that served as the paper holder.

It might be pointed out that the limitations of ascending chromatography described above are restricted to the slow moving solvents used ordinarily in paper chromatography; they do not apply to fast

moving solvents like distilled water or dilute salt solutions. As a matter of fact, ascending chromatography can be used effectively in decreasing the speed of these fast-moving solvents to the point where the adsorptive processes can become effective enough to resolve many compounds (especially larger molecules such as proteins) that cannot be resolved by the same solvent systems in descending chromatography. The successful use of this slowing-up by ascension has already been described in the chromatography of the whole native lipoprotein (cf. Fig. 4 for typical run).

Development of Small-Scale Paper Chromatography.

The small-scale paper chromatographic procedure used above to check the protein hydrolysates for the removal of the lipid interference was only one of several modifications of the larger scale ascending type of paper chromatogram described by Karler[20], Williams and Kirby[21], and Crumpler and Dent[22]. It was thought that working on a smaller scale than that involved with 18 in. x 22 in. sheets might have the following advantages over ordinary paper chromatography:

1. Save time. It requires about 48 hours to develop and dry an ordinary (18 in. x 22 in.) two-dimensional chromatogram. Any time saved would be important when labile compounds or radioactive materials were being chromatographed.
2. Simpler, cheaper apparatus. Less of the carefully purified and prepared solvent required.

3. Small papers are easier to manipulate, especially during the drying process.

4. Smaller, more intense spots resulting in an increase in in sensitivity of the proceudre and consequently much smaller amounts of protein hydrolysate might be analyzed.

The test-tube procedure of Rockland and Dunn[23] provided the initial stimulus for this small-scale procedure. Rockland and Dunn, however, restricted themselves to small-scale one-dimensional work; it seemed to this investigator that a small-scale two-dimensional paper chromatogram would be a very useful extension of Rockland and Dunn's fast, sensitive test-tube procedure.

The one-dimensional runs of Rockland and Dunn were first studied to check their claims for speed, sensitivity, and effectiveness in resolution. A piece of filter paper (Whatman #1) 150 mm long was cut with a taper 24 mm wide at the top and 16 mm wide at the bottom. The sample to be chromatographed was placed 25 mm from the bottom within a circle of 6 mm diameter. The samples were dried in a stream of warm air (30° C). Each strip was placed in a test tube (22 x 175 mm), the bottom of the strip just touching the bottom of the test tube. The unit was placed in a constant temperature room (30° C) for equilibration. Water-saturated (at 30° C) phenol, 1.0 cc* was added to the bottom of the tube by means of a calibrated transfer pipette. The

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* Later 5.0 cc was used as the standard volume of solvent for these test tube chromatograms.

tube was placed in a stable upright position and lightly stoppered with a cork (cf. Fig. 12). In 2 hours, the solvent had climbed 2/3 of the distance; in 4 1/2 hours the solvent had reached the top of the strip. The R_f's and the resolution obtained by this ultramicro procedure were comparable to those obtained with descending one-dimensional chromatograms 500 mm long requiring five times as much time. Rockland and Dunn's results were quickly confirmed; all in all, there seemed to be no reason why this micro modification could not be successfully extended to two-dimensional chromatrography*.

The first small scale two-dimensional runs were made in pyrex glass jars 8 3/4 in. deep and 4 3/4 in. wide (Leyden Jars). The cover for the jar was made of a piece of 1/4 in. wood, 5 1/4 in. x 5 1/4 in.; a #12 cork (3 1/2 in. diam.) was attached to a block of wood 2 in. x 2 in. of such thickness that the bottom of the cork was about 8 in. from the bottom of the Leyden jar when the unit was nailed to the cover. A piece of slashed rubber tubing, 1/4 in. diameter, pushed onto the rim of the jar served as a seal when a lead brick was placed on top. Sheets of Whatman #1 filter paper were cut in squares (8 in. x 8 in.). The sample position was marked by a circle 6 or 10 mm in diameter located in one corner 1 in. from each edge. The samples were applied in 5 - microliter aliquots, each aliquot

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* The use of a tapered piece of filter paper in a test tube worked so well that the technique was extended to running full length one-dimensional paper chromatograms in large 18 in. test tubes[24].

dried in a stream of warm (30° C) air prior to the addition of the next 5 - microliter aliquot. The tops of two undeveloped chromatograms were thumb-tacked to the perimeter of the corks; 50 cc of water-saturated (30° C) phenol was added to the bottom of the Leyden Jar, and the jar's covers with the attached cork and papers lowered onto the top of the jar and weighted with a lead brick. As usual, the runs were made at 30° C. In 2 hrs the solvent had risen 4 in., in 4 1/2 hrs about 6 in. After 4 1/2 hrs the cover with its attached papers were placed in a small drying chamber and dried in a stream of warm air (30° C) for 2 hrs. The papers were then removed, rotated 90° and attached as before to a new* wood-cork cover. One hundred** milliliters of the water saturated (30° C) n-butanol-acetic acid mixture were placed at the bottom of a clean Leyden jar; the cover with the attached papers was lowered and sealed as before. The solvent rose 6 in. up the papers in about 4 hrs; the papers were removed as before and dried for 1 hr. Each pair of chromatograms were run as duplicates in each jar; one of the developed chromatograms was placed in an air-oven set at 110° C in order to bring out the ultra-violet fluorescence of the amino acids, and the other was sprayed 3 times with 0.25 percent ninhydrin in n-butanol. The results were encouraging: When sprayed with ninhydrin, chromatograms of the original delipidized

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* It was necessary to avoid contamination of the second solvent system with any traces of phenol that might be associated with the phenol-absorbing cork and wood.

** It was thought 100 ml would speed up the run.

6 S_f-lipoprotein (22R-10T_{0-2}) hydrolysate revealed 11 very distinct, intense, and varied-colored ninhydrin spots--the spots possessed more characteristic shapes and colors* than the normal large descending 2-dimensional chromatogram. The untreated paper that had been placed in the oven (110° C) for three hours to develop the ultraviolet fluorescence gave spots corresponding to every spot in the ninhydrin developed chromatogram; in addition, there were several long fluorescent streaks. Despite the fact that some more work would have to be done to eliminate the fluorescent streaks**, this fast small-scale sensitive modification of the larger two-dimensional paper chromatographic procedure seemed on the whole more than adequate for qualitative study of protein hydrolysates.

Before further work was done, it was decided to change the apparatus to run four or six chromatograms in each chamber; for this purpose 2 1/2 and 3 gallon glass aquaria were used, thus permitting the papers to hang free in the solvent (Figs. 13 and 14). Using four aquarium chambers it was found convenient to complete 16 or more two-dimensional chromatograms per day.

A comparative qualitative amino acid study was made using the small 2-dimensional chromatogram. The acid hydrolysates of the following proteins were studied:

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* cf. last section for application.

** Subsequent investigation revealed that careful distillation of the commercial (C. P. grade) n-butanol used resulted in removal of most of these fluorescing streaks.

1. 41 R, 2-10 T_4 Highly purified 6 S_f lipoprotein delipidized.
2. 37 R, 9-15 T_1 S_f = 13, supernatant.
3. 37 R, 9-15 T_1 (ppte) S_f = 13, pptn. from above sample.
4. 41 R - T_1 S_f = 17 to 40.
5. 26 R - C5 Contains everything in human blood serum.
6. Albumin (Armour Co.) - diluent for RH testing.
7. 22 R - 10 T_{o-2} Pooled 6 S_f - slightly inhomogeneous.

As might have been expected repeated chromatography of these hydrolysates revealed no real qualitative differences. There seemed to be some variation in the relative amounts of each of the amino acids; the amino acid ninhydrin spots were usually too intensely colored to give any valid comparative semi-quantitative data. Furthermore, it was rather obvious that a few of the amino acids could not be separated and/or accounted for by this procedure. Despite all this, the small-scale procedure proved very convenient in identifying most of the amono acids present; since the amino acid distribution patterns of each of the protein hydrolysates studied were essentially the same, it was only necessary to add a series of known amino acid "markers", to the starting positions of filter papers containing the various hydrolysates ready for chromatographic development. The speed and ease with which these small-scale two-dimensional chromatograms can be run made this procedure much easier to carry out than the conventional procedure of multiple large scale two-dimensional chromatography, isolation of individual amino acids, and their study by using "markers" in one-dimensional

chromatography. Actually, of course, the small scale procedure was subsequently checked by the conventional large scale procedure: no discrepancies were found--the large scale procedure did, however, permit the identification of those few amino acids poorly resolved by the small-scale chromatograms.

The "markers" technique employed so successfully for the rapid qualitative determination of most of the amino acids in these hydrolysates is described in greater detail in the last Section. Positive qualitative identification is discussed in some detail in the section on quantitative studies.

Study of Basic Hydrolysates

While the qualitative study of the acid hydrolysates was in progress, it was thought advisable to modify the hydrolytic millieu in order to determine those amino acids which are partially or wholly destroyed during the acidic hydrolysis. The small-scale two-dimensional chromatograms immediately indicated that most of the tryptophen had been destroyed and at least some of the cystine lost in the form of multiple spots near the main cystine spot*. Hydrolysis by means of a basic and/or enzymatic hydrolytic agent followed by small scale

* Although it is true that there is some decomposition of pure cystine in paper chromatography with phenol, nevertheless, the destruction is trivial compared to that which seems to occur in acid hydrolysis.

paper chromatography seemed to be the simplest way of determining those amino acids wholly or partially destroyed by acid hydrolysis*.

A saturated aqueous solution of barium hydroxide was chosen as the basic hydrolytic agent in preference to sodium or potassium hydroxide; it would be easier to remove the excess barium hydroxide after hydrolysis without the volume increases resulting from the neutralization of the excess sodium or potassium hydroxide and the difficulty of determining the endpoint with concentrated reagents in the centrifuge hydrolytic tubes used for all the hydrolyses in this investigation.

A saturated solution of barium hydroxide was prepared by shaking (at room temperation, 25° C) a great excess of $Ba(OH)_2 \cdot 8\ H_2O$ (325 gm.) in 500 cc distilled water intermittently over a period of a couple of days. The solid undissolved material was permitted to settle out overnight and the supernatant was pipetted off as needed for hydrolysis.

Since our interest in hydrolytic agents other than hydrochloric acid was limited to supplementing the information obtained by acid hydrolysis, each basic hydrolysis was usually performed simultaneously with duplicate acid hydrolysis of the same protein, and both hydrolysates

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* Hydrolysis of proteins by hydrochloric or by sulfuric acid leads to the destruction of most, if not all, of the tryptophan and the possible dismutation of some of the cystine, serine, threonine, aspartic acid, and glutamic acid[25,26,27]. The main advantage the bases have over the acids is that they do not cause the formation of humin; on the other hand, they cause complete or partial destruction of cystine, arginine, and lysine[25]; Sahyun claims threonine and certain other amino acids are also partially or completely destroyed.

chromatographed at the same time under the same conditions. The first proteins studied were albumin (Armour's RH diluent) and 26 R - C5 (whole human serum). Duplicate aliquots of each of these protein solutions were placed into centrifuge-hydrolysis tubes, precipitated and "delipidized" by the ethyl alcohol extraction procedure used for the lipoprotein components, and hydrolyzed with 1 cc 6 N HCl or 1 cc satd. $Ba(OH)_2$. The proteins were hydrolyzed on a steam bath--24 hours for the acid and 36 hours for the base.

An attempt was made to develop satisfactory chromatograms from the whole barium hydroxide hydrolysate without removal of any of the barium hydroxide prior to sampling; it was hoped thereby to avoid possible losses of amino acid in the removal of the barium as the insoluble, voluminous carbonate. Ninhydrin spraying of the developed chromatograms revealed rather distinct, brightly orange and yellow colored spots[29] whose distribution pattern differed from that obtained with the hydrochloric acid hydrolysates. Judging from the yellow-orange colors instead of the ordinary red-purple colors and from the modified distribution pattern, it can only be concluded that the amino acids chromatographed as the corresponding barium salts or carbamates. The individual spots were not identified, but chromatography of the barium salts of the amino acids might be useful for separating certain amino acids that are not easily separable as the free bases or the hydrochlorides. A comprehensive qualitative study is indicated here.

The barium ions were removed by passing a slow stream of carbon dioxide gas through the barium hydroxide hydrolysates until the precipitation was complete. The hydrolysates were centrifuged and the supernatant removed. The white barium carbonate residue was analyzed further for any traces of amino acids* and the supernatants were chromatographed as the hydrochlorides**. The presence of tryptophan was established; no new amino acids were found different from those whose presence was established by work with acid hydrolysates; some of the amino acids seemed to have undergone appreciable destruction, especially cystine which was almost completely destroyed.

Determination of Amino Acids on the Paper Chromatogram by the Ninhydrin Method.

Now that successful qualitative analysis on various components had been carried out be means of amino acid markers on small-scale two-dimensional chromatograms without revealing any significant differences in amino acids present, it was obvious that a quantitative or at least a semi-quantitative study would have to be made. Since paper chromatography was the only practical method of separation of

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* There were some amino acid losses but they did not seem large enough to affect the preliminary qualitative study. The barium carbonate residues were first thoroughly washed with water, and redissolved with dilute HCl. The barium slowly reprecipitated with sulfuric acid and the sulfate washed with hot water until amino acid free.

** The free amino acids were converted to the hydrochlorides by exposing the wet sample spot of the paper chromatogram to copious hydrochloric vapors.

the amino acids, the next problem was to decide whether the amino acids could be determined on the paper or whether they would have to be removed to secure the required sensitivity and accuracy.

A considerable effort by many investigators[30] had already been expended on the attempted quantitative determination of amino acids on the filter paper chromatogram. Direct determination of the amino acid without removal from the filter paper would be ideal provided sufficient accuracy could be achieved. Most investigators seemed to have concentrated on attempts to produce a consistent ninhydrin-amino acid color on paper and its subsequent determination by any one of several densitometers. Unfortunately, most of the workers failed to appreciate the extraordinary difficulties in controlling the color reproducibility on paper of the ninhydrin-amino acid reaction. Over the past five years, this investigator has observed, semi-quantitatively at least, that the following "variables" seem involved in ninhydrin color development on paper:

1. Size of Spot. Both the total amount and the surface concentration (amount per unit area) are important. With increasing surface concentrations, for example, the colors tend to shift from the purples and blues to rusty reds, pinks, oranges, and yellows.
2. Temperature of Color Development. Development at room temperatures versus development at 70 - 100° C: As the temperature is increased the colors tend to shift from the purples to the reds and yellows as above.

3. Kind of Filter Paper.

4. Previous Handling of the Filter Paper. Pre-treatment by washing with water or dilute HCl sometimes shifted the colors toward the deep blues and purples.

5. Kinds of Solvents used during Development.

6. Manner of Application of the Ninhydrin. Single versus multiple spraying of the ninhydrin solution(s): Multiple spraying is usually more effective.

7. Kind of Ninhydrin Spray Vehicle. Colors vary with the particular solvent used and increase with the water-saturation of any of the solvents.

8. Humidity during Color Development. Increasing the humidity during color development (at 70 - 100° C, at least) increased somewhat erratically the color yield.

9. Rates of Color Development and Color Stability. Color development on paper seems to be a complex time-dependent reaction always accompanied by appreciable color fading. Not only does the rate of color development seem to vary greatly for the various amino acids, but the rate of color fading also varies greatly.

All these variables cast real doubt on the work done thus far in attempting to assay by color density the various amino acids on a paper chromatogram. The fact remains that the kind of color (wave length) and the amount of color cannot be satisfactorily con-

trolled at present. Patton's improved method[31] (cf. last Section for application) represented a definite step forward but was still unsatisfactory for a truly quantitative procedure. Some work will have to be done on the fundamental mechanism(s) involved in the ninhydrin-amino acid color development on filter paper before color density can be used with confidence as a quantitative measure of amino acids separated on paper chromatograms.

There was still another possibility: That of developing the ninhydrin color on paper, eluting off the pigment, and determining the color (optical density) in a spectrophotometer. This approach was rejected on the basis of the above unevaluated variables in color development*.

Tentative Procedure for the Quantitative Recovery of Amino Acids on Paper Chromatograms.

In view of the considerations just discussed, the procedure selected as best for the handling the chromatograms was as follows:

1. Heating the chromatograms just enough to permit location of all the amino acid spot by ultraviolet fluorescence.
2. Cutting out the spots.

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* Later, in the summer of 1951, Singer and Carpenter (of the Virus Laboratory, University of California) attempted to make this elution process quantitative but failed. Actually, of course, once color development on paper can be more satisfactorily controlled, this procedure will be ideal for quantitative paper chromatography of protein hydrolysates, thus eliminating the frequent uncertainties in spot location and delination.

3. Removing the spots by elution chromatography.

4. Determination of the amino acid by some sensitive procedure such as that involving the Moore-Stein ninhydrin reagent.

The experiences of this investigator with paper chromatography had been such that the first three steps in this procedure seemed to offer no insurmountable difficulties; in fact, these three steps had been studied somewhat in previous problems from 1947 to 1950[32,33,34,35]. It was thought best to concentrate at this point on the development of a sensitive ninhydrin reagent or some other sensitive reagent specific for amino acids.

Actually four methods for the ultramicro analysis of amino acids were evaluated:

1. Copper complex method of Woiwood[36].

2. Titrimetric methods: indicator and oxidation-reduction endpoints[37].

3. Dye oxidation method of Gordon[38].

4. Ninhydrin procedure of Landua and Awapara[39].

In the final study, the ninhydrin procedure was adopted principally because the quantitative ninhydrin reagent had already been studied and successfully applied[39] to the recovery of ultramicro amounts of amino acids separated by filter paper chromatography, whereas the other three procedures had not as yet been evaluated chromatographically.

Despite some rather obvious criticisms the experiments of Awapara[40] with the Moore-Stein ninhydrin reagent seemed at the very least a promising starting point. The key to the alleged success of their procedure was the substitution of a pyridine-water ninhydrin reaction with the more sensitive and more consistent ninhydrin reagent of Moore and Stein. Although the Moore-Stein ninhydrin reagent and procedure was designed for use in analyzing the batches of the hundreds of samples obtained by means of automatic fraction cutters in column chromatography, Landua and Awapara and this investigator found it possible to use the Moore-Stein procedure with few modifications for analysis of the amino-acid spots in paper chromatography.

Reagents for the Moore-Stein Ninhydrin Procedure.

Ninhydrin. A very good grade of ninhydrin (or triketohydrindent hydrate) is required in order to insure low blank readings. The ninhydrin obtained commercially from Eastman Kodak Company (# 2495) gave satisfactorily low blanks except occasionally when the batches possessed a color darker than the normal light yellow of carefully recrystallized ninhydrin. If the ninhydrin gave too high a blank, it was necessary to recrystallize the batch until the desired blank

had been obtained*.

Citrate Buffer. The citrate buffer (0.02 M) is prepared by adding 21.0 gm of citric acid (reagent grade) to 200 cc of 1 N NaOH diluted to 500 cc. When diluted with an equal volume of water, the pH of this buffer should be 5.0 ± 0.1; if not correct, the pH can be adjusted either way by the dropwise addition of fairly concentrated solutions of citric acid or sodium hydroxide. It was soon discovered that the critical factor in the preparation of batches of ninhydrin reagents of exactly the same sensitivity and reproducibility was the citrate buffer; hence, it was advisable to prepare at least 2000 cc of citrate buffer and store the excess in cold with thymol or at 0° C as is**.

Methyl Cellosolve. A good grade of methyl cellosolve should be used. It should be checked 1) for impurities by mixing with an equal volume of water (clear solution should result) and 2) for peroxides

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* The recrystallization procedure was as follows: Ninhydrin, 10 gm, was added to 50 cc of hot distilled water. The hot solution is treated with 1 gm of decolorizing charcoal and filtered hot. The filtrate is stored at 4° C overnight. The crystals are washed four or five times with 5 cc portions of very cold water, dried quickly in a mild vacuum, and stored in dark glass bottles. Actually, with the impurer batches obtained from certain other manufacturers at the time, it was found necessary to recrystallize at least once more. This is a rather messy procedure and it is best not to bother with a very impure (i.e., dark orange or brown) sample of ninhydrin. Fortunately, the quality of several commercial ninhydrins has been improved to the point where they can be used without further purification.

** Moore and Stein recommend thymol but this investigator found that refrigeration at about the freezing point preserved the solution satisfactorily for at least a year and probably much longer than that.

by adding a 10 percent aqueous KI solution (no more than a faint positive test should result). A very satisfactory grade of methyl cellosolve was obtained commercially from the Griffin Chemical Company of San Francisco, California; distillation with a simple fractionating column (Vigreux) was all that was necessary for purification.

Ninhydrin Solution. It was found convenient to prepare the final ninhydrin reagent solution in 500 cc batches as needed. Reagent grade $SnCl_2 \cdot 2H_2O$, 0.40 gm, were dissolved in 250 cc of citrate buffer, pH $\sim$5; this solution was chilled to 0° C. Ninhydrin, 10 gm, was dissolved at room temperature in 250 cc methyl cellosolve in a glass stoppered 500 ml pyrex bottle; the solution was then chilled to about -50° C in an acetone-dry ice bath. While the ninhydrin solution was in the dry ice bath, the chilled citrate buffer was added and the resulting slightly brown solution rotated rapidly until the heat produced had been dissipated; when the temperature of the solution had been reduced to 0° C, the solution was flushed for several minutes in a steam of nitrogen gas, stoppered and stored at 0° C or even better

at -20° C*. The stability of this reagent was best maintained by removing aliquots for analysis as quickly as possible, flushing the solution with nitrogen and returning the tightly stoppered bottle to the refrigerator. If used in this way, the solution was stable for at least a month; in fact, the sensitivity of the reagent used under these conditions was found to decrease not more than 1 percent in six weeks--no measurable decrease was detected in four weeks.

Further Comments on the Ninhydrin Reagent.

Moore and Stein were somewhat confusing as to whether the citrate buffer was used undiluted with water or diluted with water to give a pH of 5 prior to addition of the ninhydrin cellosolve solution. As will be discussed in next section the buffering capacity of the previously diluted buffer proved to be too low for successful use: the color yields for most of the amino acids were very pH sensitive. In fact, even though the undiluted citrate buffer worked satisfactorily, the buffering capacity was not as great as would have

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* The careful chilling of the solutions before and during preparation of the ninhydrin reagent solution seemed to decrease the color blanks (at 570 mμ) and to increase the sensitivity of the reagent appreciably. Although the "browning" occurs at these low temperatures, it is rather insignificant compared to that which occurs when the solutions mixed at room temperatures. In case of the chilled solutions within five or ten minutes all the brown color has disappeared; when mixed at room temperatures, only part of the brown color disappears on standing. Sometimes the solution became progressively darker, this was especially true if the order of mixing of the two unchilled solutions were reversed. Even if chilled as described above before and during mixing the heat production is great enough to bring the temperature of solution to 25° C.

been desired; it was necessary to evaporate all samples to dryness in order to control at all times the volume of the solutions. A preliminary attempt was made to increase the buffering capacity but the sensitivity (color yields) of the reagent dropped sharply; Moore and Stein seemed to have achieved the necessary conditions for maximum color yield. This conclusion is implicitly confirmed by the more highly buffered quantitative reagents of Boissonas[41] and Fowden and Penny[42]; the color yields of their more highly buffered ninhydrin reagents were only about 50 to 80 percent of those the unmodified Moore-Stein reagent. It seemed best to use the unmodified Moore-Stein reagent even though this meant all samples would have to be evaporated to dryness prior to the addition of the ninhydrin reagent*.

Another word of caution is in order here: Acidic acid even neutral solutions will usually pick up traces of ammonia from the air. Since as little as 0.1 of a microgram of ammonia can be detected by this procedure, it is important to work in an atmosphere where ammonia is not being used and to handle the samples and blanks under strictly

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* Moore and Stein[43] were not hampered by the low buffering capacity of their very sensitive reagent because all their samples and/or aliquots were automatically adjusted to present equal volumes prior to addition of their ninhydrin reagent and subsequent color development.

parallel conditions*.

Procedure for Use of the Moore-Stein Ninhydrin Reagent.

Moore and Stein[43] used 1.00 cc of their ninhydrin reagent for 0.1 or 0.2 cc samples of eluate and 2.00 cc for 0.5 cc samples. In either case, after the development of the color, 5.00 cc of diluent (water - n - propanol, 1:1 by volume) were added to each tube and the optical density determined. It was expected that the eluate from each amino acid spot would be about 0.5 cc; hence the 2.00 cc volume of ninhydrin reagent was chosen as a reasonable volume for this procedure.

The procedure initially used for checking the sensitivity, accuracy, and precision of the ninhydrin reagent was as follows:

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* This extreme sensitivity for ammonia also means that ammonia (and other ninhydrin-reacting substances) cannot be added to the solvents used for developing the chromatograms more effectively in the first step of the analysis of the hydrolysates--unless, of course, the residual ammonia or added compound is removed by an extra laborious step (cf. Fowden).

To guide the investigator, here is a fairly complete list of substances that have been found to be quite ninhydrin reactive:

1. Free alpha-, beta-, and gamma-amino acids.
2. Nitrogen-esterified amino acids; e.g., sarcosine.
3. Peptides especially smaller ones.
4. Primary amines including such compounds as ethanolamine, choline, betaine, and taurine.
5. Secondary amines including such compounds as epinephrine and ephedrine.
6. Alpha-amino aldehydes such as the glycosamines (glucosamine and galactosamine).
7. Ammonia and ammonium salts.
8. Amides.
9. Xanthines including methylated derivatives such as caffeine and theobromine.

The desired amount of amino acid was transferred by means of a "Misco"* pipette to a very clean glass-stoppered (pyrex) 5 ml volumetric flask; the pipette was rinsed 3 or 4 times to effect quantitative transfer. Since these samples were rather small (about 0.05 cc including the rinses), approximately 0.5 cc of water was added to the flask, followed by 2.00 cc of ninhydrin reagent. Four flasks (one blank and three samples) were prepared at the same time. The set of flasks, loosely stoppered, were clamped into a 4-holed brass plate, and the bulbs of the flasks immersed in a vigorously boiling water bath (Fig. 15) for 20 minutes. The flasks were allowed to cool to room temperature (10 minutes) and then filled to the mark with the diluent (water - n - propanol, 1:2 by volume for the samples and 2:1 for the blanks). The solutions were measured in a Beckmann Spectrophotometer (Model DU) at 570 millimicrons using the standard "Corex" 1 cm cuvettes. The ninhydrin blanks were measured against water - n - propanol (2:1) and the samples against their corresponding ninhydrin blank.

Micrograms Valine	Optical Density x 1000										Spread
0	113	82	73	76	117	107	92	107	108	112	73→117
2	66	63	83	113	57	51	57	120	57	41	41→120
5	139	169	183	184	187	188	182	150	141	160	139→188
10	250	327	332	357	-	290	339	318	309	318	250→357

* Manufactured by the Microchemical Specialties Co., 1834 University Avenue, Berkeley, California

The high blanks and spread of the results indicated that something was drastically wrong. In view of the ambiguity regarding the Moore-Stein directions for preparation of the buffer and the possibility of a poor bath of ninhydrin, it was thought advisable to prepare another ninhydrin reagent solutions using a newly prepared citrate buffer and a better ninhydrin preparation*.

Micrograms Valine	Optical Density x 1000						Spread
0	77	94	68	67	113	66	66→113
2	68	52	81	83	37	71	37→83
5	176	167	172	172	131	177	131→177
10	323	383	323	362	380	332	280→382

At this point the situation seemed rather hopeless. It was decided to concentrate on a given amino acid level, running one blank and four samples of the same amount of amino acid in an attempt to eliminate one possible source of error: the possibility that Beer's law was not operative especially at this low level of amino acid. Four samples of 15 micrograms of valine were studied.

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* In the course of this preparation, the first new citrate buffer prepared had the fantastically high pH of 10 when diluted with an equal volume of water. This buffer was, of course, discarded but the error indicated that the pH of the above ninhydrin reagent solution may have been off somewhat due to a poor glass electrode in the Beckman pH meter.

Blank	15 γ Valine
0.068	0.474
	0.474
	0.474
	0.468

This is the type of results that we had hoped to secure for the final standard curves. Other amino acid levels were studied in the same fashion* and are summarized below.

Optical Density x 1000

γ Valine	Blank	Samples			
15 γ	68	474,	474,	474,	468
10 γ	67	253,	247,	257,	242
5 γ	77	132,	138,	129,	113
10 γ	83	233,	183,	183,	184
10 γ	67	227,	218,	217,	214
10 γ	63	188,	207,	221,	207
10 γ	59	197,	217,	223,	217
10 γ**	68	218,	213,	212,	199
10 γ	51	217,	191,	221,	197

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* It should be noted that it was found convenient to add three drops of water to the bottom of each flask prior to the addition of the sample to facilitate quantitative transfer; consequently only about 0.4 ml of water instead of 0.5 ml added to the sample flask prior to the addition of the ninhydrin reagent.

** Increased time of heating over steam bath from 20 to 25 min.

Thus far, only the 15 microgram (valine) level seemed to have the desired reproducibility. It was thought best to repeat this series.

Optical Density x 1000

γ Valine	Blank	Samples			
15 γ	43	262	308	298	308
15 γ	46	301	283	282	288
15 γ	51	287	287	288	298

Apparently there was about a 40 percent decrease in sensitivity or color yield of this reagent within twenty-four hours. Runs at other levels were made in an attempt to find some consistency somewhere.

γ Valine	Blank$^{x10^3}$				
5 γ	38	87	82	83	81
20 γ	47	388	392	348	368

The situation seemed somewhat better although far from satisfactory. Before further work was done at 570 millimicrons, it seemed advisable

to check the sensitivity of the proline at 440 millimicrons*.

Wave Length	γ Proline	Optical Density x 1000	
440	0	147	
	10	78	78
	20	148	148
570	0	48	
	10**	48	52
	20**	97	95

Not only was the sensitivity rather low but the blank was far too high for practical purposes. Since the color of the blanks seemed to be due partly to the color of the ninhydrin reagent solution, it was thought possible to decrease the blank by decreasing the amount of ninhydrin in the ninhydrin reagent. In view of the tremendous excess of ninhydrin over the amount of amino acid (approximately 10^5 mil. eq. ninhydrin for each mil. eq. of valine) being analyzed, a

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* The sensitivity was so low it was thought advisable to determine the maximum of the percent transmission curve. The 20 gamma proline sample above was used.

Wave Length	% Transmission
420	71.2
430	71.4
440	71.5
450	72.0
460	72.4
470	72.8
480	72.9

The percent transmission dropped off after this. At this stage the percent transmission at 440 millimicrons was satisfactory.

** Same as above samples.

new ninhydrin solution was prepared the same as before except that the cellosolve contained only 20 percent of the ninhydrin used previously. A blank and duplicates of 10 and 20 microgram samples of valine were prepared in the usual way, using the weaker ninhydrin reagent.

γ Valine	Optical Density x 1000	
0	60*	
10	138	138
20	302	318

The blank was not only not lowered but the sensitivity (or color yield) was decreased about 25 percent. The "weak" ninhydrin reagent was converted to the regular Moore-Stein reagent by adding the rest of the required amount of ninhydrin to the cold reagent; dissolution was slow, but the ninhydrin reagent seemed to have the same properties as those of the previous ninhydrin reagent.

The work on this phase of the problem was suspended to concentrate on several other aspects. It was not until about three weeks later while trying to use this procedure for determining protein recoveries from the hydrolysates that the discovery was made regarding the extremely low buffering capacity of the ninhydrin reagent.

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* At 440 mμ = 149

Elimination of Some Possible Errors Prior to Chromatogaphy of the Hydrolysate.

Hydrolytic Agents. Thus far very little thought had been given to the agents and conditions of hydrolysis of the various protein components. Only 6 N HCl and saturated Ba $(OH)_2$ had been used under rather arbitrary conditions, 24 hours and 36 hours respectively on the steam bath; as already indicated in the first hydrolyses of the delipidized proteins, only a preliminary qualitative study of the hydrolysis had been made. It was rather obvious that any modification and/or destruction of any of the amino acids during hydrolyses would be reflected in the final determination of the isolated amino acids, thus giving an inaccurate picture of the amino acid composition. Because of the complexity of proteins and the greater complexity of the hydrolyzing protein, it is an extremely difficult task--if not an impossible one--to evaluate the changes and/or losses that each amino acid undergoes in the hydrolysis of any given "pure" protein. The establishment with certainty of the exact relationship between the amino acid composition (as determined by careful analysis) and the amino acid composition of the protein from which the hydrolysate was made will have to wait for that day when wholly synthetic proteins can be made. In the meantime, the best that might be done is to study the fate of each of the amino acids in the controlled hydrolysis of proteins whose amino acid composition and structure will be qualitatively and quantitatively determined. Sanger's[44] success in the

determination of the total amino acid structure of insulin is the most encouraging work presented thus far. The well-established qualitative and quantitative amino acid composition of crystalline insulin makes it excellent as a standard for checking any method of analysis on the intact and the hydrolyzed molecule. But it must be borne in mind that the results from the insulin standard will apply with certainty only to insulin and not necessarily to any other protein. As the intra-relationships of the polypeptide chains of insulin are elucidated and as the structure of other "pure" proteins are determined, the use of such standards will become effective in the development and evaluation of amino acid methods.

There are other ways of overcoming somewhat the difficulty of evaluating losses in protein hydrolysis[45,46]. These include 1) preparing the protein hydrolysate in different ways, using different hydrolyzing agents and different hydrolyzing conditions; 2) analyzing each amino acid by several different procedures; and 3) analyzing the amino acids in the intact molecule before hydrolysis[7] and comparing these values with those obtained from the hydrolysate. Although no work could be done in evaluating any losses in hydrolyses until a scheme of analysis had been worked out for suitable synthetic protein hydrolysates[45] made from pure amino acids, it is advisable to keep in mind the possible limitation of any single set of hydrolyzing conditions. However, as far as deciding whether or not the protein moiety of the various lipoprotein components is the same or different, the

problem is somewhat simplified if each component is treated exactly the same way. If the scheme of analysis can be duplicated, it might be assumed that each protein will undergo changes that can be reproduced rather closely upon repetition of the analysis with another sample of the same protein; hence, if two lipoprotein components have the same protein moiety, any changes and/or losses in the amino acids during hydrolysis should be the same for the two similar proteins, and will not cause any error in interpretation; if, however, the protein moieties are distinctly different proteins, any changes and/or losses during hydrolysis will, if anything, most likely only enhance these differences*, thereby greatly facilitating the comparative study of the protein moiety of the lipoprotein components. Hence, in so far as the final comparative quantitative study of the ratios of some of the amino acids are concerned, a single procedure of analysis including only one carefully chosen hydrolytic agent would be sufficient to answer the question of similarity of the protein moieties of these lipoprotein components.

All known protein hydrolytic agents and conditions were considered for the comparative quantitative study. Unfortunately,

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* In the early phases of this work when only very limited amounts of rather impure lipoprotein components were available for study, it was observed in the qualitative study of what were supposed to have been the same components from different human subjects that tryptophane was not always completely destroyed under the same conditions of acid hydrolysis; in fact sometimes very appreciable amounts were detected on the chromatograms. This great variability in the destruction of tryptophane may have been due to different proteins being involved and a consequent enhancing of this difference due to the changes in hydrolysis as discussed above.

most of the hydrolysates produced by the various hydrolyzing procedures were inadequately evaluated because of the purely qualitative nature of the approach and the analytical techniques. It seemed wisest to choose those hydrolyzing conditions that had been used for the best qualitative and quantitative study of the amino acids of protein hydrolysates, preferably those employing chromatographic techniques. Conveniently enough, the recent elegant work of Moore and Stein[47] with lactoglobulin using hydrochloric acid hydrolysis, starch column chromatography and their own sensitive ninhydrin reagent constitutes some of the most accurate, careful work that has ever been done in this field.

Hydrochloric acid (6 N) had already been used for the hydrolysis of most of the proteins studied thus far in this investigation; furthermore, hydrochloric acid is a most convenient agent for use in preparing protein hydrolysates to be paper chromatographed in that the acid does not have to be removed prior to the addition of the sample to the paper, thus avoiding possible losses in removal of the hydrolytic agent and eliminating a tedious step in the procedure. It was noticed, however, that considerable amounts of "humin" were formed in the hydrochloric acid hydrolyses; subsequently, much of the humin was eliminated by more complete removal of the lipid, but some humin

was always formed* even with the most thorough delipidization. Although the humin formed did not absorb any appreciable amounts of amino acids from its hydrolysate, nevertheless, it seemed desirable to decrease as much as possible, if not eliminate, the humin formed, especially in view of the fact that at least part of the humin probably represent definite losses of amino acid; appreciable amino acid losses could not be tolerated because of the very small amounts of protein available for analysis (0 to 0.2 mg unhydrolyzed protein for some components from an individual donor**).

Moore and Stein, at the suggestion of Dr. C. F. Jacobsen, carried out their hydrolyses by dissolving the protein in a minimum volume of water or dilute hydrochloric acid, adding 200 times the sample weight of 6 N hydrochloric acid twice distilled in an all-glass apparatus*** and refluxing for sixteen hours on an oil bath.

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* Subsequent work (cf. last Section) with very low concentrations of protein revealed on visual humin; as a matter of fact, there was no coloration (browning), much less dark brown particle-formation, in samples containing about 0.2 mg or less of delipized protein per cc of hydrolytic solution. Of course this fact does not mean that some destruction of amino acid did not occur although it seems to lend support to Dr. C. F. Jacobsen's claims (cf. next footnote) regarding the desirability of using dilute protein-hydrochloric acid solutions for minimizing amino acid destruction during hydrolysis.

** Although most of this work had been done with pooled lipoprotein components, it was the ultimate objective of this investigation to develop an analytical procedure for studying components from the serum of a single subject ("homologous samples").

*** Jacobsen had unpublished experimental data (1948) demonstrating the advantages of carrying out the hydrolysis in relatively dilute solution with hydrochloric acid which is free of heavy metal impurities. Humin formation is supposed to have been reduced considerably under these hydrolyzing conditions.

This procedure was modified slightly to fit our small-scale requirements. The maximum amount of protein hydrolyzed per tube was 2 mg; 0.2 ml of hydrochloric acid would have been too small a volume to use in this type of condensing system. One milliliter of HCl or at least 1000 times the sample weight, was used. The acid used was constant boiling HCl (5.6 N) prepared by distillation and purified by twice redistilling in an all-glass apparatus. The tubes were heated on a steam bath (Fig. 2), (temperature inside the tubes was about 99° C.) for twenty-four hours.

A Device for Accurate Reading of the Liquid Meniscus in the Centrifuge-Hydrolysis Tube. As has been pointed out before, all operations prior to the placing of the aliquot of the hydrolysate on filter paper were carried out in the original centrifuge-digestion tube without any transfers to eliminate intolerable losses of the small amounts of protein available. This meant that the final volume of the hydrolysate would have to be measurable to about ± 1 percent at the one-milliliter level.

An attempt was made to use the calibration marks on the tubes themselves by adding 1.00 cc of 5.6 N HCl from a calibrated transfer pipette to a series of clear, dry centrifuge-hydrolysis tubes. It was found impossible to read the tubes reproducibly; furthermore, the meniscus is so large in this inverted conical section of the tube that it was obvious some device would have to be constructed to insure a steady reproducible meniscus. Here is a typical series of readings for 1.00 cc 5.6 N HCl:

Tube No.	Volume of 1.00 cc
1	0.98
2	0.94
3	0.94
4	0.91

The easiest solution seemed to be that of eliminating the meniscus. Some work had already been done with non-wetting silicon films (DC Silicon Resins and "Drifilm"), and the work of Moore and Stein[47] indicated that these films were very acid resistant. A thin film of "Drifilm" was applied without subsequent heating to tubes (1) and (4) above. HCl acid, 1.00 cc of 5.6 N as above, was added to each tube. Both tubes read at 1.00 cc without any of the previous difficulties. Four tubes were then treated with a thin film of Dri-film and heated for three hours at 180° C to make the film more permanent. Four lipoprotein components* were selected and carried through the entire procedure to chromatography (denaturation, delipidization, and hydrolysis). Two difficulties developed for all the samples studied: 1) the denatured lipoprotein and the delipidized protein particles adhered most tenaciously to the sides of the entire length of the centrifuge digestion tube, making it difficult to complete the delipidization and impossible to get all of the protein into the bottom part of the tube for controlled hydrolysis; and, furthermore, 2) the meniscus which had been completely flattened with pure 5.6 N HCl before was now as extensively

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* Two samples of $S_f = 6$, pooled and an individual sample; one $S_f = 13$; and one with higher flotation rate S_f probably 30-60.

curved and as difficult to read as was the untreated tube*. The use of water-repellant surfaces was rejected for the analysis of lipoproteins at least.

There seemed nothing to do but to devise an apparatus for reading the liquid level of the samples under reproducible conditions of position and lighting. A wooden file cabinet box (18 in. x 10 in. x 12 in.) was placed upright and fitted with small ring "Misco" stand nailed firmly onto the bottom. A "Misco" clamp was fastened to the ring stand to hold the regular centrifuge-digestion tube in such a way that its bottom was in the middle of the box; a slightly shorter clamp was attached below the first clamp in such a way that a straight edge of some sort (e.g. a long edge of an hexagonally-shaped pencil) could be attached adjacent to and on the same level with the one-milliliter meniscus of the tube. The straight edge was perpendicular to the rear of the upright box. A 100-watt projector lamp was mounted just outside the box within the upper third of the open side by means of a screw-type clamp. The upper half of the box, including the projector lamp, was covered with a shield of aluminum foil. A piece of black plastic or paper was placed to the rear and under side of the liquid to give the meniscus a sharp black appearance. The readings were made with a four inch reading glass by sighting along the straight edge to put the eye in a reproducible reference position and then

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* The "Drifilm" surfaces seemed to be completely covered with a thin layer of brownish material that was quite wetted by this acid protein hydrolysate.

determining the height of the lowest part of the meniscus as measured by the manufacturer's calibration on the tube. Any error due to change in eye level was easily eliminated by rapidly shifting the eye between straight edge and the meniscus. By means of this simple device it is easy to determine a 1.00 cc volume or similar volume of liquid with a precision greater than ± 1 percent. This straight edge is used as a fixed reference point and is never moved. If larger volumes are to be measured in these tubes, another straight edge is clamped above the reference edge and the calibration determined as above*.

Stirring Technique in the Centrifuge-Hydrolysis Tube. At this point in the procedure there was only one possibility for material loss in manipulation. During the early steps of denaturation-delipidization the lipoproteins adhered most tenaciously to glass surfaces, especially the glass stirring rods that were used. Although the losses on the glass rods would be negligible for macro-scale quantitative analysis, at this level (less than 2 mg protein) such losses were disastrous. It proved to be almost impossible to remove completely the protein from the long glass stirring rods without some loss.

A simple magnetic stirring arrangement was finally used with great success. A piece of soft iron, 1 cm long and about 0.1 - 0.2 cm in diameter (sharp end of a small soft iron nail is satisfactory),

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* As pointed out before all calibrations were made by introducing the required volume of liquid into the tube by means of an accurately calibrated transfer pipette.

is sealed* in a close-fitting piece of thin-walled pyrex glass tubing (about 0.2 or 0.25 cm inside diameter). The glass-covered stirrer is checked for cracks and leaks before use by subjecting it to the strain of the experimental procedure by shaking it vigorously against the bottom of a glass tube, and immersion in 5.6 N HCl and heating on a steam bath for twenty-four hours. Any cracks or defects are readily detected by the yellow $FeCl_3$ that forms. After testing, the glass-covered iron rod is placed in a centrifuge-digestion tube (Fig. 1), and the tube now calibrated by adding a 1.00 cc volume of 5.6 N HCl. The glass-covered rod remains inside this centrifuge-digestion tube throughout the procedure. The rod is manipulated by means of a small powerful horseshoe magnet ("Almico", 5/8 in. or 3/4 in. between the poles). This stirrer is very effective not only in stirring but also in removing any particles that tend to stick to the walls of the tube above the liquid level.

The Question of Completeness of Precipitation of the Protein.

In the denaturation-delipidization procedure described above for handling the lipoprotein preparations, only a small amount of work had been done to check for possible losses of protein in the supernatants. The supernatants of the first couple of samples were checked for ninhydrin-reacting substances by hydrolyzing the supernatants

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* The iron must move freely in the sealed tube; any glass-metal fusion will subject the glass to strains that are likely to crack the glass and thus ruin a sample, especially during hydrolysis with HCl.

in 6 N HCl and testing for amino acids by the usual filter paper spot test using the ninhydrin-butanol spray and heating to develop the color. The color that was developed was compared with color produced by a corresponding aliquot of the delipidized protein hydrolysate. Although only a very small fraction of the ninhydrin color of the hydrolysate was produced by the supernatants, nevertheless it did not preclude the possibility of appreciable losses* of protein for these pooled lipoprotein components and even larger proportionate losses for those components having a lower protein content. As was pointed out by Frank Lindgren, Virgil Herring, and Frederick Aladjem of this laboratory and by Prof. Harold A. Gordon (a wartime member of the Harvard Blood Fractionation Group), the comparatively mild denaturation-delipidization and the possibility of solubilization and/or denaturation protection[49] by the large quantities of complex lipid could result in appreciable losses of protein. Aladjem and Lieberman of the Laboratory encountered appreciable losses of protein especially with smaller amounts of lipoprotein and/or those lipoprotein components low in protein in their attempts to use a trichloroacetic acid precipitation prior to the determination of nitrogen by the Kjeldahl procedure. Furthermore, the writer had frequently observed a rather copious, white, flocculent

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* The experiences of numerous investigators in the hydrolysis of proteins in the presence of various impurities emphasized the dangers involved in incurring appreciable losses of at least some of the amino acids due to complex and little understood reactions with any impurities present (confer, for example, the Maillard reaction[48]: destruction of amino acids by heating in the presence of glucose and other carbohydrates).

substance that formed when the lipoprotein components were stored overnight at -20° C after the absolute alcohol had been added to effect the initial denaturation-precipitation reaction and the precipitated protein centrifuged. When the supernatant was warmed to room temperature, most of the secondary precipitation usually dissolved; there were, however, a few samples containing the flocculent precipitate that seemed to be quite insoluble. Keeping in mind the possibility of irreversible changes in the lipid of the supernatant, oxidative, polymeric or otherwise, the possibility that some of the protein had not been completely precipitated could not be disregarded. As pointed out before any such protein losses might result in a partial fractionation of the protein moiety, the precipitated protein no longer being representative of the total protein moiety and hence quite useless in so far as this investigation is concerned. Therefore, it seemed best to check the completeness of the precipitation of protein to see whether the losses, if any, were significant.

The Analytical Determination of Microgram Amounts of Protein. In checking the completeness of precipitation of protein in lipoprotein samples having 0 to 0.2 mg protein per cc, it is necessary to have a quantitative analytical procedure capable of assaying 1 to 10 micrograms of protein or protein equivalent. There are, of course, quite a few more or less satisfactory methods for the ultramicroanalysis[50,51,52,53,54] of unhydrolyzed proteins. It is to be noted, however, that all of

these methods depend upon the establishment of some sort of standard calibration curve to be determined under reproducible conditions. Such a limitation means that these methods can be applied with any degree of confidence only to the determination of different samples of the same pure protein used for the preparation of the particular standard curve. At this stage of the work, not only was there no evidence for a quantitative similarity between components (pooled and/or individual samples) but there was not enough protein available for the establishment of "standard" calibration curves. Furthermore, all of these methods depend upon the protein being in solution as the native protein or as some solubilized form produced by initial alkaline hydrolysis or some other method. It might also be pointed out that the presence of the large quantities of the complex and variable lipids found associated with these lipoproteins would interfere disastrously with many of the standard methods of protein analysis mentioned above and that any extensive delipidization procedure will

result in extensive desolubilization of the partially* delipidized protein.

All in all, it began to appear that the adaption and/or development of a satisfactory procedure for the determination of the protein moiety of the lipoprotein components was a separate project in itself and hardly worth this investigator's efforts in view of his interest being restricted to the evaluation of protein recovery in any denaturation-delipidization procedure. There was one technique that seemed most conveniently adaptable to the overall denaturation-delipidization-hydrolysis-chromatographic procedure already outlined; this was the use of a modification of the ninhydrin technique on paper used to check the end-point of hydrolysis of delipidized proteins. Although at this point in the investigation the colorimetric ninhydrin procedure of Moore and Stein had not given the precision desired, it was thought to be good enough for use in the determination of the

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* In this investigation only enough lipid was removed to avoid interference with the paper chromatography of the protein hydrolysates. Although some of the lipoprotein samples seemed to have been completely delipidized by the simple water-alcoholic extraction procedure, there was ample evidence that many samples still contained appreciable amounts of lipid albeit not enough to interfere with subsequent chromatography of their protein hydrolysates. The preliminary chromatographic studies with P^{32}-labelled whole native lipoprotein seemed to indicate that a more effective separatory procedure than that of simple batch extractions will have to be used to effect complete delipidization. Some modification of this micro-chromatographic procedure should easily strip all the lipid away from the protein moiety. The totally delipidized protein however, would be a denatured-coagulated mass probably very strongly attached to the adsorbent, and hence hardly ready for immediate protein assay.

total amino acids of protein hydrolysates; it was also hoped that some clue as to the source of error for the single amino acid might be revealed by an extension of the use of this reagent.

At this particular stage of this investigation, the feeling was current that the protein moieties of the various lipoprotein components were identical or at least very similar proteins; hence, it was felt that an arbitrary "synthetic" protein hydrolysate would serve as a good standard for the various hydrolysates; this same "synthetic" protein hydrolysate could also be used for checking the efficacy of the proposed qualitative and quantitative paper chromatographic procedures.

All the available evidence indicated that the lipoprotein components were globulins or globulin-like proteins[55,56,57]. A synthetic gamma globulin hydrolysate was prepared by adding the calculated amount of each of the purest, dry amino acids available. The amounts of amino acids added were calculated directly from the data published by Edsall[58] based upon the work of Brand and Edsall[59] and Brand[60]. No special attempts were made to purify the commercial amino acids used. Since previous paper chromatographic work with all of these commercial preparations had indicated single-spot homogeneity for each, further purification at this time seemed rather pointless. All of the amino acids used were dried <u>in vacuo</u> at 110° C. for forty-eight hours in a large, heated desiccator[61] using a "mega-vac" pump.

THE COMPOSITION OF THE SYNTHETIC GAMMA-GLOBULIN HYDROLYSATE

	Sample Used	Form of sample	Grams** of Amino Acid per 100 G. Protein	Milligrams of Amino Acid*** Added to 50 Ml Water (Final Soln.)
1. Glycine	Paragon #2384		4.2	21.0
2. Alanine	EK*845	dl	---	----
3. Valine	EK 1678	dl	9.7	48.5
4. Leucine	Merck 44341	l(-)	9.3	41.5
5. Isoleucine	GBI*16152	dl	2.7	13.5
6. Proline	GBI 16306	l(-)	8.1	40.5
7. Phenylalanine	GBI 16179	dl	4.6	23.0
8. Cysteine	GBI 16394	l(+) HCl	0.7	3.5
9. Half-cystine	EK 667	l	2.4	12.0
10. Methionine	EK 3167	dl	1.1	5.5
11. Tryptophane	GBI 16376	l(-)	2.9	14.5
12. Arginine	Merck .12371	l(+)lHCl	4.8	24.0
13. Histidine	EK 4274	l lHCl	2.5	12.4
14. Lysine	GBI 16267	l(+) lHCl	8.1	40.5
15. Aspartic Acid	EK 1010	dl	8.8	44.0
16. Glutamic Acid	Merck .36991	l(+)	11.8	59.0

* Eastman Kodak Company and General Biochemicals, Inc.

** Data from Brand[60].

*** Calculated as the free amino acid.

	Sample Used	Form of sample	Grams of Amino Acid per 100 G Protein	Milligrams of Amino Acid Added to 50 Ml Water (Final Soln.)
17. Serine	GBI 16308	dl	11.4	57.0
18. Threonine	GBI 7283	dl	8.4	42.0
19. Tyrosine	EK 666	l	6.8	34.0

This ad hoc mixture of amino acids simulating a gamma-globulin hydrolysate was then used for establishing a standard color curve for globulin or globulin-like protein hydrolysates.

As pointed out before, the first task was to eliminate the final source (s) of error in the Moore-Stein ninhydrin procedure as applied to these amino acids. The ninhydrin procedure as used for the valine standard was applied without change to aliquots of the synthetic gamma globulin hydrolysate. The first set of four samples run simultaneously looked very encouraging:

Hydrolysate equiv. to Microgram of Unhydrolyzed Protein	Optical Density x 1000
0	47
10	137
10	133
20	317
20	293

Since these results looked encouraging, it seemed best to determine the standard curve without further tampering with the procedure. The results were obtained by running a set of five samples simultaneously, each containing an amount of hydrolysate equivalent to 0, 10, 20, 30, and 40 micrograms of unhydrolyzed protein. The results of a series of such runs are summarized in the accompanying table. The only satisfactory results in this table were the consistently low blanks. Something was drastically wrong, but it seemed advisable to proceed with the application of the procedure to an actual delipidized lipoprotein hydrolysate before further modification of the ninhydrin reagent and procedure.

Amt. of Unhydrolyzed Protein the Sample is equivalent to →	Optical Density x 1000				
	0 (blank)	10	20	30	40
Set # 1	46	142	298	433	684
# 2	38	216	278	600	705
# 3	38	138	297	483	654
# 4	47	133	273	476	618
# 5	37	145	298	494	657

All four samples of each "hydrolysate" level prepared and run simultaneously.

The hydrolysate used was obtained in usual way be denaturation, delipidization, and subsequent hydrolysis (6 N HCl) of duplicate aliquots of a pooled beta-lipoprotein. The final hydrolysate contained 1.28 mg hydrolyzed protein per 1.00 cc. The final hydrolysate was centrifuged and the volumes found to be 1.00 cc. Four 100 microliter samples* were taken for the ninhydrin analysis; each sample was neutralized by adding 100 microliters of 6 N NaOH. This was followed by the regular ninhydrin procedure using 2.00 ml ninhydrin reagent. Here is a summary of the striking results:

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* It had been intended to analyze for less protein, but the calculated concentration was only one-fourth of what it should have been.

Sample #	Description	
0	(blank contained 100 microliters of 6 N HCl and 100 microliters of 6 N NaOH) Orange color.	
#1	Green color--the solution was found to be very basic; had to add 75 microliters before color slowly darkened (purple) at room temperature. Green color indicated some extensive production of a yellow compound. Deep purple color in about 10 min.	
#1	Green color	
#2	Green color	Added 50 microliters of 6 N HCl to each of these tubes.
#2	Green color	

Then all five flasks were returned to the steam bath for 15-20 minutes. The blank was unchanged but the four samples were a very deep purple. This run was the first check on the buffering capacity of the Moore-Stein reagent; obviously the buffering capacity was extremely low.

The buffering capacity was further checked using only 20 microliter aliquots of four other 6 N HCl hydrolysates that contained much lower concentrations of amino acids; no NaOH was added to "neutralize" the excess HCl.

Hydrolysate-Sample Designation	Type of lipoprotein	Run # 1	Run # 2	Run # 3*	Run # 4
Blank	(20 microliters 56 N HCl, const. boiling)	6	12	55	68
84 R-LT$_3$	Pooled beta-lipoprotein[1]	22	13	408	393
69 R-PT$_1$	$S_f = 13$	16	0	203	192
94 R-6T$_1$	Nichols' "low protein"	16	8	288	286
86 R-6T$_3$	Nichols' "high protein"	22	18*	405	433

All the blanks and samples of Runs # 1 and # 2 looked alike. Obviously, no more than 20 microliters of constant boiling HCl (5.6 N) was needed to shift the pH so far to the acid side that absolutely no color was produced. As a matter of fact, it was soon shown that even such slight variations of ± 0.05 cc in the 0.4 cc volume of water added to the dry sample in the flask prior to the addition of the 2.00 ml volume of ninhydrin reagent were readily detected in the final spectrophotometric determination. With these facts in mind Runs # 3 and # 4 were performed as indicated in the table.

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* Runs # 1 and # 2 finally indicated that the most likely source of error for all previous ninhydrin determinations using the unmodified Moore-Stein ninhydrin reagent lay in the assumption of the existence of appreciable buffering capacity at pH 5. Hence, in Runs # 3 and # 4, the 20 microliter samples were evaporated to dryness (air oven 135° C--20 minutes was sufficient) in the 5 ml volumetric flasks, this was followed by 0.40 ml water and 2.00 ml Moore-Stein ninhydrin reagent.

Another attempt was made to secure a suitable calibration curve for proteins using the synthetic gamma globulin hydrolysate as the standard hydrolysate. The results are summarized in the accompanying table and averages of the optical density were plotted on linear graph paper. All four values for each point were determined simultaneously. An almost linear plot of optical density vs. micrograms of globulin was obtained in the range of 10-40 micrograms (Fig. 16). This curve was used to check the protein recoveries in those four protein hydrolysates used to demonstrate the low buffering capacity of the Moore-Stein ninhydrin reagent:

Hydrolysate-Sample Designation	Optical Density x 1000			Equiv. Micrgrs. of Gamma Globulin	Ninhydrin protein equivalent	TCA (Trichloroacetic Acid) Protein Mg.-%	Kjeldahl N protein Mg.-%
	Run #3	Run #4	Mean				
Blank	55	68	--				
84 R-LT_3'*	408	393	401	25.4	254	250	310
69 R-PT_1	203	192	198	13.6	136	174	374
94 R-$6T_1$	288	286	287	19.4	194	187	252
86 R-$6T_3$	405	433	419	26.4	264	342	444

Protein Calibration Curve Using Ninhydrin Solution No. 2

Micrograms of Unhydrolyzed Protein equiv.	Blank	Optical Density x 1000	Mean - Std. Deviation from Mean
10	58	133, 133, 133, 142	135 ± 4
20	54	295, 297, 296, 302	298 ± 3
30	48	475, 486, 490, 480	483 ± 6
40	52	691, 664, 690, 680	682 ± 12
100	47	1270, 1270, 1230, 1240	1250* ± 18

Although the recoveries as determined by the ninhydrin procedure on hydrolysates obtained from alcohol-treated lipoproteins compared rather well with those obtained from the corresponding the TCA-treated lipoproteins, both series of results were very low compared with those obtained from the total Kjeldahl nitrogen by subtracting the lipid Kjeldahl nitrogen. If the Kjeldahl nitrogens were reliable, the losses during alcoholic denaturation and/or delipidization were far greater than expected**.

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* This value was equivalent to a %-Transmission = 5.6% and hence supposedly beyond the "workable" limits of the Beckman spectrophotometer. The particular instrument used in this investigation functioned so well that accurate, reproducible results down to 3%-transmission were obtained and used routinely despite theoretical limitations.

** Gross losses during hydrolysis could not have been great enough to account for the discrepancies. About 20 to 60 percent of the protein could hardly have been destroyed under these conditions of hydrolysis although 10 to 20 percent might have been. There is also the possibility that the phospholipid nitrogen values were in error (cf. last Section) and/or that non-protein nitrogen (such as that from glycosamines suspected to be present in these components) as yet unevaluated might eventually decrease the discrepancies between the three methods.

These preliminary runs indicated that further study of protein recoveries was in order. Although at the time there was no concern with the question of complete recoveries *per se*, there was interest in securing a representative sample of the protein moiety for amino acid analysis; if as much as 60 percent of the protein was being lost with the lipid fractions, there was always the possibility that some selective splitting of the molecule or fractionation of the component had occurred removing only certain specific parts of the polypeptide chains, leaving a non-representative "protein" for subsequent hydrolysis and analysis.

Another ninhydrin solution had to be prepared before further work could be done. This Solution (3) was checked by drawing up another calibration curve for the standard synthetic *gamma*-globulin hydrolysate. The results are summarized in the accompanying table. Although the precision was slightly less than that obtained for ninhydrin Solution 2 (the data was obtained over a period of a week), the results gave a very straight line plot. This Solution 3 and graph were then used for further study of protein recovery. These results are summarized in the next table.

Amount of Syn. Gamma Globulin Hydrolysate	Optical Density x 1000				
	0 (Blank)	10 γ	20 γ	30 γ	40 γ
Set # 1	33	138	318	499	667
Set # 2	33	139	292	500	692
Set # 3	28	139	302	477	653
Set # 4	32	133	307	496	698
Set # 5	33	141	327	524	672
Set # 6	37	132	298	468	678
Set # 7	38	144	303	506	685
Mean and Std. deviations	34 ± 2.1	138 ± 2.6	307 ± 7.4	496 ± 11.3	678 ± 9.4

This ninhydrin solution was then used to check the protein recoveries of a large series of lipoprotein components against the recoveries obtained by the Kjeldahl and the trichloroacetic acid (TCA) procedures. The results of this study are adequately summarized in the table below.

COMPARATIVE SUMMARY OF PROTEIN RECOVERIES FROM VARIOUS LIPOPROTEIN SAMPLES BY THREE METHODS

Sample Designation	S_f Value or Range	Mg.-% Protein by Ninhydrin (Delipidized Protein Hydrolysate)	Mg.-% Protein by Kjeldahl (Total Nitrogen minus Lipid Nitrogen)	Mg.-% Protein by Kjeldahl (TCA Nitrogen minus Lipid Nitrogen)
84R-LT$_3$	6	254	365	250
69R-PT$_1$	13 and above	136	374	174
94R-6T$_1$	17 and above	194	252	18.7
86R-6T$_3$	6	264	444	342
90R-15P	17 to 25	94	139	114
90R-6T$_o$	80 to 200	50	84	(no TCA ppt.)
90R-15T$_o$	100 and above	75	191	10.6
90R-15T$_1$	45 and above	92	166	64.4
6A (same as 84R-LT$_3$	6	272	365	250
85R-1T$_2$	10	253	(sample lost)	(sample lost)
85R-1T$_5$	8	357	332	(no TCA ppt.)
98R-T$_3$ 1/1	6	233	269	175
2A	(serum albumin)	837	795	807
96R-T$_o$	300 and above	25	24.4	(no TCA ppt.)
96R-T$_1$	60 and above	86	115	100
105R-1T$_1$	13	73	123	48.2
105R-1T$_2$	10	60	151	67.5

Sample Designation	S_f Value or Range	Mg.-% Protein by Ninhydrin (Delipidized Protein Hydrolysate)	Mg.-% Protein by Kjeldahl (Total Nitrogen minus Lipid Nitrogen)	Mg.-% Protein by Kjeldahl (TCA Nitrogen minus Lipid Nitrogen)
$105R-1T_3$	10	47	118	66.9
$105R-2T_2$	(centrifugal serum supernatant)	43	193	65.0
$105R-2T_3$	(same type of sample as $105R-2T_2$)	49	20.5	61.9
$106R-1,2T_o$	100 and above	87	(no result)	(no result)
$110R-5T_o$	(chylomicra)	11	(method too insensitive)	(no TCA ppt.)
$110R-5T_6$	(chylomicron background)	23	61.4	37.5
$111R-T_4$	30	40	32.5	73

As a result of the discrepancies between the various protein recovery methods the following modified, slightly more drastic denaturation-delipidization procedure was checked as satisfactory and was used in all subsequent work:

The precipitated lipoproteins are heated in the presence of their 75-80 percent alcohol-denaturing medium just below reflux temperatures (about 80° C.) for 6 to 24 hours. It was, however, soon noticed that, for some lipoprotein samples especially those obtained in very samll amounts for analysis, a single heating may not be sufficient for avoiding significant loss of protein; it is necessary to check all samples for completeness of precipitation by repeatedly chilling (-20° C., 10-20 hrs.) and heating (75° C., 6-8 hours) until all the "lipid" that precipitates out upon chilling readily redissolves upon heating. The alcoholic precipitating medium is removed by thorough centrifugation and very careful pipetting, great care being used to avoid material losses.

The precipitated lipoprotein is extracted with 5 ml of 75 percent ethanol-water by heating at 75° C. for four hours on a modified air-oven* with frequent, vigorous stirring. The extracting medium is removed with great care by centrifugation and pipetting. This heat extraction is repeated twice.

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* Twenty holes (five-eights inch) were drilled in the top of an ordinary asbestos-insulated oven.

The partially delipidized protein is then subjected to three or four extractions with 5 ml absolute ethanol, the extraction procedure being the same as that for the 75 percent ethanol above.

The delipidized protein is then gently warm-air dried to remove the last traces of solvent and is now ready for hydrolysis.

If the delipidization is still insufficient for any reason or purpose, the alcoholic heat extractions may be followed by a similar triple extraction by heating with a chloroform-ethanol (1:1 by volume) mixture. It might be pointed out that the chloroform-ethanol extractions are usually unnecessary except for larger amounts of some lipoprotein components (e.g., pooled samples) or for those studies where more complete delipidization may be desired beyond the requirements of semi-quantitative or quantitative amino acid analysis using paper chromatography. A single chloroform-ethanol heat extraction can be conveniently used to eliminate the possibility of insufficient delipidization especially for those lipoprotein components of higher lipid content ($S_f > 35$).

Qualitative Analysis of the Protein Hydrolysates

A preliminary qualitative analysis of the synthetic gamma-globulin hydrolysates and the delipidized lipoprotein hydrolysates was effected by means of small-scale (8 in. x 8 in.) two-dimensional paper chromatography using known amino acids as markers. The procedure described below was based upon reliable reproducibility of the relative positions of the various amino acids from a protein hydrolysate (or

other complex mixture of amino acids) subjected to two-dimensional paper chromatography under carefully controlled experimental conditions[63]; the use of the absolute distance of movement for each amino acid (R_f value)[18] in a complex mixture of amino acids had already been shown[64] to be subject to many unknown uncontrollable variables and hence had to be disregarded in this investigation. The procedure was as follows:

The synthetic gamma-globulin hydrolysate is chromatographed two-dimensionally using increasing amounts of "hydrolysate" until the number of amino acid spots (located by fluorescence and/or ninhydrin) corresponds to the known number of amino acids added or until no further spots appear. The amino acid spots are then tentatively identified by running a large series of small and large two-dimensional chromatograms, judiciously adding selected one, two, or three known amino acids to the synthetic gamma globulin hydrolysate spot prior to development of the chromatogram; the large scale (18 in. x 22 in.) two-dimensional chromatograms are used only for the identification of trace amounts of amino acids and where greater resolution may be required for some of the amino acids. Once a satisfactory qualitative analysis of the standard synthetic gamms globulin has been achieved by means of amino acid markers, the entire series of runs is repeated for the unknown delipidized protein hydrolysate. Thus far the procedure is quite simple and rapid provided most of the two-dimensional chromatography is performed with the small scale (8 in. x 8 in.) papers, and the results obtained are usually quite reliable. Positive identification is a

much more involved procedure involving as it does extensive use of the slower large-scale two-dimensional paper chromatography, elution of each amino acid, and the chemical and/or physical identification of each amino acid for further checks on purity.

Here is the procedure used for the conclusive identification of the amino acids of an unknown protein hydrolysate: All the spots of the hydrolysate's amino acid pattern tentatively identified by the "marker" procedure descrived above. Four to eight large-scale (18 in. x 22 in.) two-dimensional paper chromatograms are run containing an amount of hydrolysate equivalent to 0.2 - 0.3 mg of protein per chromatogram. Each set of corresponding spots in these chromatograms are cut out and eluted chromatographically between glass slides (cf. procedure used in quantitative elution of amino acids from paper in the quantitative Section). All the eluates are rechromatographed on paper (one- or two-dimensionally) using a solvent system entirely different* from the two solvent systems used in the isolative chromatogram. This step is necessary to prove that all the spots of a given set represent the same substance and also to effect a further purification of the substance. The substance is eluted chromatographically and placed in one or more small thick-walled tubes with 5.6 N HCl and sealed. The tubes are then heated at 110° C. for 6 to 12 hours to check for

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* For example, if H_2O-phenol and HOAC-BuOH-H_2O were used in the original chromatography, then an effective basic solvent system such as collidine, lutidine - H_2O (1:1:2 by volume) might be indicated for most of the amino acids except cystine which is destroyed by a basic solvent system in chromatography.

completeness of hydrolysis; the hydrolysate is then rechromatographed. If more than a single spot is obtained*, the original protein hydrolysis was obviously incomplete and is repeated using the more rigorous hydrolyzing conditions used above (sealed tube). On the other hand, if a single spot is obtained, the substance is eluted chromatographically once. Small aliquots of the amino acid are rechromatographed with a known sample of the amino acid it is suspected to be and with any other suspected amino acids with similar R_f's. If the unknown and known give a sharp single spot and the other substances give two spots, the compound can be said to be identified. If, however, any doubt remains, it can be eliminated by means of a seried of specific spot teste on filter paper for functional groups; if enough of the amino acid remains, it can be further checked by the preparation of a suitable chemical derivative, or the compound can be crystallized and studied as a pure crystal.

In this investigation, the qualitative identification of the amino acids was carried only to the point of checking the results of the second more rigorous hydrolysis by chromatography with those amino acids each spot was most likely to be identical. The delipidized protein hydrolysates of several components representative of the human serum lipoprotein "spectrum" were studied. The main components of these lipoprotein samples possessed the following flotation rates (S_f units): 6, 13, 20, and 35 to 100.

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* The possibility of multiple spots for a single pure compound must, of course, be watched for and eliminated by changing solvent systems[63,65].

The following amino acids were in found in every component qualitatively investigated:

1. Cystine.
2. Cysteine*.
3. Aspartic.
4. Serine.
5. Glutamic.
6. Glycine.
7. Threonine
8. Alanine
9. Tyrosine
10. Methionine.
11. Valine.
12. Tryptophan.
13. Isoleucine.
14. Phenylalanine.
15. Leucine.
16. Lysine.
17. Histidine.
18. Arginine.
19. Proline.

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* Almost certainly present, but not definitely established. Presence indicated by characteristic faint ninhydrin spot near cystine. These spots are almost certainly decomposition products. There is too much destruction during hydrolysis and chromatography for identification. Qualitative identification can probably be best effected by studies on the intact, and/or delipidized lipoprotein component.

QUANTITATIVE COMPARATIVE STUDY OF SOME OF THE AMINO ACIDS OF TWO LIPOPROTEIN COMPONENTS

All experimental details that were considered to be a necessary basis for the development of a final quantitative paper chromatographic procedure for protein hydrolysates seemed to have been studied quite adequately as detailed in the previous Section. Despite the convenience, rapidity, and apparent reliability of the semi-quantitative marker-dilution technique, any visual comparative procedure would have to be substantiated by a more objective and a more quantitative method especially in those cases where the proteins differed only slightly from each other or were the same proteins. Furthermore, the slightest variation in reagents and/or experimental conditions might partially invalidate the marker-dilution technique.

In view of the fact that the final quantitative procedure was yet to be worked out and that it was important for the general problem of atherosclerosis to secure quickly some idea as to the comparative nature of the protein moieties of the various lipoprotein components, it was thought best to run simultaneously the chromatograms of a synthetic beta-globulin hydrolysate and the hydrolysates of the delipidized components, to develop the final quantitative procedure using the chromatograms of the synthetic beta-globulin, and to apply the final quantitative procedure to the developed chromatograms of the unknown proteins.

Two ultracentrifugally homogeneous lipoprotein components were

obtained for the quantitative study. One of these was a pooled sample of "$6S_f$-lipoprotein"*; the other component was a very homogeneous single sample** of $13^S{}_f$ lipoprotein (prepared in two parts[9] that were ultracentrifugally identical, No. 181R-$5T_1$ and 181R-$5T_2$). These samples were appropriately aliquoted into calibrated centrifuge-hydrolysis tubes. Each aliquot was made up to 75 percent ethanol by adding absolute ethanol, and then heat denatured by heating for 8 hours at 75-80° C. on air oven under reflux conditions. The lipoproteins were then delipidized by extracting three times with 75 percent ethanol*** and three times with absolute ethanol, heating 3 to 8 hours for every extraction. The various aliquots were then hydrolyzed as needed with 5.6 N HCl on a steam bath for 24 hours.

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* Sample prepared by pooling the lipoprotein fractions of about 20 individual donors (non-atherosclerotic) and purifying by repeated differential ultracentrifugation.

** This sample was obtained from a single donor (female) who had pronounced atherosclerosis.

*** It was found possible to effect the same delipidization by dispensing with the three 75 percent ethanolic extractions and going directly to four extractions with absolute ethanol with heating as above. Such a modification, however, fails to remove much of the NaCl used in the preparation of the lipoproteins; hence, if the salt concentration is such that appreciable amounts of it are left on the walls of the tube and/or in the gelationous protein as large crystals, it is necessary to desalt the protein with 75 percent ethanol to avoid a variability in hydrolyzing conditions. For most preparations the usual 75 percent ethanol extractions were necessary.

The synthetic beta-globulin hydrolysate was prepared in 50 ml. batches using just enough constant boiling HCl to dissolve the amino acids. The amino acids were dried *in vacuo* as was done in the preparation of the synthetic gamma-hydrolysates used thus far. The composition and other pertinent data are summarized in the following table.

THE COMPOSITION OF THE SYNTHETIC
BETA-GLOBULIN HYDROLYSATE

		Sample Used	Grams[a] of Amino Acid per 100 G Protein	Milligrams of Amino Acid[d] Added to 50 Ml Water (Final Soln.)
1.	Glycine	Paragon #2384	5.6	28.0
2.	Alanine	City Chemical Corp., N.Y.	6.0[b]	30.0
3.	Valine	EK 1678	7.0	35.0
4.	Leucine	Merck 44341	7.9	39.5
5.	Isoleucine	GBI 16152	5.0	25.0
6.	Proline	GBI 16306	7.1	35.5
7.	Phenylalanine	GBI 16179	4.7	23.5
8.	Cysteine	GBI 16394	(0.8)[c]	4.0
			3.5	
9.	Half-cystine	EK 667	(2.7)	13.5
10.	Methionine	EK 3167	1.7	8.5
11.	Tryptophan	GBI 16376	2.0	10.0
12.	Arginine	Merck .12371	6.8	34.0

a. Data from Brand[60]

b. This value was obtained by this investigator in preliminary semi-quantitative studies by means of markers.

c. The percent-ratio data for gamma-globulin was used: cysteine/half-cystine is 0.7/2.4.

d. Calculated as the free amino acid. The form of the amino acid used (d and/or l optical forms, free base or hydrochloride) is the same as that in the corresponding table for the synthetic gamma-globulin hydrolysate.

THE COMPOSITION OF THE SYNTHETIC
BETA-GLOBULIN HYDROLYSATE CONTD.

		Sample Used	Grams[a] of Amino Acid per 100 G Protein	Milligrams of Amino Acid[d] Added to 50 Ml Water (Final Soln.)
13.	Histidine	EK 4274	2.8	14.0
14.	Lysine	GBI 16267	6.6	33.0
15.	Aspartic acid	EK 1010	9.8	49.0
16.	Glutamic acid	Merck .36991	14.5	72.5
17.	Serine	GBI 16308	7.1	35.5
18.	Threonine	GBI 7283	6.1	30.5
19.	Tyrosine	EK 666	6.0	30.0

The most satisfactory procedure for quantitative recovery of at least some of the amino acids seemed to be as follows: The two-dimensional paper chromatogram was to be developed and dried at or only slightly above room temperature (25-35° C.). The amino acids were to be detected by their fluorescence under an ultra-violet light; if the fluorescence of any particular amino acid was insufficient for accurate location of all of compound, heating at 80° C. was to be used until the fluorescence was sufficient. Once the spot was satisfactorily located, it was to be removed by elution chromatography in the apparatus (Fig. 18) developed by Karler[66] and analyzed for amino acid content by the usual quantitative ninhydrin procedure (modified Moore-Stein procedure).

It was decided to check the procedure for blanks and recoveries using valine spots on unwashed filter paper without chromatography. Here is a summary of a typical run:

Spot No.	Micrograms of Valine	Amount of Heating	Optical Density X1000
0	0	none	179
1	20	none	373
2	20	none	372
3	20	80° C, 6 hrs.	353
4	20	80° C, 6 hrs.	332

In view of the very large blank, the "losses" of valine upon heating were more apparent than real.* The ordinary ninhydrin blank is

* After the sample had been removed by elution chromatography, the papers of the spots were dried and examined for possible valine losses by ultra-violet fluorescence and the ninhydrin spray reagent. No residual amino acid could be detected. This test procedure was used for all other amino acids studied whenever there was the slightest possibility of amino acid losses in elution chromatography.

about 46 x 10^{-3}; this means a ninhydrin blank equivalent to above 9.6 micrograms of valine instead of 2.5 micrograms. It was thought best to concentrate on the lowering of the blank of elution.

It was pretty obvious that the large and variable blank coming from the eluted impurities of the unwashed filter paper strips used for carrying the eluting liquid (H_2O) from trough to spot and from the spot to the volumetric flask was responsible for the high blank. This was confirmed by using carefully water-washed filter paper (papers actually washed by chromatographing off the impurities[67,68]; the values for washed blanks varied from 60 to 80 x 10^{-3}, the blanks being very reproducible when run simultaneously.

The elution procedure seemed satisfactory for valine, at least. The procedure was then applied to the recoveries of valine from two-dimensional chromatograms prepared by chromatographing the synthetic γ-globulin hydrolysate using the small-scale (8 in. x 8 in.) two-dimensional chromatograms. Four small-scale two-dimensional chromatograms were setup each containing an amount of synthetic γ-globulin hydroysate equivalent to 100 micrograms (9.7 micrograms valine) and developed under exactly the same conditions using the usual water-saturated phenol and water-butanol-acetic solvent systems; the valine was located by its ultra-violet fluroescence after heating for 6 hrs. at 80° C. The four spots* were cut out

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* A corresponding blank spot for each valine spot was also cut out and eluted simultaneously. Each blank spot was adjacent to and approximately the same size as the valine spot. It was thought such blanks might be necessary correction factors for the blank of the paper after the development by the two solvent. The valines for the blank spots were 27, 17, 13, 25 (X10^{-3}) respectively. Satisfactory recoveries were obtained without the use of these blank values; hence they were not used. Further work on these blank paper spots was performed in the final quantitative study.

and eluted chromatographically (2 hrs.) with water. Here is a summary of the results:

No. of Chromatogram	Optical Density x 10^3
Washed Blank	52
1 (9/23/50)	190
2 (9/23/50)	175*
3 (9/23/50)	198
4 (9/23/50)	197

The following series of large-scale two-dimensional chromatograms were run for the quantitative study: **

(1) Ten chromatograms for each of these amounts of synthetic beta-globulin hydrolysate (unhydrolyzed protein equivalent): 100, 150, 200, and 250 micrograms.

(2) Twelve chromatograms for hydrolysate of pooled $6S_f$ lipoprotein using duplicate samples of hydrolysate.

(3) Eight chromatograms for hydrolysate of sample No. 181R-$5T_1$ (S_f =13).

(4) Eight chromatograms for hydrolysate of sample No. 181R-$5T_2$ (S_f = 13). Ot[illegible]

Other small (8 in. x 8 in.) and medium (11-1/2 in. x 11-1/2 in.) scale two dimensional chromatograms were run for qualitative

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* The lack of great precision here is not significant as these results were secured prior to the appreciation of the very low buffering capacity of the ninhydrin reagent.

** All of these chromatograms were eventually duplicated by the time the final quantitative results were secured. Hence, the final quantitative results actually represented the best of at least sixteen chromatograms.

identification of the amino acids; the amino acids already found for components with S_f's of 6 and 13 were readily identified.

All the chromatograms for the quantitative study were prepared under the same experimental conditions using portions of the same batch of solvents. The chromatograms were individually wrapped in clean white paper to eliminate dust, and stored in a dark room until ready for analysis.

The first problem in the study of the developed paper chromatograms was that of location of the amino acid spots by means of ultraviolet light with a minimum of destruction of the amino acids. It was known that several of the amino acids, if present in appreciable quantities, fluoresce on paper without heating. An attempt was made to find these amino acid spots without heating, using the reflected light of a "Mineralight"* ultraviolet lamp; it was found that the outer edges of the spots were almost impossible to detect. It was also observed that the fluorescence of the amino acid spots increased upon standing at room temperature[69] over a period of several months (2 to 4 months), but not enough for quantitative detection of the amino acids. Standing for a year or so might well bring out the necessary fluorescence, but such a step would be impractical and would almost certainly result in the slow destruction of at least some of the amino acids. It seemed necessary, therefore, to subject the apapers to some sort of minimal heat treatment to hasten the development of the fluorescence.

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* This ultraviolet source is the "Mineralight", Model No. R51 (110 volts, 17 watts), manufactured by the Ultraviolet Products Corporation, Los Angeles, California.

A series of heating experiments were set up using some of the two-dimensional chromatograms prepared for the qualitative identification of the amino acids. Each of these sheets were heated at different temperatures for different lengths of time. A batch of the duplicate two-dimensional chromatograms were set aside as standards for a rough qualitative measure of the degree of destruction of any of the amino acids. A chromatogram was heated at a specified temperature until the amino acids fluoresced* satisfactorily for ready identification; the heated chromatogram and one of the unheated standards were sprayed uniformly three times with a 0.25 percent ninhydrin solution (water-saturated butanol) and heated at 60° C in a water-saturated atmosphere until the maximum color had developed. The lowest practical temperature seemed to be 80° C for 6 hours; however, even at this temperature tyrosine and possibly several other amino acids seemed to have been "destroyed", or at least converted into a non-reactive ninhydrin form. Nevertheless, it was thought that enough amino acids would be stable under these conditions to proceed with the elution of the amino acids and their assay. A new, more sensitive ninyhdrin reagent solution was prepared by careful control of the pH, and standard curves for the various amino acids of interest in this investigation were drawn up (Figs. 19 and 20).

Four of the developed two-dimensional chromatograms of the synthetic beta-globulin hydrolysate were selected for each amount of protein used per chromatogram: 100, 150, 200, and 250 micrograms. Each

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* "Mineralight" used here.

paper was heated for 6 hours at 80° C to bring out the fluorescence of the amino acids. The spots were located by means of reflected ultraviolet light, outlined with a soft pencil, cut out, and subjected to the usual elution chromatography; each eluate was evaporated just to dryness at 105-110° C, and the amino acid determined by the modified Moore-Stein ninhydrin procedure. The results are summarized in the accompanying tables.

It will be noted that the only blanks used were those of the usual washed paper blanks as were used successfully in the preparation of the amino acid ninhydrin standard curves. As in the preliminary studies of the recovery of valine, an attempt was made to use as blanks pieces of paper cut from the same chromatogram as were the spots. Here are a typical series of runs; all "blanks" and amino acids are measured against the "washed" paper blank.

CHROMATOGRAM NO.	AMINO ACID	OPTICAL DENSITY X 1000		
		Washed Blank	Paper Blank	Amino Acid
8*	Aspartic	78	76	463
7	Aspartic	78	33	365
8	Glutamic	94	43	1036
7	Glutamic	94	10	884
8	Threonine	93	42	251
7	Threonine	93	48	175
8	Tyrosine	83	138	138
7	Tyrosine	83	134	98

* Numbers 8 and 7 were typical chromatograms of the synthetic beta-globulin hydrolysate (chromatograms heated for 6 hours at 80° C).

The blanks secured from the paper chromatograms varied from 0.010 to 0.138; these variable blanks were similar to those obtained in the preliminary study of the recovery of valine. It was thought best to reject the idea of the use of this type of correction blank and to cut out all the spots without any excess paper.*

The amino acids seclted for the study of quantitative recovery were chosen with the following considerations in mind: (1) sharpness of the spot--no impurities, no tailing; (2) ease of development of fluorescence of the spot; (3) position of the amino acid relative to adjacent spots.** Aspartic, glutamic, clycine, threonine, alanine, and tyrosine

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* The exact nature of the ninhydrin-reacting material obtained by elution chromatography of these blanks was not determined. Part of the material was almost certainly due to impurities present in the paper and/or to substances produced by the reaction of phenol (or less likely the butanol-acetic acid-water mixture) used in the development of the chromatogram and its subsequent air-drying. The possibility that some of the blanks were due to the traces of amino acids lost due to tailing or poor chromatography was not eliminated; in fact, extensive tailing had frequently been observed in this laboratory. The possibility of tailing was important in the choice of which of the 19 amino acids of a protein hydrolysate to be studied for quantitative recovery in this investigation.

** It was frequently observed that extensive tailing of many of the amino acids in different solvent systems could be suppressed or eliminated by adding amino acids that would run close to the tailing amino acids, and that the amino acids used for "tailing" elimination would have their own tendency toward tailing minimized. Such action seems analogous to the type of mutual displacement[70] that is used in column chromatography. It is simple and convenient to visualize a similar displacement mechanism operative in paper chromatography where the spots move closely together, especially in second dimensional development of complex mixtures of amino acids as in protein hydrolysates. Such a mutual displacement mechanism helps account for the extraordinary sharpness of separation of many of the very close-moving amino acids studied in this investigation, such as glutamic and glycine, threonine and alanine, and occasionally aspartic and serine.

were chosen as those amino acids most likely to give quantitative recoveries. Valine was not chosen because methionine was found to move about half the time with the valine spot. Isoleucine, leucine, and phenylalanine were rejected because they move too close together, usually overlapping. Tryptophane and cysteine are largely destroyed in hydrolysis. Not only is cystine contaminated by some of the destroyed cysteine (oxidation to cystine) but some of the cystine is known to be destroyed during hydrolysis and during chromatography with phenol. Histidine and lysine are not effectively separated; lysine tails too much. Arginine is too diffuse a spot for quantitative studies. The front of the serine spot sometimes "tails" badly although serine might be a good one to study in the future. The proline spot is often quite diffuse and, furthermore, the color yield with the modified Moore-Stein ninhydrin reagent is too low for working at these protein levels.

The results of analyzing the amino acids obtained from 16 two-dimensional chromatograms (synthetic beta-globulin amino acid mixture) developed in the usual manner and heated at 80° C for 6 hours for adequate development of amino acid fluorescence are summarized in the accompanying tables. Recovery values were obtained by the use of Figs. 19 and 20.

The recoveries were much lower than had been anticipated especially for threonine, alanine, and tyrosine. In view of the care used in the preparation and handling of this whole series of chromatograms, it was obvious that there were three possible sources of losses:

AMINO ACID RECOVERIES FOLLOWING PAPER CHROMATOGRAPHY OF A SYNTHETIC BETA-GLOBULIN HYDROLYSATE AND DEVELOPMENT OF FLUORESCENCE BY HEATING AT 80° C FOR 6 HOURS

Amino Acid	Micrograms per Chromatogram	Theoretical Optical Density x 1000	Experimental Optical Density x 1000 Std. Dev.	Recoveries Micrograms	Percent
Aspartic Acid	9.8	212	145 ± 34	6.5	66
	14.7	313	284 ± 46	13.3	90
	19.6	415	342 ± 12	16.0	82
	24.5	515	440 ± 43	20.8	87
Glutamic Acid	14.5	350	269 ± 21	11.0	76
	21.75	519	452 ± 25	18.9	87
	29.0	679	618 ± 21	26.1	90
	36.25	814	762 ± 67	33.3	92
Glycine	5.6	280	177 ± 8	3.5	62
	8.4	412	291 ± 11	5.5	69
	11.2	539	450 ± 74	9.2	82
	14.0	661	603 ± 19	12.7	91
Threonine	6.1	181	34 ± 3	1.1	19
	9.15	271	82 ± 4	2.8	30
	12.2	361	235 ± 55	7.9	65
	15.25	452	243 ± 44	8.2	54
Alanine	6.0	248	92 ± 12	2.4	40
	9.0	372	104 ± 9	2.7	30
	12.0	491	160 ± 26	3.9	32
	15.0	605	138 ± 30	3.4	23
Tyrosine	6.0	111	31 ± 4	1.7	28
	9.0	166	74 ± 18	4.0	45
	12.0	222	76 ± 13	4.1	34
	15.0	277	114 ± 15	6.2	41

1. destruction of amino acids during development with either or both solvent systems; 2. destruction during warm air drying of the wet chromatograms; and 3. destruction during the development of the fluorescence by heating.

It had been noticed during the qualitative study that the sensitivity of the ninhydrin-spray reagent decreased roughly with extensive heat-treatment of the dried, developed chromatogram. Some destruction of amino acids in their extremely reactive physical state on the filter paper fibers[71,72] was to be expected not only during the removal of the developing solvents[73,74] but also during the rather extensive heating (80° C for 6 hrs.) that had been thus far required to develop adequately the fluorescence of the amino acids.

It was felt that the heating required for the development of the fluorescence might be drastically reduced by devising a better system for ultra-violet examination of the developed chromatograms than that provided by the reflected light of a "mineralight" lamp. Thus far, the reflected light of a "mineralight" lamp seemed to give the maximum fluorescence per microgram of each of the nineteen amino acids of the synthetic beta -globulin hydrolysate. A more extensive study of all available ultra-violet light sources and of the various conditions for observing the fluorescing amino acid spots revealed that all the available ultraviolet light sources were much more effective fluorescing agents if the papers containing the amino acids were viewed by transmitted light. It was further found that more powerful ultraviolet light sources could

be used with detection by transmitted light rather than by reflected light, provided effective filters* were used to cut down much of the increased visible light. Two types of sources were used: the diffuse source provided by tubular germicidal lamps** and the intense source by the spot projector type lamp***. The diffuse source was found to be effective for overall examination of the entire chromatogram whereas the spot type was essential in the examination of individual spots. By use of these two transmitted light sources it was found possible to increase the sensitivity while decreasing the heating period appreciably: 15 minutes at 100° - 105° C gave a very satisfactory fluorescence for all nineteen amino acids.[75]

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* One or two thicknesses of the following filter gave very satisfactory results: 6-1/2 inch, red-purple, molded, square, 1/4 inch thick. Corning 5874. $5.00. Corning Glass Co., Corning, New York.

** Two or four 18-inch G.E. germicidal lamps (tubular, "G15 T 8"), 15 watts, mounted in ordinary fluorescent lighting fixtures (18 inches). The entire units ("Rad-I-Air" lamp fixtures) can be secured from the Tru-Air Ultraviolet Products Co., Los Angeles, California.

*** This lamp was the General Electric, 100-watt, CH-4 Projector Spot lamp and was mounted in the football-shaped reflector of a "Zyglo Black Light for Zyglo or Magnaglo Inspection Materials", Model ZB-23, 110 volts. (Manufactured by Magnaflux Corporation, Chicago, Illinois.)

The work was then repeated using duplicates of the sixteen chromatograms described above. They were all heated in a large air-over at 100° - 105° C for 15 minutes and the amino acids studied by transmitted light. The results are summarized in the accompanying tables.

The recoveries for aspartic acid, glutamic acid, and glycine were all within the 100 ± 5 percent limit felt necessary for effective use of this procedure. The recoveries, however, for threonine, alanine, and tyrosine were hardly increased by the decreased heating. Until further work is done, it will have to be assumed that the losses for these amino acids are occurring during development of the chromatogram and/or during solvent removal.*

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* Subsequent work on the semi-quantitative procedure (cf. last Section) revealed that the color yield, on paper at least, of tyrosine and several other amino acids was increased by developing the chromatograms at 16° C instead of 29° C followed by ether-washing of the phenol instead of air-drying.

AMINO ACID RECOVERIES FOLLOWING PAPER CHROMATOGRAPHY OF A SYNTHETIC BETA-GLOBULIN HYDROLYSATE AND DEVELOPMENT OF FLUORESCENCE BY HEATING AT 100-105° C FOR 15 MINUTES

Amino Acid	Micrograms per Chromatogram	Theoretical Optical Density x 1000	Experimental Optical Density x 1000 Std. Dev.	Recoveries Micrograms	Recoveries Percent
Aspartic Acid	9.8	212	208 ± 4	9.7	99
	14.7	313	317 ± 5	14.9	101
	19.6	415	411 ± 8	19.4	99
	24.5	515	518 ± 9	24.6	100
Glutamic Acid	14.5	350	343 ± 7	14.1	97
	21.75	519	515 ± 10	21.6	99
	29.0	679	685 ± 8	29.3	101
	36.25	814	807 ± 13	35.8	99
Glycine	5.6	280	285 ± 5	5.7	102
	8.4	412	410 ± 8	8.4	100
	11.2	539	540 ± 7	11.2	100
	14.0	661	657 ± 12	13.9	99
Threonine*	6.1	181	70 ± 3	2.4	39
	9.15	271	129 ± 3	4.4	48
	12.2	361	148 ± 24	5.0	41
	15.25	452			
Alanine*	6.0	248	93 ± 5	2.3	38
	9.0	372	208 ± 30	5.0	56
	12.0	491	227 ± 20	5.5	46
	15.0	605			
Tyrosine*	6.0	111	28 ± 7	1.5	25
	9.0	166	69 ± 5	3.7	42
	12.0	222	87 ± 15	4.7	39
	15.0	277			

* The experimental optical densities of threonine, alanine, and tyrosine were the averages of only two spots. The theoretical optical densities of threonine and tyrosine were obtained by calculation from the 10 microgram levels (10 microgram threonine and tyrosine had optical densisites of 0.296 and 0.185, respectively); the optical densities of alanine were obtained by means of a standard curve.

All developed chromatograms of the proteins of the three sample components (the pooled $6S_f$ lipoprotein, and the $13S_f$ lipoproteins, 181R-T_1 and 181R-T_2) were heated simultaneously at 100-105° C for 15 minutes. They were then carefully examined under the ultraviolet light sources as described above. There were about 16 of these large-scale two-dimensional paper chromatograms for each sample component; the best four chromatograms of each set of 16 was chosen for quantitative assay of aspartic acid, glutamic acid, and glycine. The results are listed in the next two tables. The second table contains the experimental aspartic acid-glutamic acid-glycine ratios for the three lipoprotein samples and the synthetic beta-globulin hydrolysate as well as the corresponding ratios for the various plasma proteins isolated by the Harvard plasma fractionation process. Unfortunately, there was no quantitative analytical data available for the amino acid composition of the $beta_1$-lipoprotein ($S_f = 2 \rightarrow 20$) isolated by the Harvard process.

The protein moiety of our pooled $6S_f$ lipoprotein seemed to be rather similar to the protein of the gamma-globulin. The proteins of the other two samples, however, seemed to be decidely different from any of the other proteins. Furthermore, these two ultracentrifugally homogeneous ("identical molecules") $13S_f$ samples*, 181R-$5T_1$ and 181R-$5T_2$, were decidedly different from each other. This unexpected difference indicated that the component was not homogeneous, that the ultracentrifuge was "fractionating" the component despite the sharpness of the

* These samples were obtained from a single donor (female) who has pronounced atherosclerosis.

THE AMOUNTS OF ASPARTIC, GLUTAMIC, AND GLYCINE FOUND IN THE HYDROLYSATES OF TWO LIPOPROTEIN COMPONENTS AFTER PAPER CHROMATOGRAPHY

Sample Designation, Tube No.	Amino Acid	Optical Density x 1000 at 570 Mu	Corresponding Values in Micrograms	Mean Std. Deviation, Percent Deviation
Tube #57	Aspartic	459, 467, 479, 447	21.7, 22.1, 22.7, 21.2	21.9 ± 0.549, 2.5 %
(S_f = 6	Glutamic	678, 702, 684, 697	29.0, 30.2, 29.2, 29.9	29.6 ± 0.49, 1.7%
pooled)	Glycine	437, 425, 445, 419	8.9, 8.7, 9.1, 8.6	8.8 ± 0.19, 2.2%
Tube #53	Aspartic	462, 469	21.9, 22.2	22.1
(S_f = 6	Glutamic	690, 677	29.6, 28.9	29.3
same as 57)	Glycine	436, 428	8.9, 8.8	8.9
181R-5T_1	Aspartic	675, 668, 692, 669	32.0, 31.7, 32.8, 31.5	32.0 ± 0.5, 1.5%
Tube #75	Glutamic	1011, 1013, 986, 1025	51.1, 51.2, 48.9, 52.4	50.9 ± 1.27, 2.5%
(S_f = 13)	Glycine	747, 749, 769, 765	16.2, 16.3, 16.9, 16.8	16.6 ± 0.31, 1.9%
181R-5T_2	Aspartic	685, 714, 710, 697	32.5, 33.9, 33.7, 33.1	33.4 ± 0.54, 1.6%
Tube #80	Glutamic	1055, 1031, 1041, 1050	55.1, 52.9, 53.8, 54.7	54.1 ± 0.69, 1.3%
(S_f = 13)	Glycine	960, 974, 980, 971	23.2, 23.7, 23.9, 23.6	23.6 ± 0.255, 1.1%

RATIOS OF ASPARTIC/GLUTAMIC/GLYCINE FOR THE THREE SAMPLES AND THE SYNTHETIC BETA-GLOBULIN HYDROLYSATE ANALYZED HERE AND FOR SEVERAL OTHER PLASMA PROTEINS REPORTED IN THE LITERATURE

HYDROLYSATES ANALYSED HERE

Tube No.	Sample Designation	Aspartic/Glutamic/Glycine Analytical Amts.	Ratios
Tube #57	Pooled lipoprotein, $S_f = 6$	21.9/29.6/8.8	1/1.35/0.40
Tube #75	Single donor, 181R-5 T_1, $S_f = 13$ (T_1 is major part of this component 181R)	32.0/50.9/16.6	1/1.59/0.52
Tube #80	Single donor, 181R-5 T_2, $S_f = 13$ (T_2 is the rest of this component 181R)	33.4/54.1/23.6	1/1.62/0.71
Synthetic	Beta-globulin hydrolysate (at the 250 microgram "protein" level)	24.6/35.8/13.9	1/1.46/0.58

CORRESPONDING RATIOS FOR OTHER PLASMA PROTEINS FROM LITERATURE DATA*

Albumin		10.4/17.4/1.6	1/1.67/0.15
Gamma-Globulin		8.8/11.8/4.2	1/1.34/0.48
Beta-Globulin**	(synthetic mixture same as above "synthetic beta-globulin hydrolysate)	9.8/14.5/5.6	1/1.48/0.57
Alpha-Lipoprotein		9.0/21.6/3.1	1/2.40/0.34
Fibrinogen		13.6/14.3/5.6	1/1.05/0.42

* Data from Edsall[58] based on samples obtained by the Harvard Group, analyzed by Brand's group at Columbia.[60]

** This is not the "$beta_1$-lipoprotein" of the Harvard Plasma Fractionation Group. No quantitative amino acid data is available for this protein.

refractive index gradient peak. In spite of this apparent inhomogeneity, the two parts (samples) of the component were decidedly different from the pooled $6S_f$ lipoprotein isolated from about 20 normal individuals. In view of our inability at the time to isolate the $6S_f$ lipoprotein homologous* to the atherosclerotic (181 R-T_1 and -T_2, S_f = 13) sample above, it was impossible to say whether this difference between the atherosclerotic component (S_f = 13) and the normal pooled $6S_f$ lipoprotein was a real difference or merely a reflection of an overall serum lipoprotein disturbance in the artherosclerotic patient.

In conclusion, this limited quantitative study emphasized the need for characterizing a statistically significant number of carefully chosen homologous lipoproteins. The last phase of this investigation was concerned with the first part of such an investigation.

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* "Homologous" in that all the components of a given lipoprotein series or spectrum come from the same donor subject. (cf. next Section)

COMPARATIVE STUDY OF THE PROTEIN MOIETIES OF SEVEN SETS OF HOMOLOGOUS LIPOPROTEINS

Seven sets of homologous* lipoprotein components were prepared by Frank T. Lindgren by differential ultracentrifugation;[9] a sample of the B_1-lipoprotein prepared by the Harvard Plasma Fractionation Group was also characterized on the analytical ultracentrifuge. Appropriate aliquots of all of these components were placed in calibrated centrifuge-hydrolysis tubes and then denatured, delipidized and hydrolyzed as previously described. The protein concentrations were determined by use of the quantitative Moore-Stein ninhydrin reagent, the optical densities were evaluated by means of a calibration curve (Fig. 21) prepared from a synthetic β-globulin amino acid mixture. These values along with the concentrations obtained from ultracentrifugal plates and other pertinent data are summarized in the next table. The concentration data are in fair agreement except for the components designated I-1 ($S_f = 25 \rightarrow 60$) and AK-3. It was obvious that, despite the small amounts of protein hydrolysate (100 to 250 micrograms protein per chromatogram) required, there was hardly enough protein in two or three of the components for adequate quantitative study by the procedure described in the previous Section.

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* "Homologous" indicates that each lipoprotein component of a given pair or set came from the same individual as contrasted with the "pooled" components obtained by pooling the serum of two or more individuals prior to isolation and purification.

SUMMARY OF PROTEIN RECOVERIES OBTAINED BY THE NINHYDRIN METHOD AND BY THE USE OF ULTRACENTRIFUGAL DATA

Sample Designation	S_f Value or Range	Mg.-% Protein by Ninhydrin Method (Delipidized Protein Hydrolysate)	Mg.-% Protein from Ultracentrifugal Data*
1-4	6 (4 to 8)	932	800
1-1	40 to 70	91	100
2-4	6	440	370
2-1	40 to 70	53	40
I-3	6	720	500
288R-T_1	10 to 20	644	440
I-1	25 to 60	107	700
AK-1	6	596	870
AK-2	40 to 70	139	350
AK-3	40 to 70	15	440
AK-4	6	450	730
AK-5	40 to 70	83	190
AK-6	6	489	890
$beta_1$-lipoprotein (Harvard)	2 to 20	610	970

* The lipoprotein concentrations were obtained from the plates of the analytical ultracentrifuge (Spinco Model E); the protein concentrations were then calculated by assuming protein percentages of 30 for 4-8S_f lipoproteins and of 15 for 20-100S_f lipoproteins.

It had been observed in the preliminary and qualitative stages of this investigation that very satisfactory two-dimensional chromatograms with considerably less than 100 micrograms protein could be obtained using 8 in. x 8 in. papers. It was known from the 18 in. x 20 in. chromatograms produced for the quantitative study that any significant differences in the amino acid composition were readily detected not only by ninhydrin spraying but even by the much less sensitive fluorescence with ultraviolet light; as a matter of fact, the final careful quantitative study only confirmed those differences. Furthermore, it was thought that differences in composition might be confirmed and/or revealed by studying the order of disappearance of the amino acids as the sample sizes were decreasd progressively to a few micrograms.*

A series of small-scale (8 in. x 8 in.) two dimensional chromatograms were prepared with standard synthetic β-globulin and γ-globulin amino acid mixtures in order to ascertain the feasibility of, and the best levels for, the comparative study and the order of disappearance of the amino acids. It was found that satisfactory chromatograms could be obtained with amounts of a synthetic protein hydrolysate equivalent to 5 micrograms or even less. Unfortunately, despite all attempts to establish and maintain uniformity in the preparation, development, and handling of the chromatograms the disappearance of the various amino acids was rather difficult to establish because of the significant variations in

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* Berry and Cain[76] restricted their semiquantitative study to only four amino acids.

area and for color density of each of the separated amino acids in quadruplicate, triplicate and even duplicate chromatograms at the low levels (0-10 micrograms protein) required for such a study. However, many of the differences in synthetic β-and γ-globulin amino acid mixtures could readily be detected all the way from the 10 to 300 microgram-protein level. This preliminary comparative study indicated that the running of triplicate or even duplicate small-scale (8 in. x 8 in.) two-dimensional chromatograms with equivalent amounts of each amino acid mixture (or hydrolysate) ranging from 10 to 20 micrograms of protein would be more than satisfactory for detecting any significant amino acid differences and hence any protein differences.

Before any work could be done on the hydrolysates of the components, it was necessary to redetermine the relative positions of each of the amino acids and the resolving power of the solvent systems in order to select those amino acids most suited for visual comparison. To do this, 50 chromatograms were prepared with 100 micrograms of synthetic β-globulin amino acids; these chromatograms were used as reference standards by adding each of the following amino acids to each of two of these 50 chromatograms prior to chromatographic development:

1)	20	micrograms	leucine	10)	20	micrograms	alanine
2)	20	"	isoleucine	11)	20	"	threonine
3)	20	"	leucine	12)	20	"	glycine
	20	"	isoleucine	13)	20	"	glutamic
4)	20	"	phenylalanine	14)	20	"	aspartic
5)	20	"	tryptophane	15)	20	"	serine
6)	20	"	valine	16)	20	"	cystine
7)	20	"	methionine	17)	20	"	ornithine
8)	20	"	valine	18)	20	"	histidine
	20	"	methionine	19)	20	"	lysine
9)	20	"	tyrosine	20)	20	"	arginine

21) 20 micrograms histidine
20 " lysine

22) 20 micrograms arginine
20 " lysine

The ninhydrin spots of the following amino acids seemed to be the most reproducible and hence the best for comparative studies:

For both 100 γ and 20 γ levels	For 20 γ levels*
1. Phenylalanine and leucine and isoleucine	1. Threonine
2. Valine and methionine	2. Glutamic
3. Tyrosine	3. Aspartic
4. Arginine	4. Serine
5. Alanine	

The above series of chromatograms supplemented previous experiences in substantiating the amino acid pattern for synthetic β- and γ-globulin amino acid mixtures at the 100 microgram protein level (Fig. 22); furthermore, this study indicated that not only is the color intensity of each spot related to the amount of each of the amino acids present but that (1) the size and shape of each of the spots is related to the amount of the particular amino acid and (2) each spot can be modified by the adjacent amino acids. Hence, it soon became obvious that any distortions in the amino acid pattern, especially in those regions of poor resolution, can be of definite help in detecting** amino acids may be poor.

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* Valid only if the resolution is good and the spots are quite sharp.

** Some very interesting effects were observed in some of the cases where 20 γ of an amino acid was added to 100 γ synthetic β-globulin amino acid mixture; for example, 20 γ serine forced all the aspartic, glycine, glutamic, and serine as well as almost all the histidine and lysine to move as one large uniform spot whereas 20 γ histidine increased greatly the resolution of aspartic, glycine and glutamic. Hence, in some of the chromatograms, it was possible to correlate "distortion" with drastic shifts in the relative amounts of amino acid in a given section of the amino acid pattern.

Obviously, any postulated adsorption mechanism that assumed each of the amino acids of a mixture of a large number of amino acids (as in a protein hydrolysate) to move as though independent of the others would be highly naive, to say the least; very likely a situation obtains where an extensive series of mutual displacements occurs in which the paper surface is completely saturated or covered with one or more amino acids giving a series of changing adsorbing surfaces and the paper surface forces playing the role of a more passive carrier (partition chromatography) than might be expected from the chromatography of single components or simpler mixtures than those used in this investigation.

There still remained the problem of determining what amounts or levels of protein hydrolysate per chromatogram to use in order to bring out any existing amino acid differences. Another series of pairs of duplicate chromatograms containing the following amounts of synthetic β- and γ-globulin amino acid mixtures were run simultaneously:

Set I	Set II	Set III
1 micrograms	10 micrograms	150 micrograms
2 "	15 "	200 "
3 "	20 "	250 "
4 "	40 "	300 "
5 "	80 "	400 "
6 "	100 "	
8 "		

In the range 1 to 8 micrograms many of the spots disappeared, the disappearance varying with the ninhydrin spot size of each amino acid. The 10 to 20 microgram runs were quite consistent in their spot sizes and possessed sufficient color intensities to permit easy detection of any differences between the two mixtures in the following amino acid spots: phenylalanine - leucine - isoleucine, valine - methionine, alanine*, threonine, arginine, proline, tyrosine, and usually serine (although those amino acids with R_F's <0.4 were usually at this level too variable in shape, size, (and too low in color intenisty for use in detecting differences). Nothing was gained by working with amounts of protein

*Note that there was no alanine in the synthetic γ- globulin amino acid mixture.

beyond 20 γ to 80 γ; however, at the 100 γ level the general distortion of the amino acids with R_f's < 0.4 became reproducible and quite characteristic for each of the β-globulin and γ-globulin amino acid mixtures. From 150 γ to 400 γ the overloading of the chromatograms became obvious; resolution became increasingly poorer and the color* of practically all spots became so great that irrespective of the resolution only the grossest differences in amino acids were discernible. Hence, it was decided to set up a series of duplicate chromatograms containing 20 and 100 micrograms of protein (equivalent) for each hydrolyzed protein component.

The results obtained by careful visual comparison at daily intervals from 18 hours to 7 days after spraying with ninhydrin** may be summarized as a comparison of homologous components:

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* Not only did the color density increase beyond the point of usefulness in visual comparison, but the colors themselves tended to shift toward the reds and yellows especially in the regions of the highest amino acid concentration usually the center of the spots.

** New ninhydrin spray solution prepared according to Patton.[31] Patton reported 6% fading per year for all of these amino acids but the results in this laboratory were closer to 6% fading per day; furthermore, the rate of fading varied slightly for the various amino acids. Nevertheless, Patton's procedure represented a tremendous improvement over all previous methods.

A. Tube No. 82 (Sample 1-4, S_f = 4-8) and Tube No. 85 (Sample 1-1, S_f = 40-70) from patient V.H.

Chromatograms were quite similar; at most only the following dubious slight differences could be noticed

Glutamic	82 $\geq$ 85
Threonine	85 $\geq$ 82
Alanine	85 $\geq$ 82
X_1*	82 $\geq$ 85
X_2*	82 $\geq$ 85

Distortion of patterns similar.

Conclusion: The protein moieties of this set are essentially identical.

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* These ninhydrin-reacting compounds were never identified: Their R_f values were X_1 $R_f = 1.0_I$, $0.4\text{-}0.5_{II}$ - X_2 $R_f = 0.9 - 1.0_I$, $0.8\text{-}0.9_{II}$. (Note: X_1 moves adjacent to the top and front edge of the proline spot.) They were present in comparatively small relative amounts (about 1 - 2 percent of the total ninhydrin color) and were found in all the hydrolysates of this series including "β_1-lipoprotein" (Tube No. 119) but were completely absent from the synthetic β- and γ-globulin amino acid mixtures. They had been noticed only occasionally in previous study of delipidized protein hydrolysates but never with such reproducibility. The very large R_f values (in water-saturated phenol) of these two spots indicate that these compounds are small polypeptides (cf. Dr. Fred Sanger's investigations on insulin).

B. Tube No. 86 (Sample 2-4, S_f 4-8) and Tube No. 90 (Sample 2-1, $S_f \rightarrow 70$) from patient B.P.

Chromatograms were decidedly different; here are at least some of the differences, the more definitely discernible ones:

Leucine+isoleucine +phenylalanine	86 >> 90
Valine + methionine	86 > 90
Glutamic	90 > 86
Arginine	86 >> 90
Aspartic	86 > 90
Threonine	86 > 90
Tyrosine	86 > 90
Glycine	90 > 86
Proline	90 > 86
X_1	86 >> 90
X_2	86 >> 90

Distortion of patterns: quite different distortions especially at the 100 γ levels; e.g., no resolution for glutamic-threonine-alanine in No. 90 but definite separation of alanine from glutamic + threonine in No. 86. There were also definite differences in cystine + aspartic spot but distortions were very great.

Conclusion: The protein moieties of this set are almost as different as the synthetic β- and γ-globulin amino acid mixtures. Different protein moieties seem involved here.

C. Tube No. 93 (Sample I-3, S_f = 4-8) Tube No. 96 (Sample 288R-T_1, S_f = 10-20) and Tube No. 97 (Sample I-1, S_f = 25-60) from patient R.W.

No discernible differences at either the 20 γ or 100 γ levels.

Conclusion: Identical proteins are involved.

D. Tube No. 99 (AK - 1), Tube No. 104 (AK-4), Tube No. 109 (AK - 6), Tube No. 112 (AK - 2), Tube No. 114 (AK - 3), and Tube No. 116 (AK - 5) were chromatographed as unknowns.

No significant amino acid differences were discernible. The distortion of No. 114 in the R_f values <0.4 was very great*, but there seemed to be no significant amino acid differences between No. 114 and the rest.

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* In fact, the distortion was not eliminated until the sample size was decreased to 10 micrograms. This distortion, especially at the 100 microgram level, was so great as to be reminiscent of the unsuccessful early attempts in this investigation to remove the brownish "lipid" (?) interferences; examination of these papers under ultraviolet light prior to ninhydrin color development seemed to confirm this. The failure to remove as much of the "lipid" as was removed in all the other components of this series is all the more remarkable in view of the fact that this component was present in the lowest concentration throughout the denaturation-delipidization procedure and hence should have been the most completely delipidized of all the components. This would suggest some structural "abnormalities" and/or possibly some unusual lipid composition.

Conclusion: There are no significant differences in the protein moieties of these components. After completion of this chromatographic study, the identity of each of the "AK" samples was revealed to be as follows:

1. AK - 1 (S_f = 4-8) and AK-2 (S_f = 40-70) homologous set from patient A.K.
2. AK-6 (S_f = 4-8) and AK-3 (S_f = 40-70) homologous set from patient B.S.
3. AK-6 (S_f = 4-8) and AK-5 (S_f = 40-70) homologous set from patient F.A.

E. Tube No. 119 (β_1-Lipoprotein from Harvard Plasma Fractionation Laboratory, S_f - 2-20*).

Chromatograms of this component were first compared to those of synthetic β- and γ-globulin amino acid mixtures at corresponding levels. The following differences were readily discernible:

Valine + methionine	$\gamma > \beta >$ No. 119
Tyrosine	$\gamma > \beta >$ No. 119
Proline	γ and β both $\gg$ No. 119
Arginine	No. 119 $\geq \beta > \gamma$
Serine + histidine	$\gamma > \beta >$ No. 119
Glycine**	No. 119 $> \beta > \gamma$
Alanine***	$\beta >$ No. 119

* Sample has been preserved in distilled water at 0° C for 1 year; during this period there was no change in the appearance and the ultracentrifugal pattern of this sample.

** The glutamic acid spot of No. 119 $> \beta$, but threonine interference with glutamic spot of γ mixture made comparison rather dubious for the glutamic of γ mixture and No. 119.

*** Gamma-globulin mixture has no alanine.

X_1, X_2 } Both spots present in No. 119 but completely absent in the synthetic amino acid mixtures (β and γ) even at the 300 and 400 micrograms levels.

All the chromatograms for all these components at their respective levels were then compared with each other using those prepared from the hydrolysate of Tube No. 119 as an arbitrary standard of reference. With the exception of the chromatograms of Tube No. 90, there appeared to be no gross reproducible differences between any of the entire series of chromatograms, and those of Tube No. 119, irrespective of the floatation rates (S_f values) of the components.

On the basis of this investigation and in so far as comparative chromatographic amino acid studies of the hydrolysates of the protein moieties are a measure of the nature of these delipidized proteins, it can only be concluded that for five of the six sets of the homologous lipoproteins studied there are no gross differences in the protein moieties and that for twelve of the thirteen components (the hydrolysate of tube No. 90 being the exception) there are no gross differences in their protein moieties and no gross differences between them and that of the "standard" β_1-lipoprotein (Tube No. 119).

SUMMARY

The significant achievements of this investigation may be listed as follows:

1. Development of a satisfactory small-scale procedure for denaturing and delipidizing homologous sets of serum lipoproteins.
2. Development of a technique for the chromatography of whole native lipoproteins and other proteins.
3. Development of a new quantitative ultramicro procedure for the determination of protein after hydrolysis.
4. Development of satisfactory qualitative, semi quantitative and quantitative chromatographic techniques for analyzing the protein hydrolysates of homologous sets of serum lipoproteins.
5. Preliminary application of these techniques to the analysis of a limited number of homologous sets of human serum lipoproteins.

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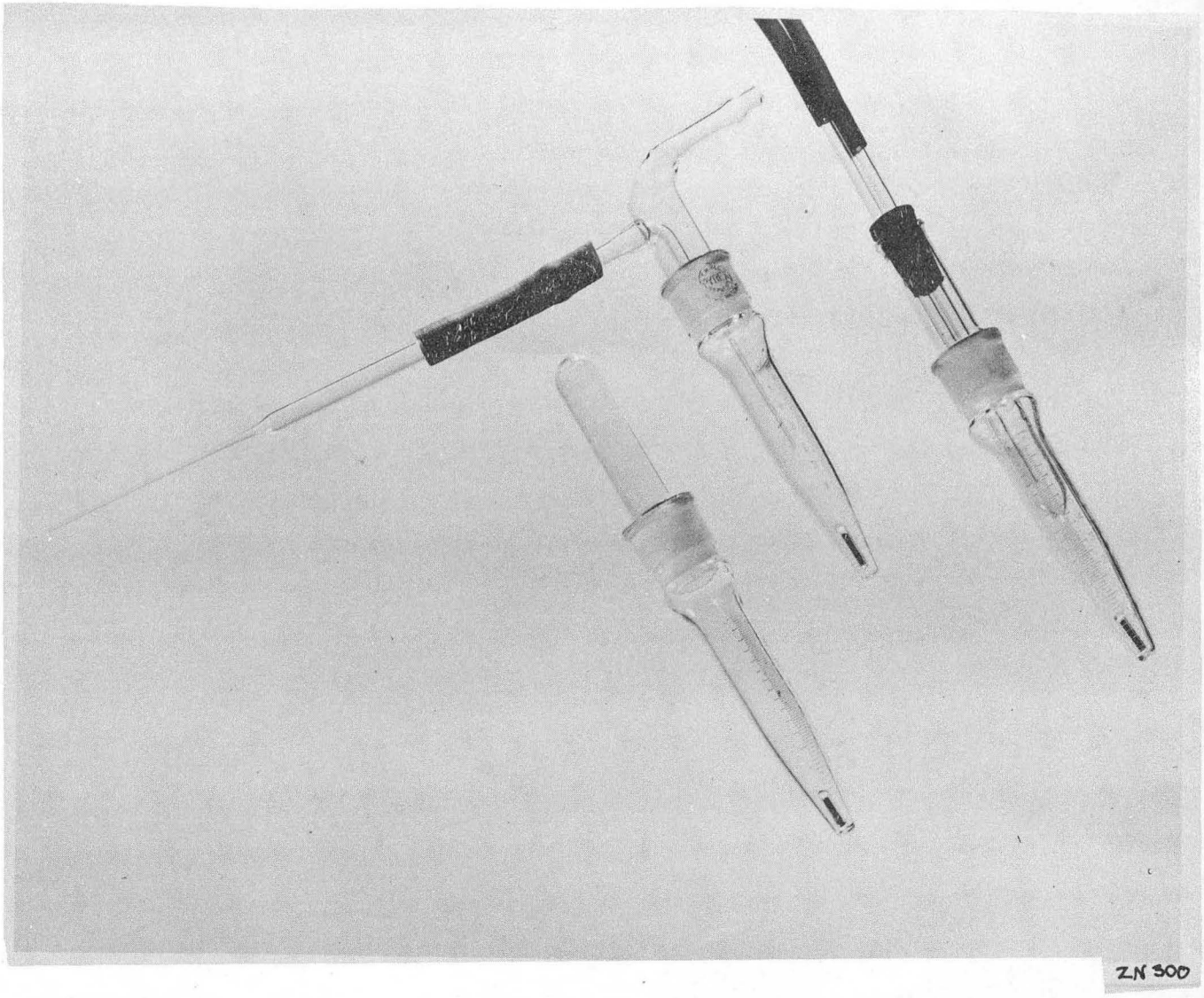

Fig. 1 Centrifuge-hydrolysis tubes as used in denaturation, delipidization, and hydrolysis of lipoproteins. From left to right, tubes equipped with an air condenser, solvent remover, and water condenser.

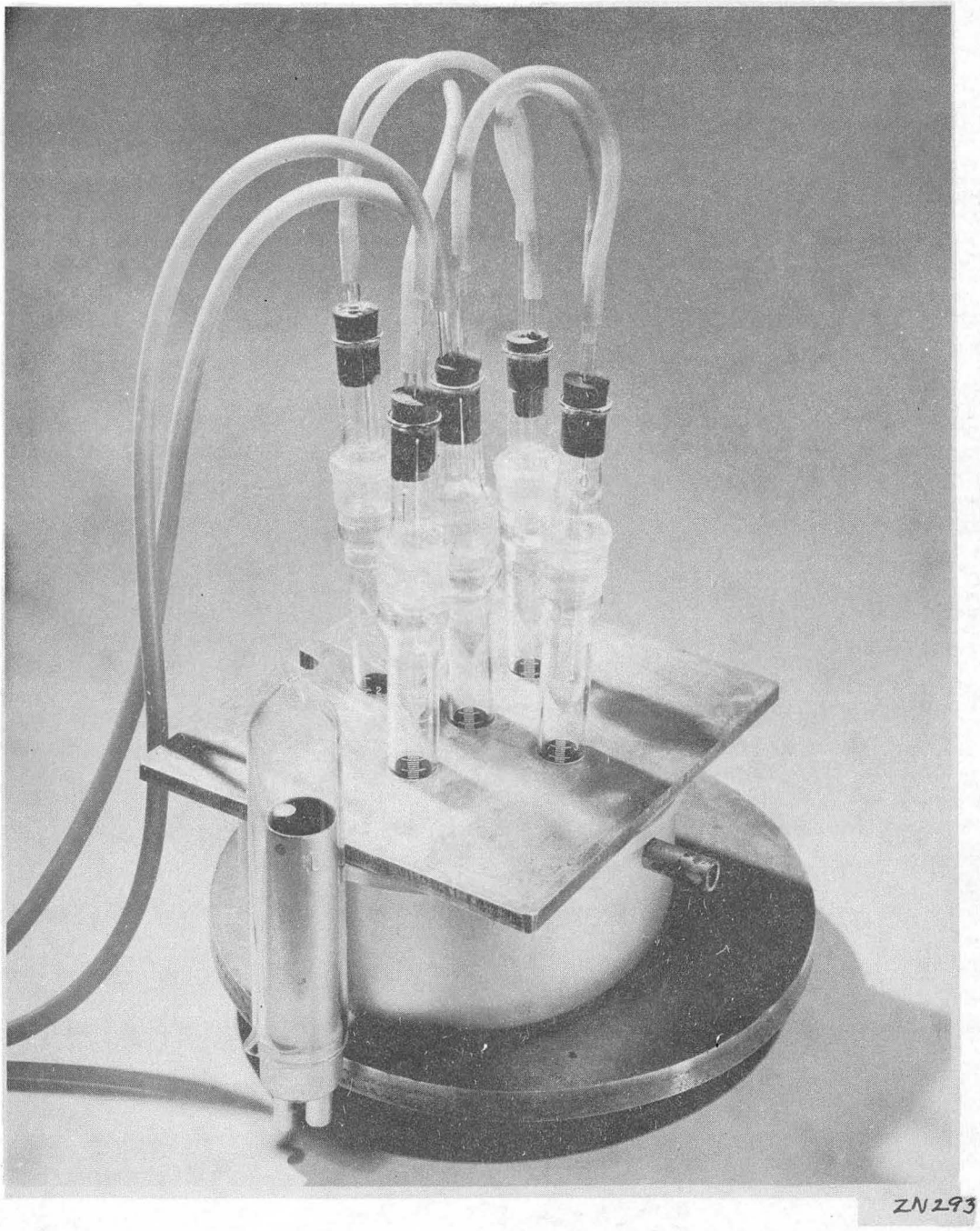

Fig. 2 Protein Hydrolysis. Use of the "centrifuge-hydrolysis" tube for the hydrolysis of small amounts of proteins, 12 ml. graduated centrifuge tubes, modified, equipped with condensers.

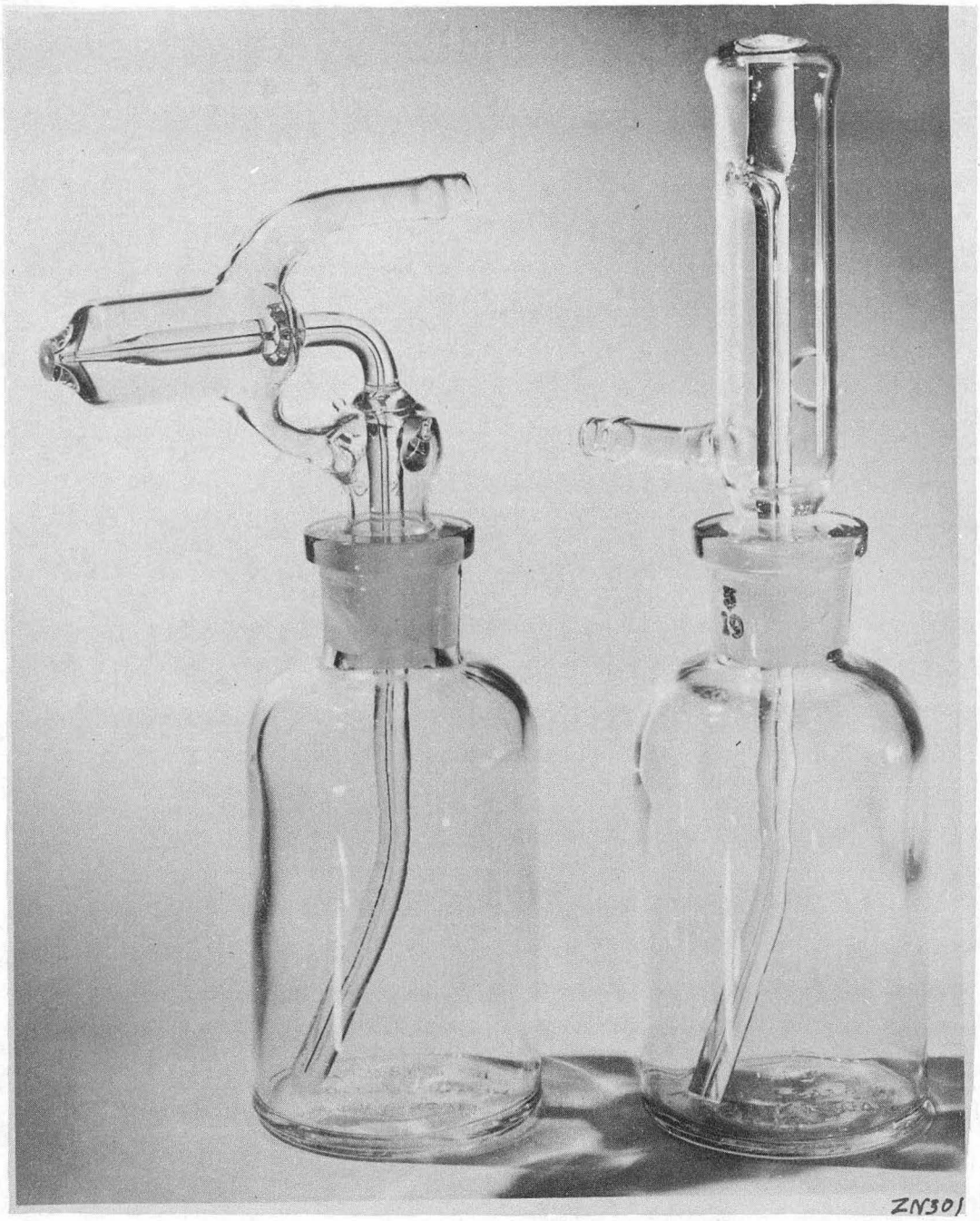

Fig. 3 All-glass sprayers or atomizers for use in paper chromatography. Designed and made by Mr. Harry Powell of the U.C.R.L. Glass Shop.

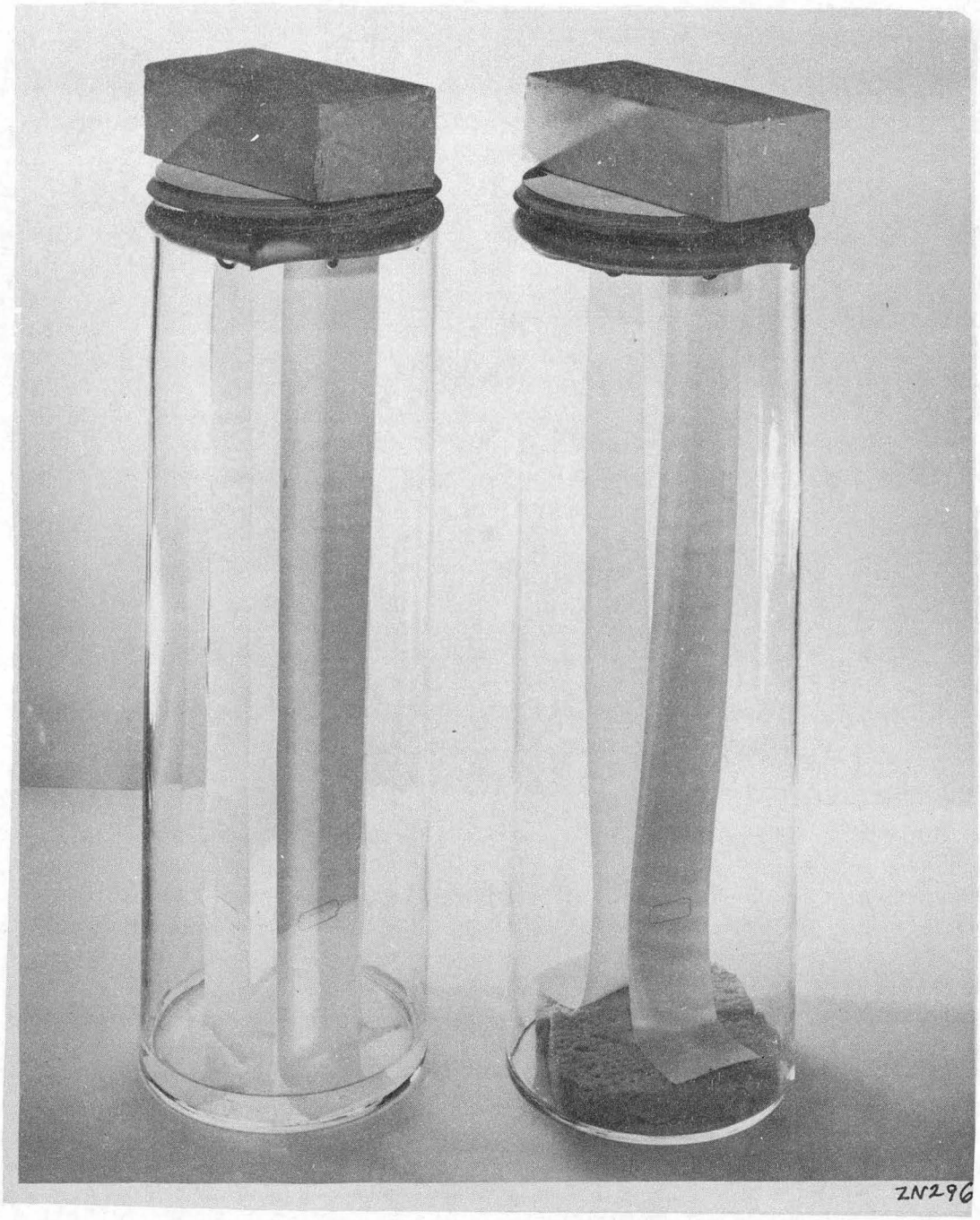

Fig. 4 Paper chromatography. Large-scale, one-dimensional, ascending, 6 in. x 18 in. jars. Jar on right represents arrangement for preventing drying of the samples prior to chromatographic development.

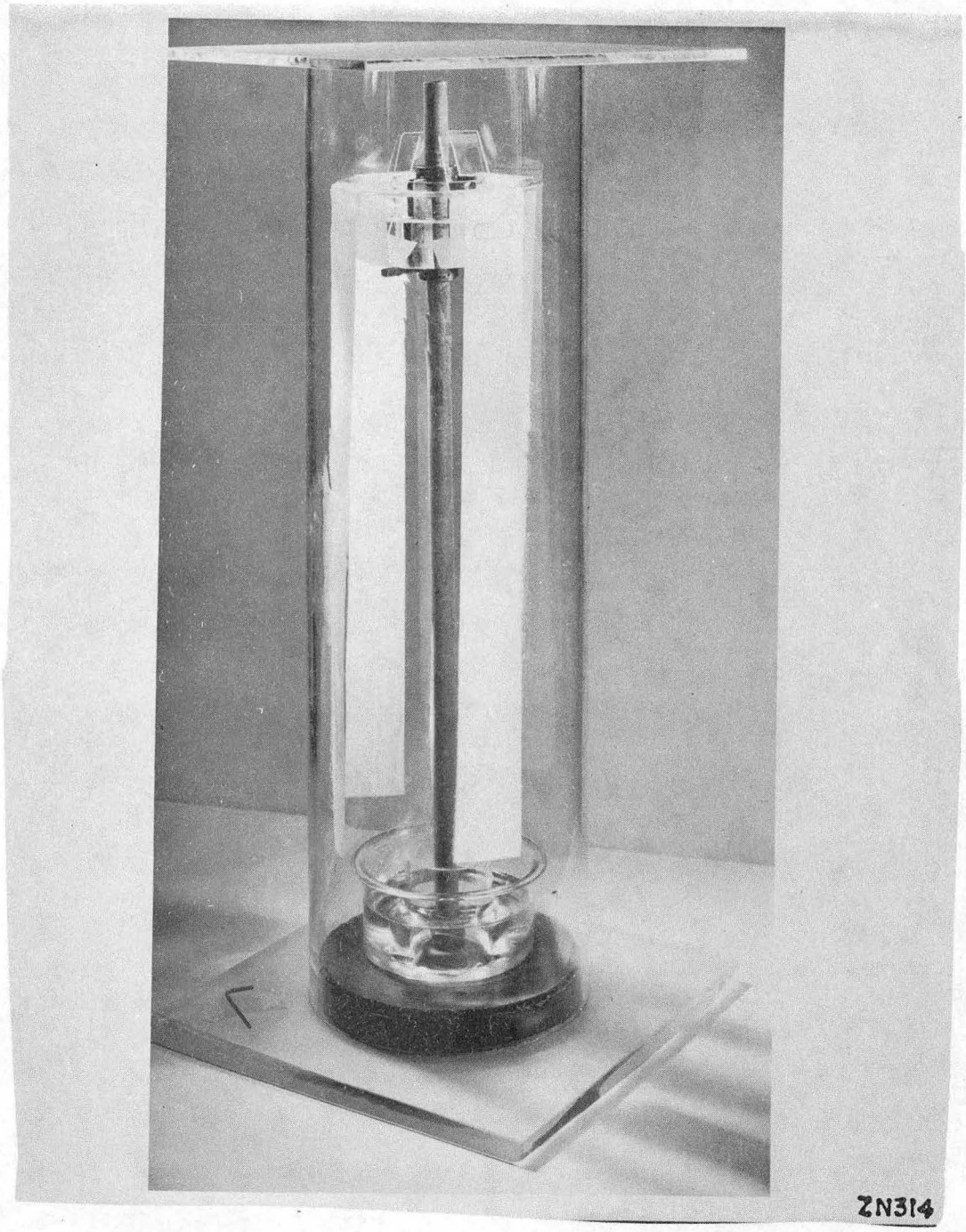

Fig. 5 Paper chromatography. Large-scale, one-dimensional, descending. Glass "doughnut" type trough.

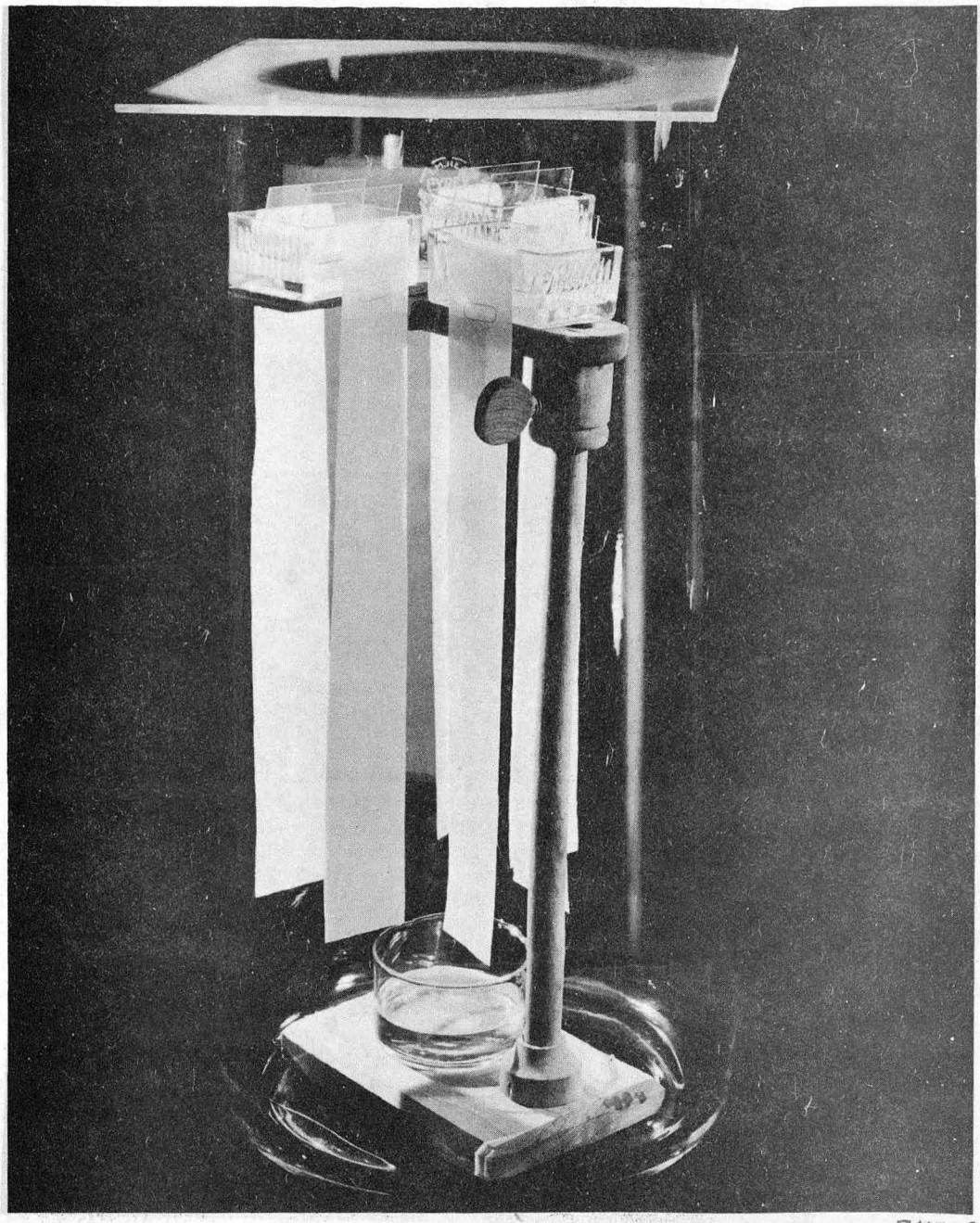

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Fig. 6 Paper chromatography. Large-scale, descending, one-dimensional using staining dishes and glass jar (12 in. x 18 in.).

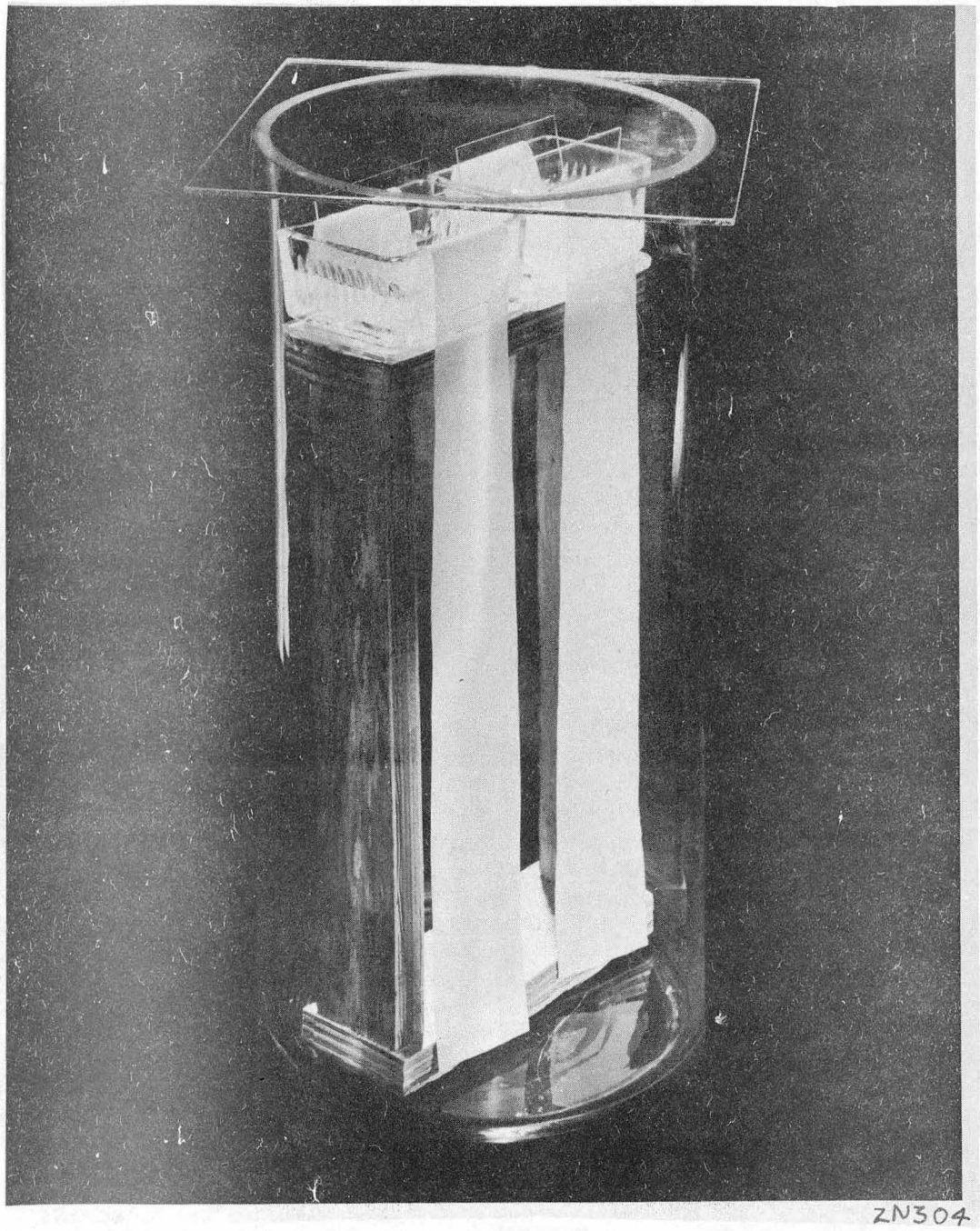


Fig. 7 Paper chromatography. Large-scale, descending, one-dimensional using staining dishes and glass jar 8 in. x 18 in.

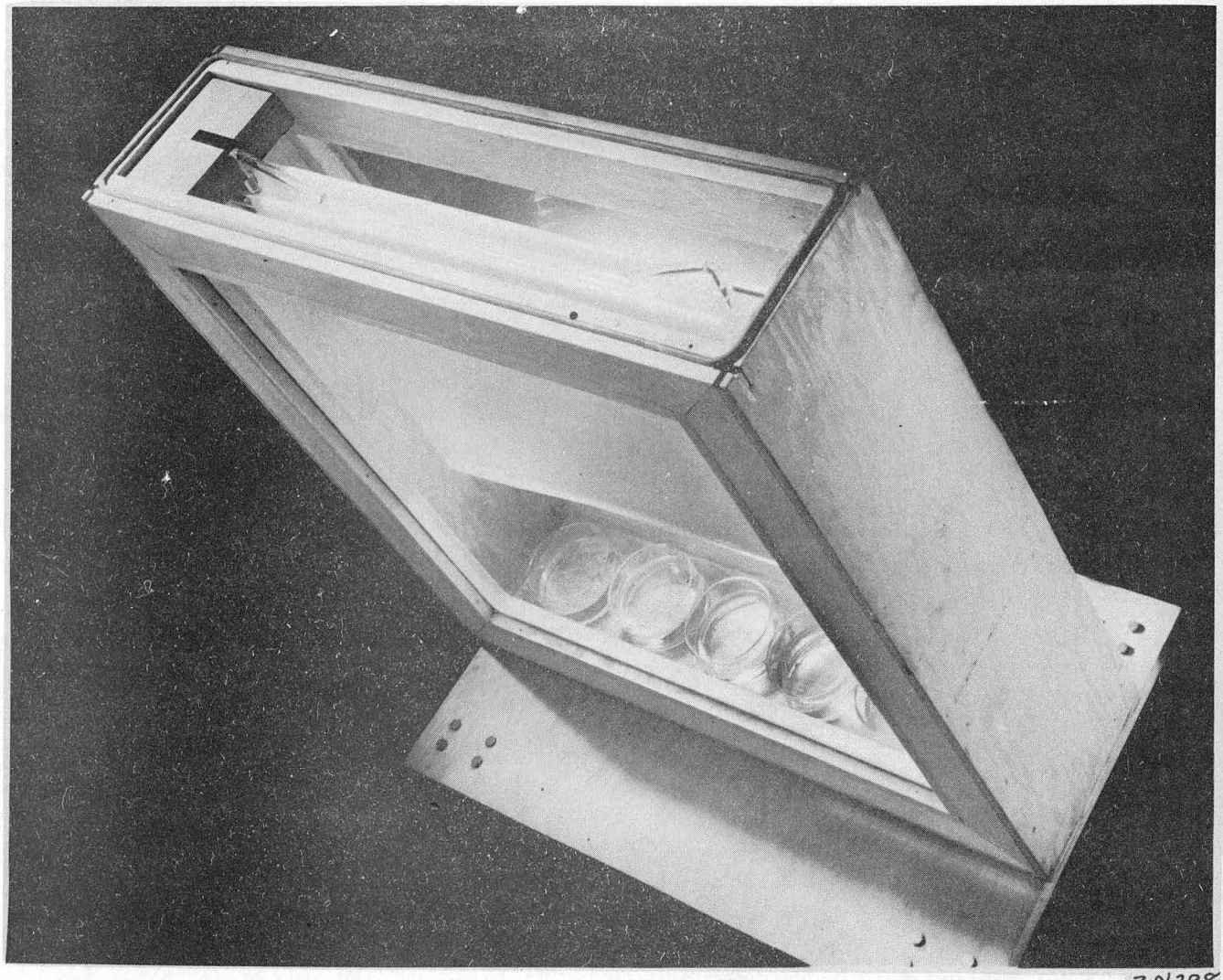


Fig. 8 Paper chromatography. Large-scale, two-dimensional, descending "window sash" type, top removed.

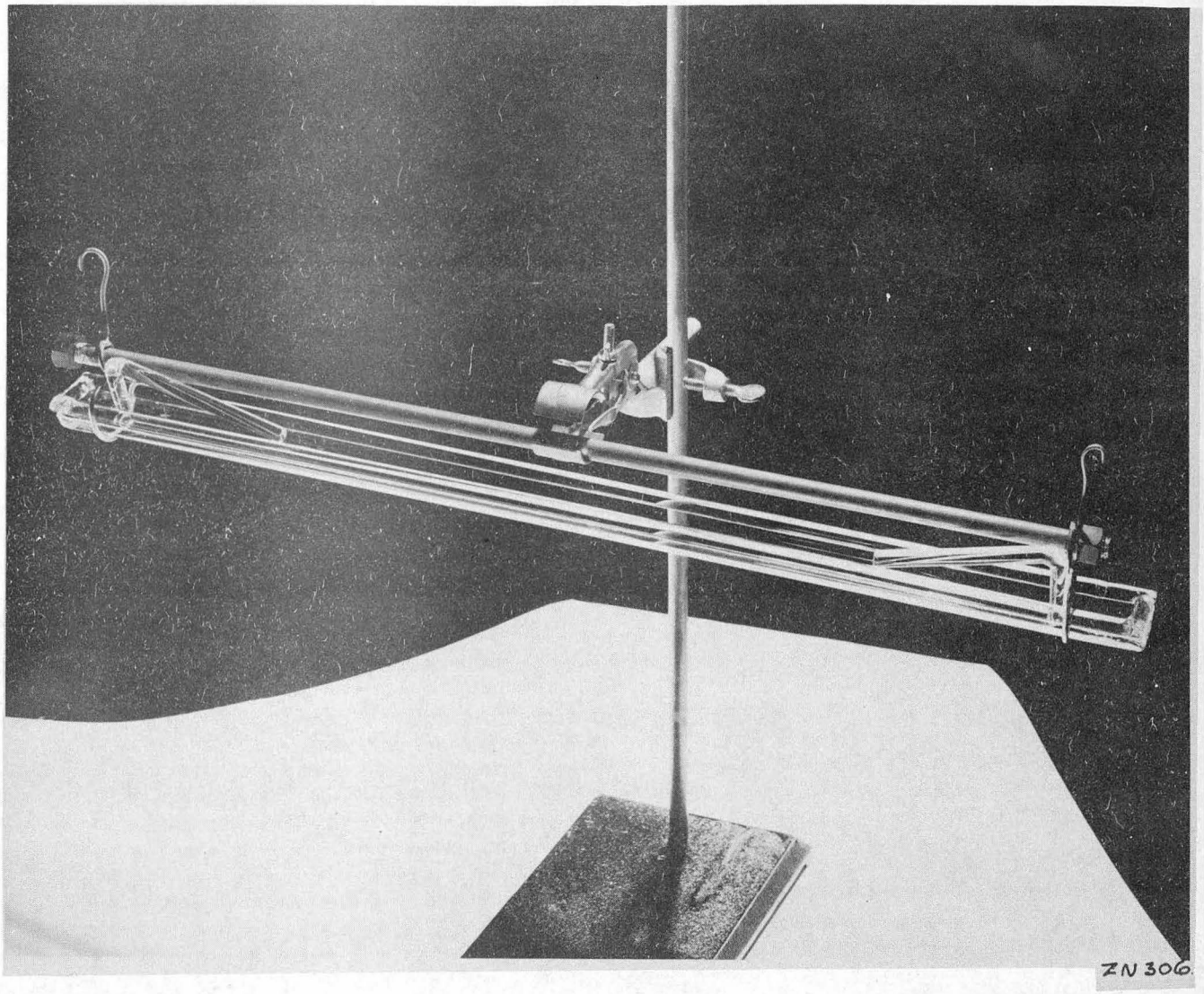


Fig. 9 Paper chromatography. Frame used to hold solvent trough and accessories during the drying of the large-scale, two-dimensional, descending paper chromatograms.

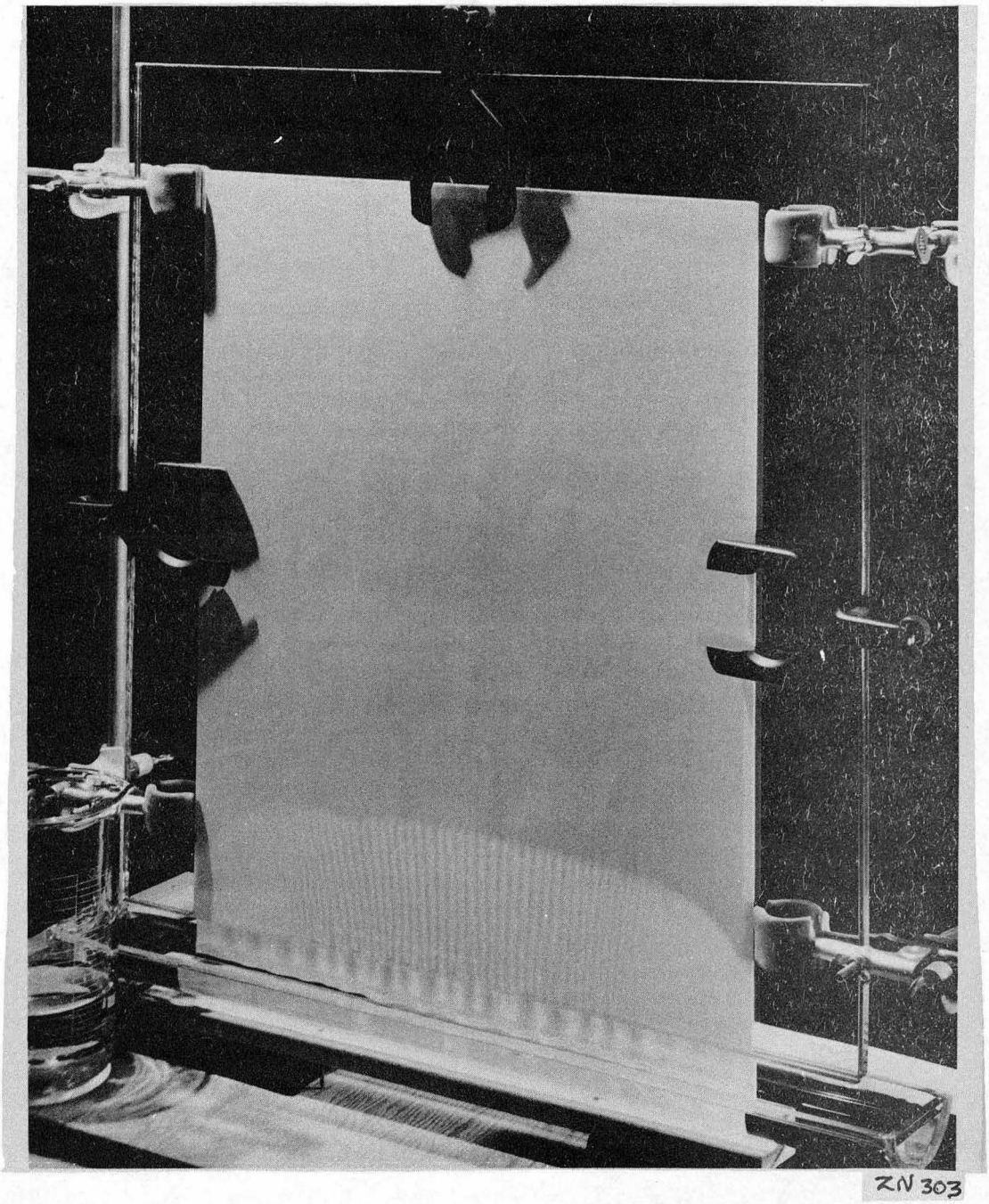


Fig. 10 Paper chromatography. Experimental set-up used to check the mechanism of ascending chromatography, to chromatograph volatile compounds, and to increase the capacity of paper chromatograms by the use of stacks of filter paper. A stack of five sheets of Whatman No. 1 filter paper is being used here.

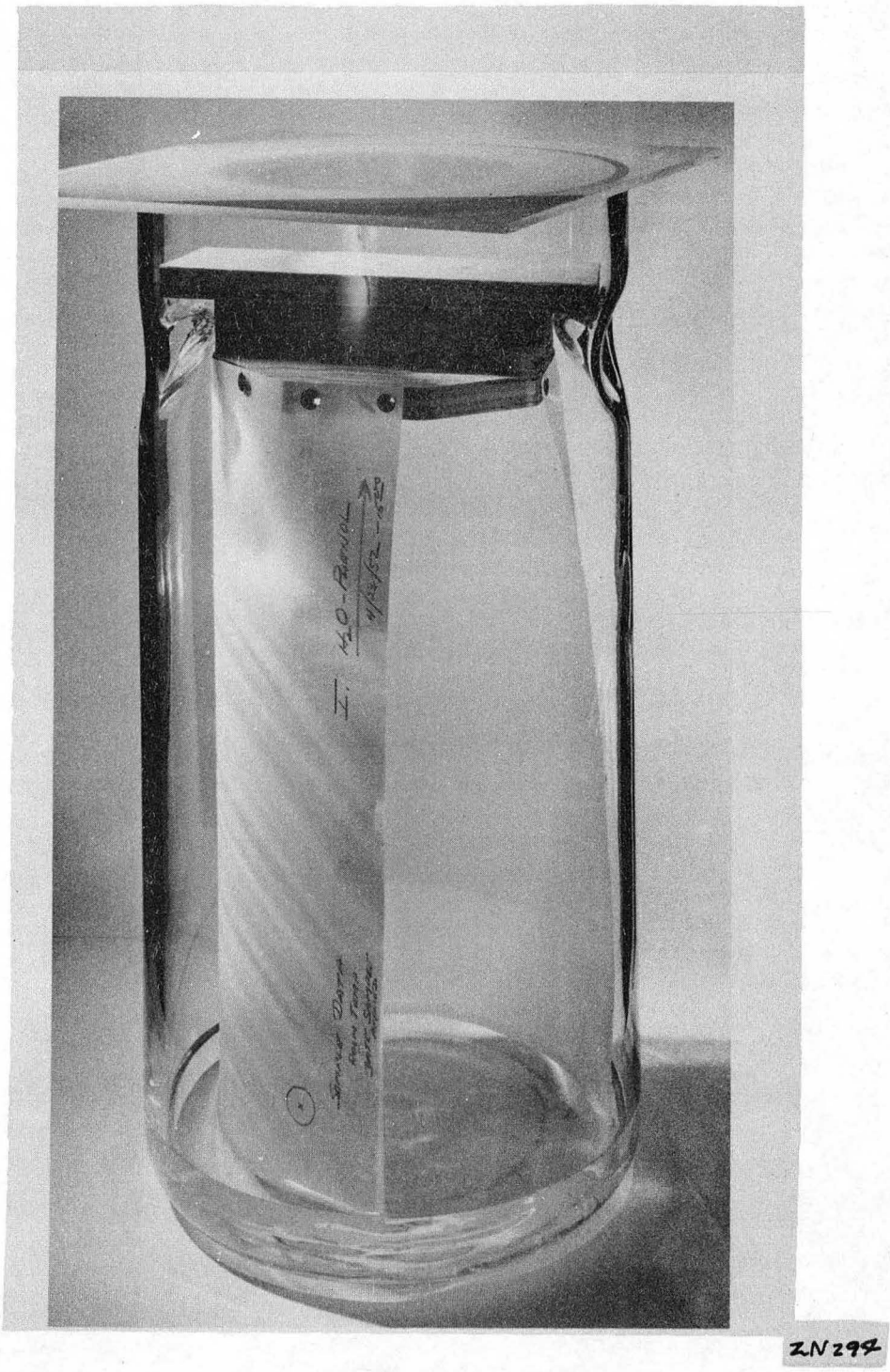


Fig. 11 Paper chromatography. Large-scale, two dimensional, ascending. Jar 9-1/2 gallons.

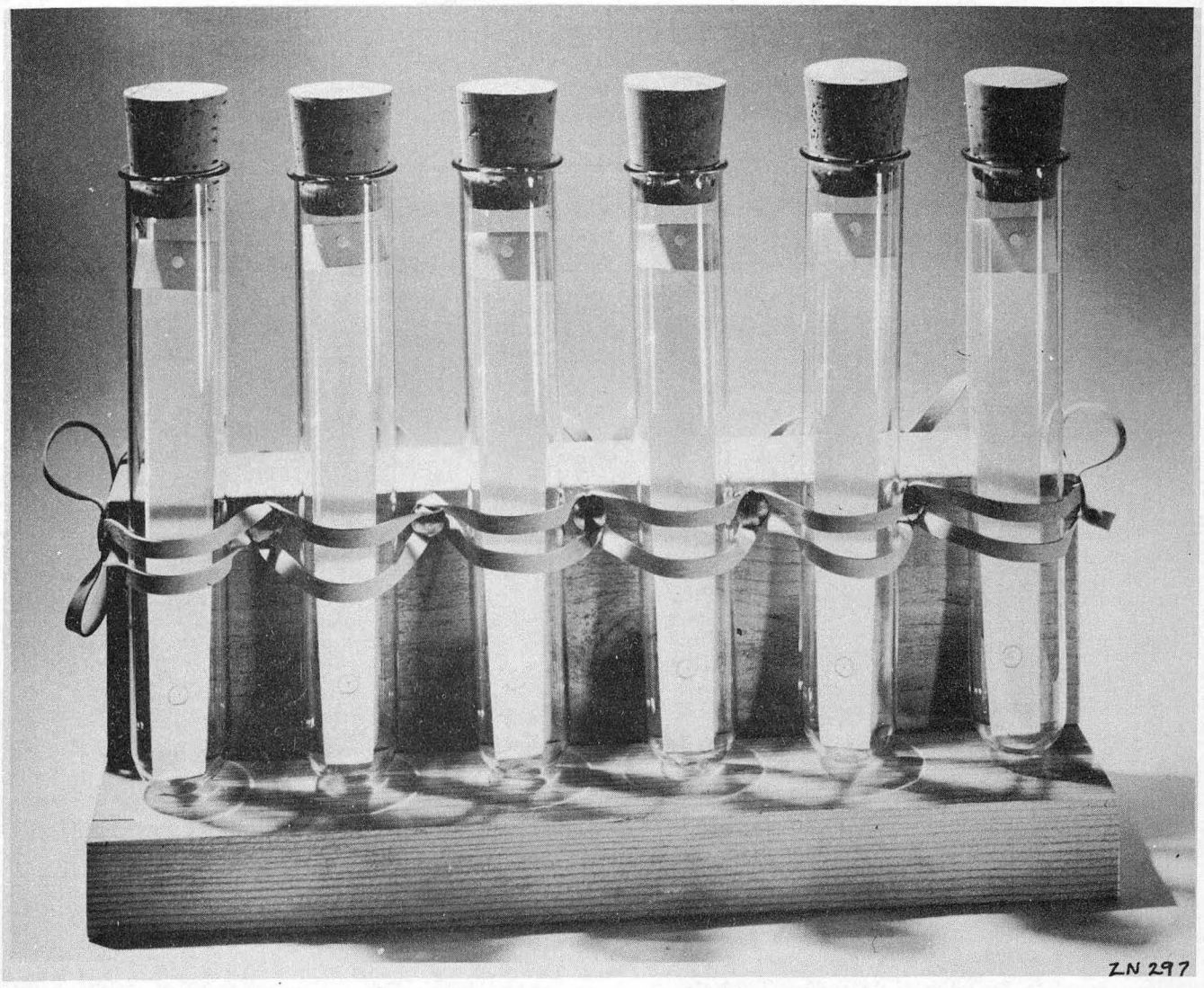


Fig. 12 Paper chromatography. Small Scale, one-dimensional, ascending, 25 x 200 mm test tubes.

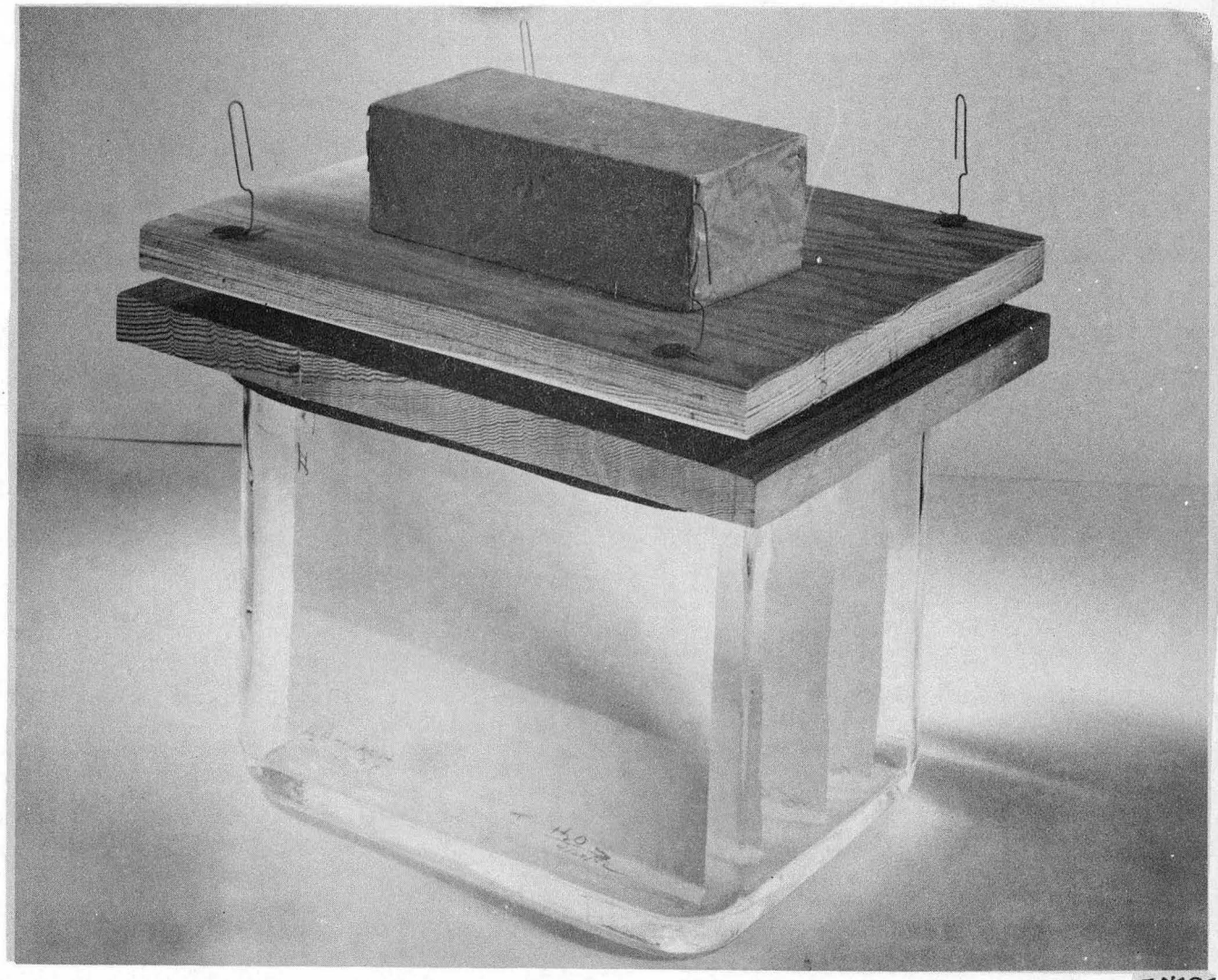


Fig. 13 Paper chromatography. Small-scale, two dimensional, ascending. Fish bowl chamber, 2-1/2 gallons.

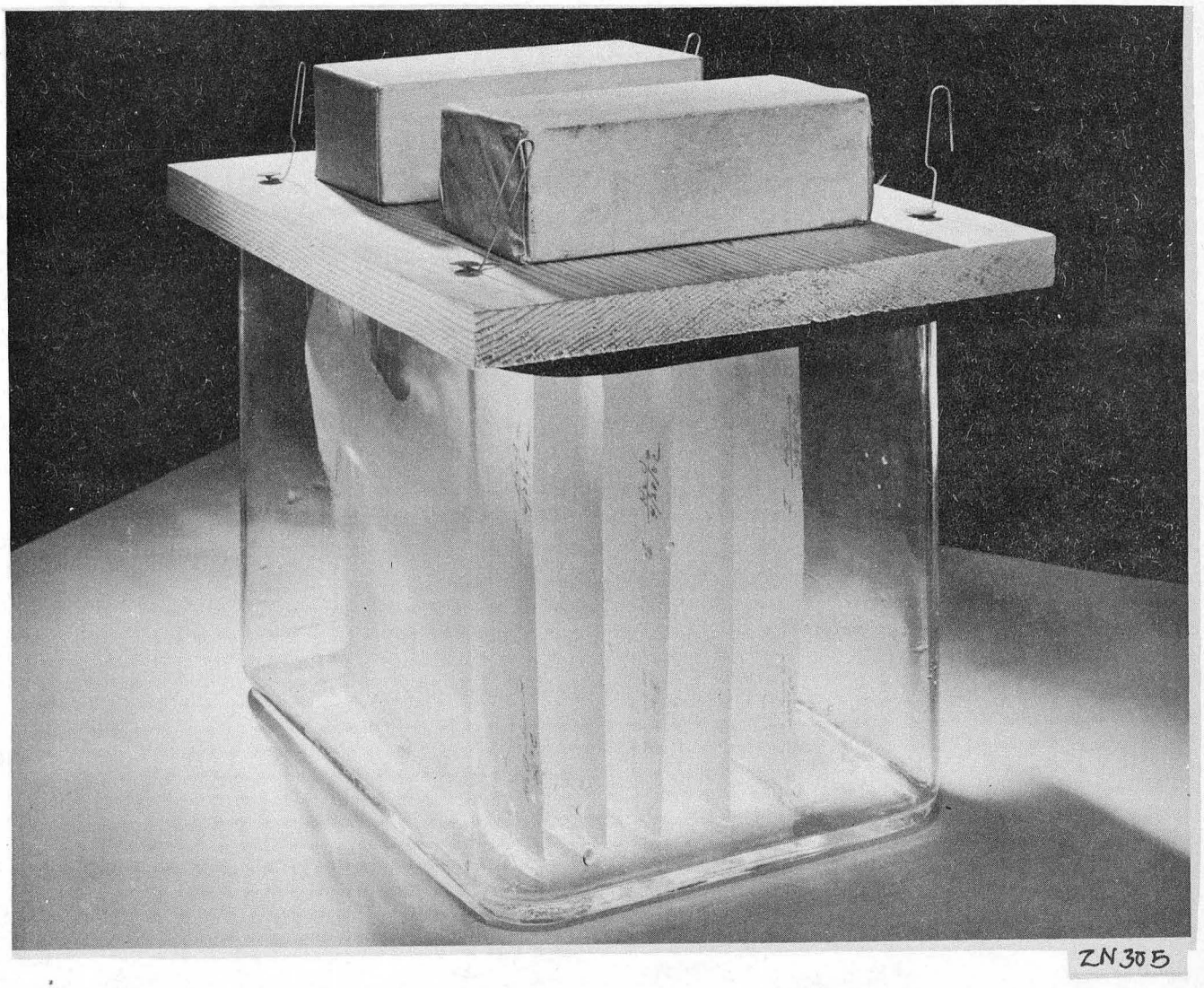


Fig. 14 Paper chromatography. Small-scale, two-dimensional ascending. Fish bowl chamber, three gallons. (one paper is missing in this illustration.)

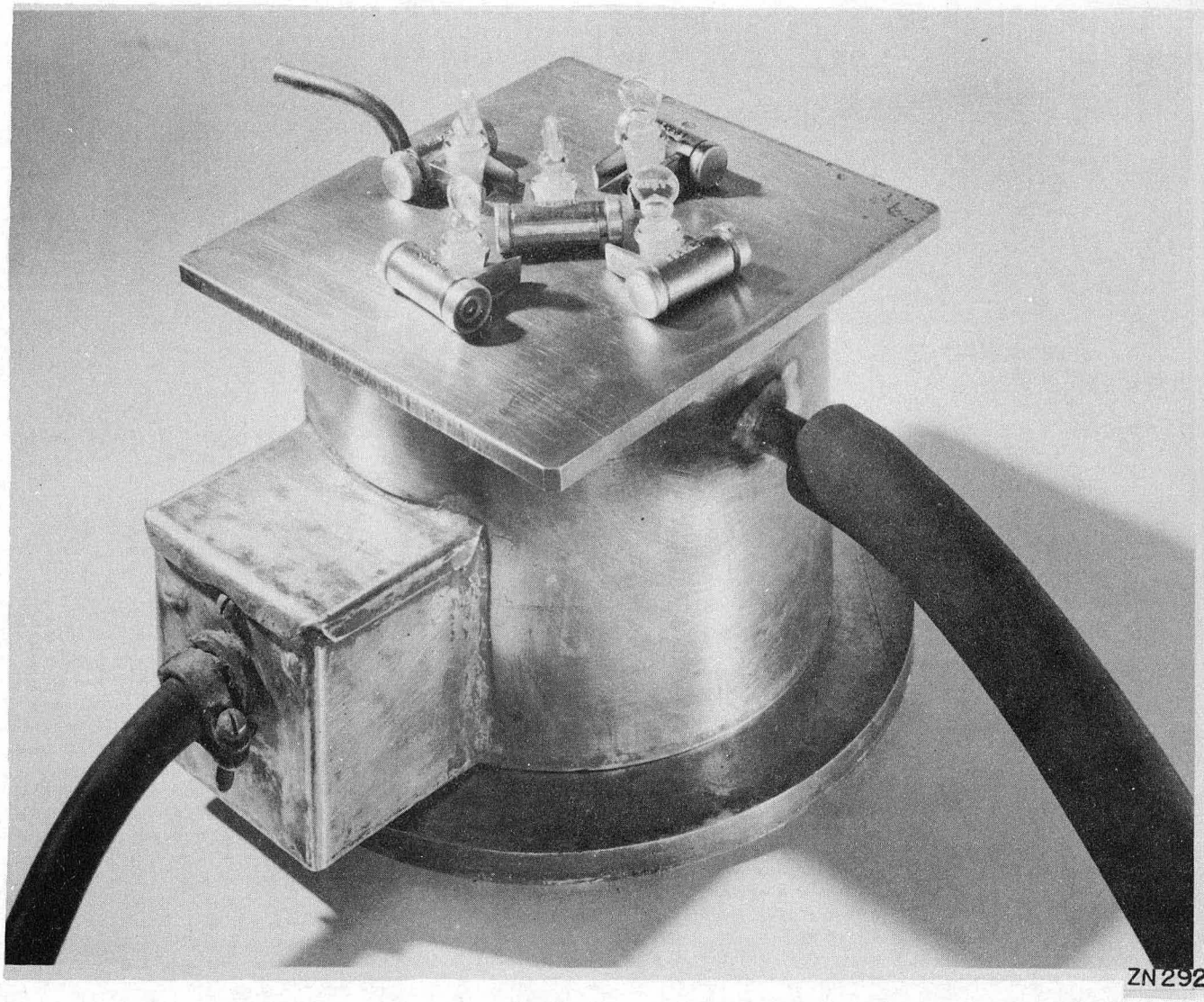


Fig. 15 Ninhydrin Color Development-Brass plate and clamps for holding 5-ml. volumetric flasks and a steam bath containing a 1000-watt immersion heater.

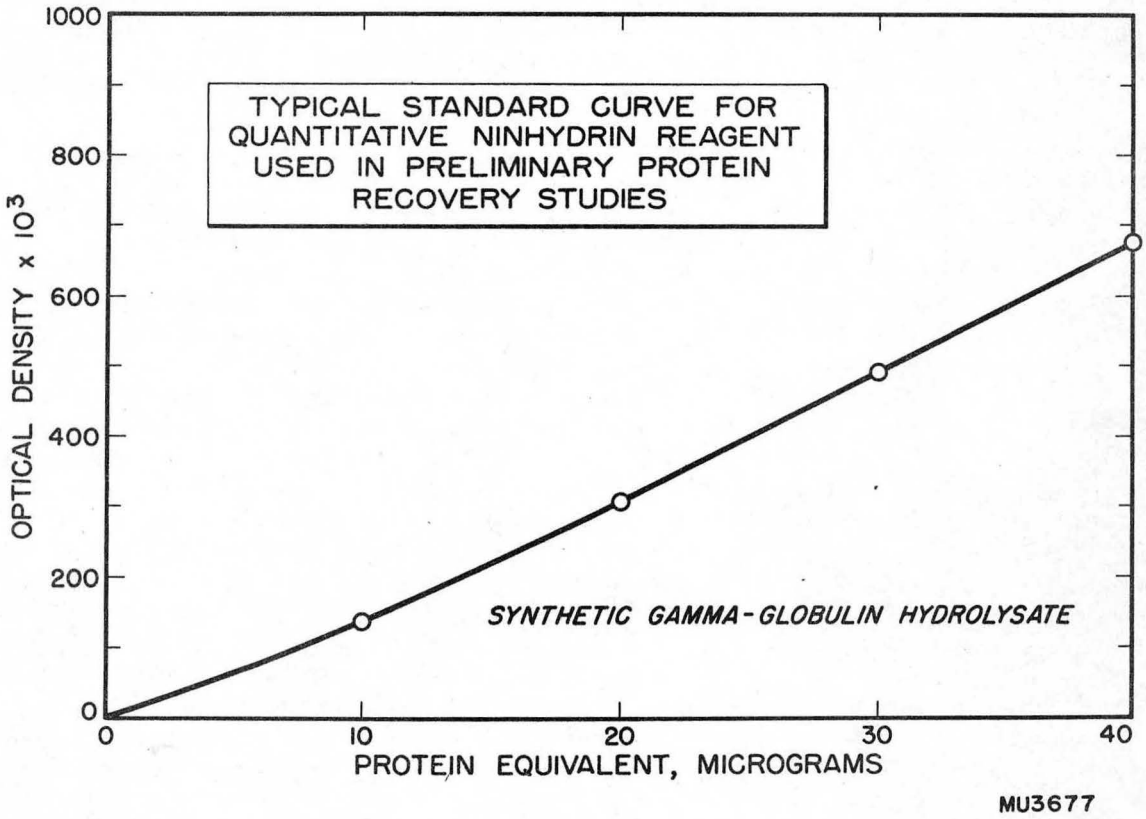
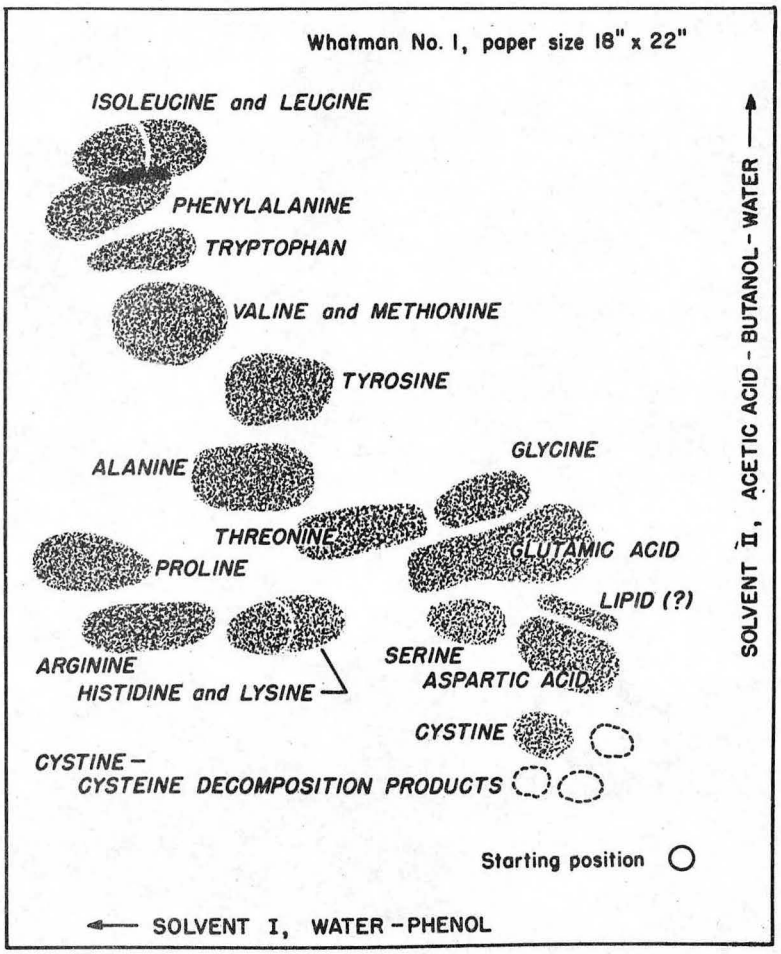

TYPICAL STANDARD CURVE FOR QUANTITATIVE NINHYDRIN REAGENT USED IN PRELIMINARY PROTEIN RECOVERY STUDIES
SYNTHETIC GAMMA-GLOBULIN HYDROLYSATE
OPTICAL DENSITY x 10^3
1000
800
600
400
200
0
0
10
20
30
40
PROTEIN EQUIVALENT, MICROGRAMS
MU3677

Fig. 16



MU3681

Fig. 17 - A typical amino acid distribution pattern for a protein hydrolysate obtained by descending chromatography using Whatman No. 1 filter paper and run at 29 5° C. The solvent systems used were (I) water-saturated phenol and (II) a mixture of acetic acid, n-butanol, and water (1.0: 4.6: 2.7 parts by volume).

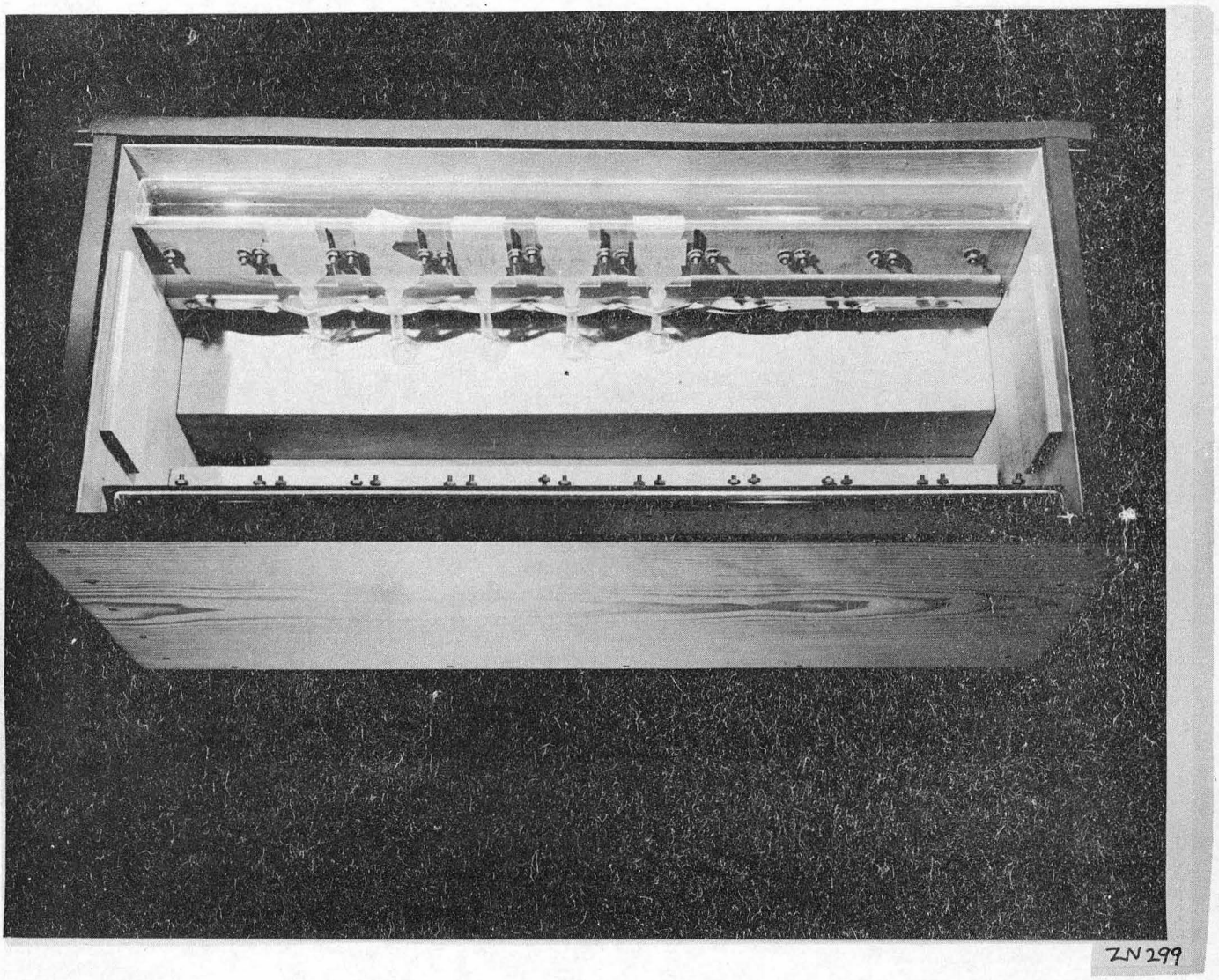


Fig. 18 Paper chromatography. Device for chromatographic elution of compounds separated by paper chromatography.

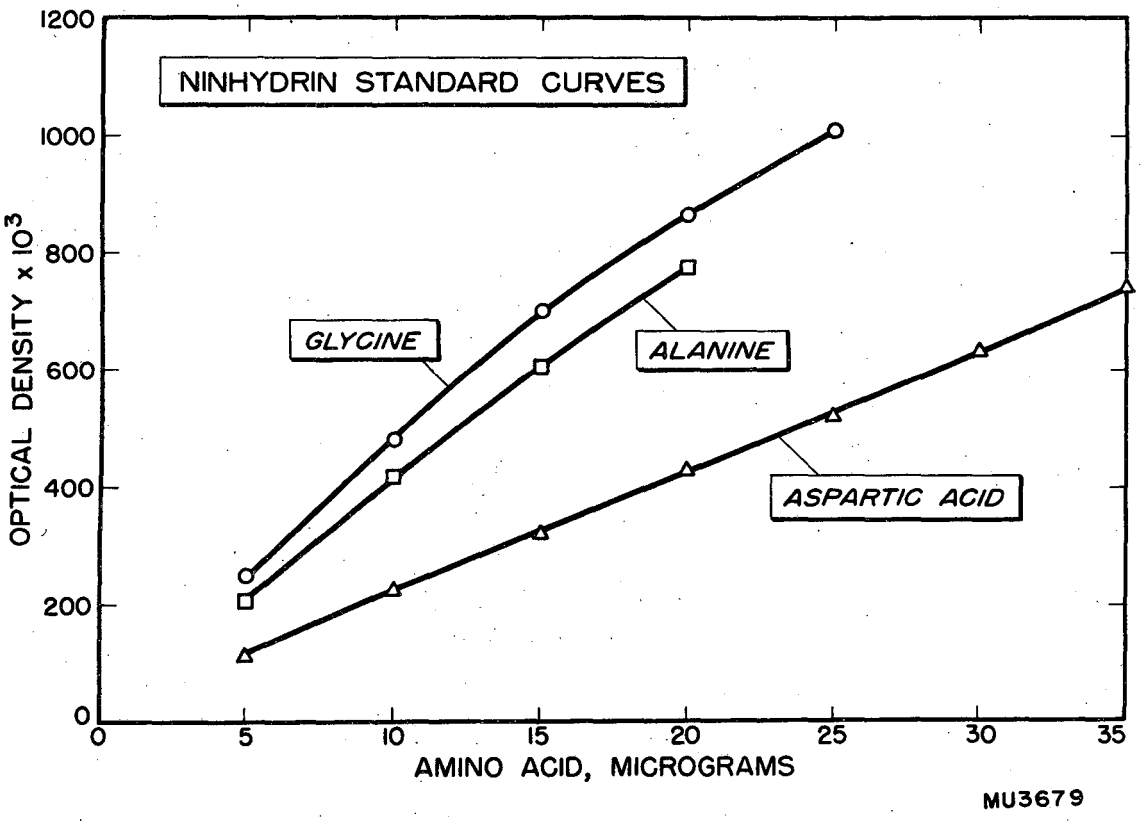

NINHYDRIN STANDARD CURVES
GLYCINE
ALANINE
ASPARTIC ACID
OPTICAL DENSITY x 10³
AMINO ACID, MICROGRAMS
0
200
400
600
800
1000
1200
0
5
10
15
20
25
30
35
MU3679

Fig. 19

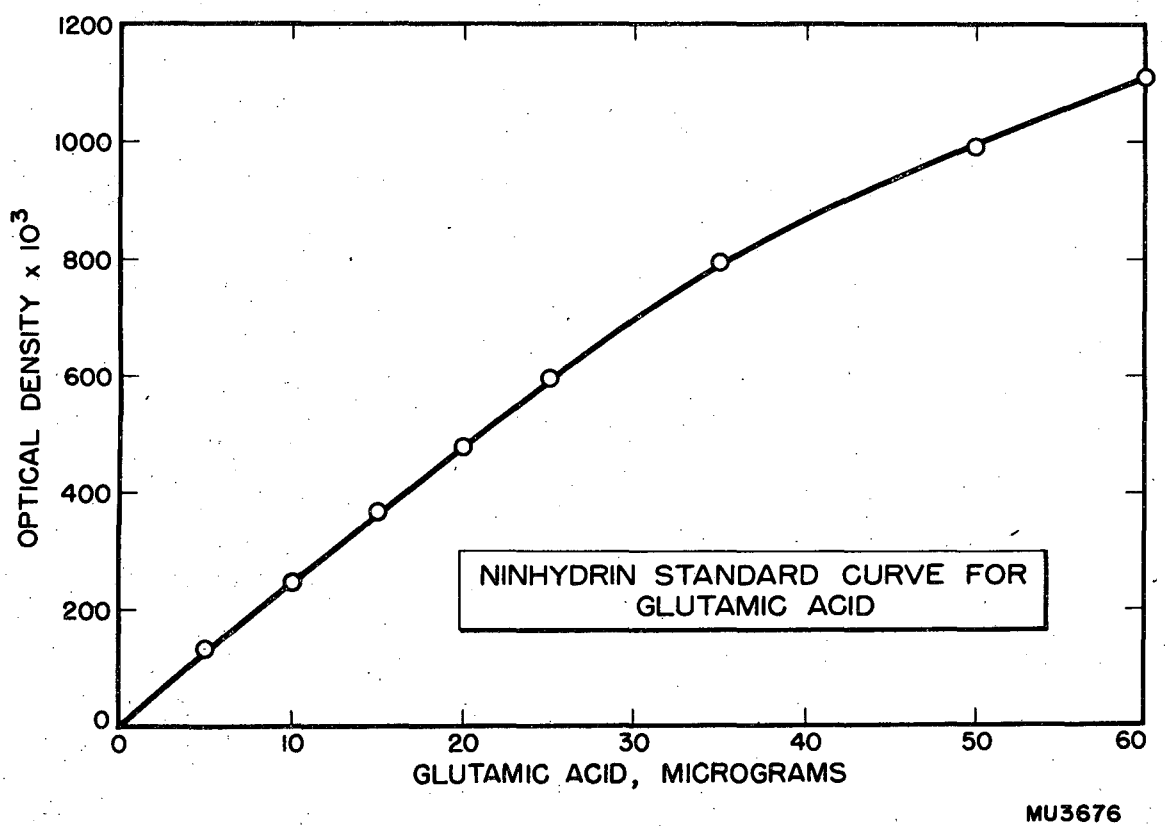

NINHYDRIN STANDARD CURVE FOR GLUTAMIC ACID
OPTICAL DENSITY x 10^3
GLUTAMIC ACID, MICROGRAMS
0
200
400
600
800
1000
1200
0
10
20
30
40
50
60
MU3676

Fig. 20

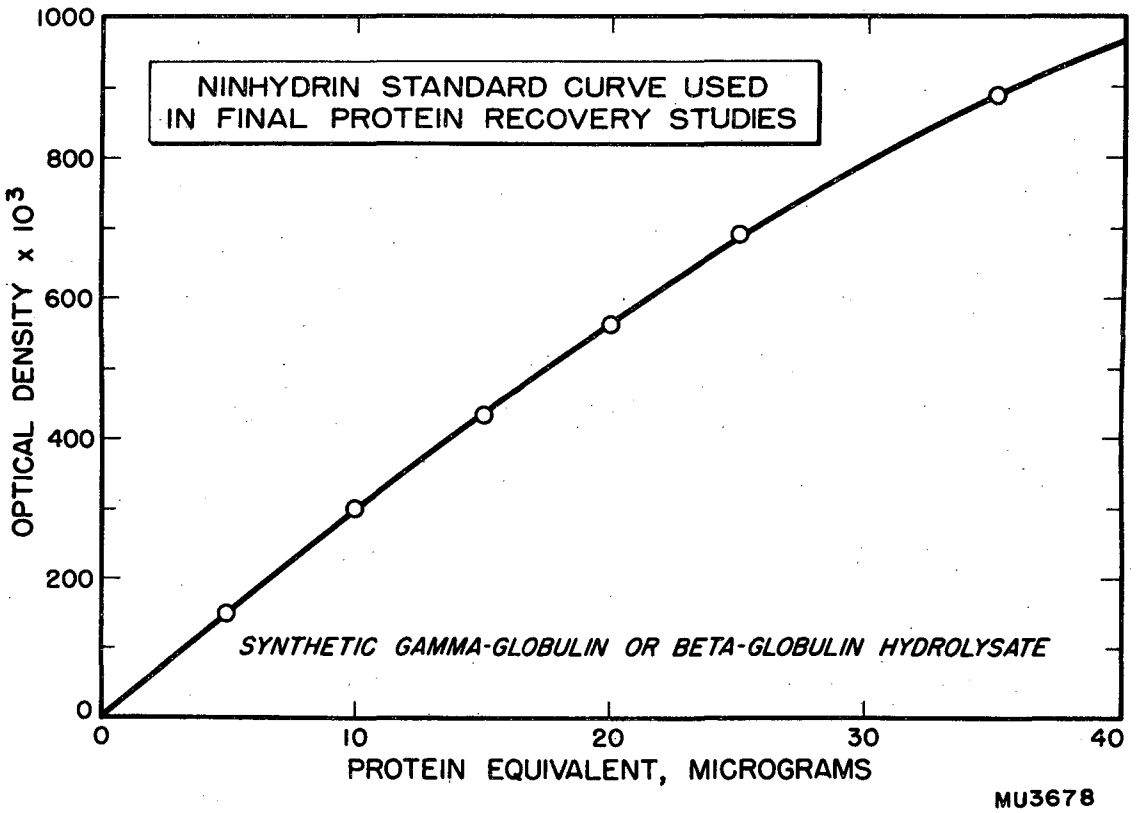
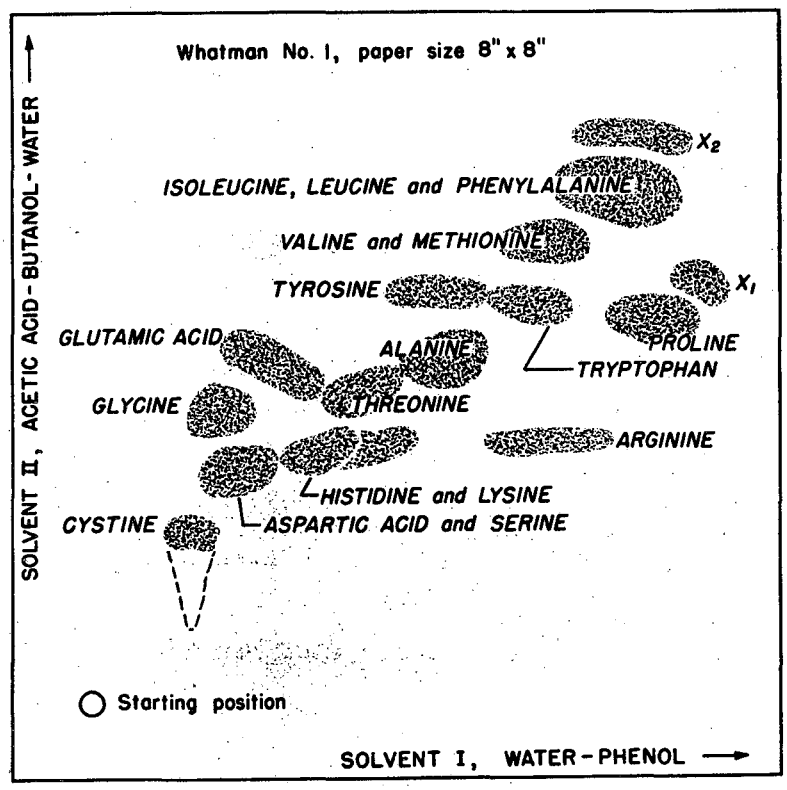

NINHYDRIN STANDARD CURVE USED
IN FINAL PROTEIN RECOVERY STUDIES
SYNTHETIC GAMMA-GLOBULIN OR BETA-GLOBULIN HYDROLYSATE
OPTICAL DENSITY x 10^3
1000
800
600
400
200
0
0
10
20
30
40
PROTEIN EQUIVALENT, MICROGRAMS
MU3678

Fig. 21



MU3680

Fig. 22 - A typical amino acid distribution pattern obtained with synthetic β- and γ-globulin mixtures. Experimental Conditions: Ascending Chromatography, Filter Paper 8 in. x 8 in. (Whatman No. 1); temperature 16° C Solvents (I) Water-saturated Phenol and (II) Acetic Acid-Butanol-Water (ratio by volume 1.0:4.1:2.7). Running time 8-9 hrs. for each solvent.