with the content of the same weight of plasma protein has been recorded.

TABLE VIII

ASSAY FOR PROTHROMBIN AND PLASMINOGEN ACTIVITY

Values are in units/cc. plasma

Run	Plasma	(II + III)	II + III Susp.	II + IIIW	III	III-2	III-3
			Prothombin				
171	82	63	75	70	46	..	..
173	100	54	91	60	..	..	..
175	...	95	89	50	41	..	..
176	58	83	86	60	37	..	..
177	72	61	38	17	..	..	..
178–179	88	58	18	190	35	..	..
180–181	56	68	32	55	37	34	..
182–183–184	68	73	24	18	27	..	..
185–186	63	78	53	31	21	..	..
S362-1,2	...	..	62	73	81	78	..
			Plasminogen				
S-362-1,2	...	..	32	27	30	(21)	14

Typical assays for prothrombin and plasminogen activity are recorded for the various fractions in Table VIII.[61] Assays for both of these substances are somewhat uncertain, probably due largely to the presence of inhibitors which make it difficult to completely convert prothrombin to thrombin and plasminogen to plasmin without decomposition. Little or no prothrombin and plasminogen is found in any fraction other than III-2 and III-3.

(61) These studies have been made by Miss S. G. Miller and one of us (D. A. Richert), under the supervision of Professor J. T. Edsall. The methods used are recorded elsewhere.[34,35]

Summary

1. The properties of various protein components of Fraction II + III of normal human plasma are reviewed.

2. Methods are outlined for the separation of Fraction II + III into a series of subfractions by low-temperature ethanol precipitation, in which careful control of the *p*H, temperature, and concentration of ethanol, salt and protein have been achieved. The subfractions so obtained have led to satisfactory separation and concentration of the following components: γ-globulin antibodies in Fraction II, isoagglutinins in Fraction III-1, prothrombin in Fraction III-2, plasminogen in Fraction III-3 and β_1-lipoprotein in Fraction III-0.

3. Studies of the distribution of components into these subfractions are tabulated. They have involved measurements of protein nitrogen, dry weight of protein, cholesterol, prothrombin activity, plasminogen activity and various antibody activities.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

Filter Paper Chromatography

BY HENRY B. BULL, J. WILFRID HAHN AND VICTOR H. BAPTIST

Consden, Gordon and Martin[1] reported a very ingenious method for the separation of amino acids by filter paper chromatography in which phenol or some other appropriate organic solvent sweeps the applied amino acids along the filter paper, capillarity causing the organic solvent to move. The positions of the amino acids are located by spraying the filter paper with a solution of ninhydrin and heating the filter paper strips. The amino acids develop colors, the intensity and tint of which depend upon a number of factors. A review of filter paper chromatography has recently appeared.[2]

The present paper reports an attempt to make filter paper chromatography of amino acids quantitative. The percentage light intermission along the chromatogram is measured and this transmission has been plotted on semi-log graph paper against the distance along the filter paper strips, and the areas of the segments above the plotted curve measured with a planimeter. It has been found that the areas so determined are over a limited concentration range a simple function of the concentration of the amino acids. The various factors which influence the reliability of this method are reported.

(1) Consden, Gordon and Martin, *Biochem. J.*, **38**, 224 (1944).

(2) Consden, *Nature*, **162**, 359 (1948).

Experimental

Carl Schleicher and Schuell quantitative filter paper number 507 was cut against the machine direction into strips 7.20 mm. wide and 60 cm. long. One end of the filter paper strip was placed in the bottom of an oblong butter dish and a thick glass strip placed on top of the paper to hold it in position. It was found convenient to use 5 such strips hanging at about 2 cm. intervals over each side of the butter dish. The lower ends of the filter paper strips were attached to small metal clamps affixed to a metal rod. 0.0135 cc. of the amino acid solution adjusted to a *p*H 5 to 7 was placed on a marked spot on the filter paper strip with a Blodgett pipet about 4 cm. from the edge of the butter dish. The filter strips were in a horizontal position during this operation. The amino acid solutions were air dried on the filter paper and the butter dish, with the filter paper strips hanging vertically, was placed on a stage in a tall glass jar. The jar had a layer of 80% aqueous solution of Merck reagent grade of phenol on the bottom. Eighty per cent. aqueous solution of phenol was poured into the butter dish to cover the ends of the filter paper strips. A second glass jar was inverted over this jar and the joint between the jars sealed with vaseline.

After about forty-eight hours, the phenol solution had nearly reached the ends of the filter paper strips. The strips were removed and the flow distance of the phenol marked with a pencil. The strips were allowed to air dry until they were no longer "wet" with phenol. They were then placed in an oven provided with a circulating fan for two minutes at 90°. The filter paper strips were removed and stretched in a horizontal position and sprayed with a solution containing 0.40 g. of ninhydrin, 10 g. of phenol and 90 g. of *n*-butyl alcohol. After the "wet" appearance of the strips had disappeared, they were sprayed on the reverse side and allowed to dry until the "wet" appearance had disappeared. The strips were then placed in an oven at 90° for ten minutes. They were then placed over an enclosed steam-bath for five minutes. The color had now reached its maximum.

The filter paper strips were mounted on a glass slide 4 cm. wide, 0.5 cm. thick and 70 cm. long blacked out except for a groove 7.2 mm. wide in which the filter paper strip was placed and fastened at both ends with Scotch tape. The glass strip was then passed between an illuminated slit 6 mm. high and 5 mm. wide and the phototube. An electronic photometer model 512 and photometer tube "D" of the Photovolt Corporation of New York was used in this work. The Photovolt Corporation has since generously placed at our disposal a densitometer specially constructed for this type of measurement, the details of which can be obtained from this Corporation.

The percentage light transmission was read every 5 mm. along the filter paper; thus the entire filter paper strip was scanned except for 0.6 mm. along each edge. A light filter whose maximum transmission was at 570 mμ was placed in front of the slit.

The light transmission through the filter paper was then expressed in per cent. of the light transmitted through a "blank" filter paper strip which had been carried through the entire procedure except that no amino acid had been applied to it. The per cent. transmission was then plotted on semi-log graph paper against the distance along the filter paper strips. The areas above the plotted curve and 100% transmission were measured with a planimeter and this area multiplied by the standard color-area of the particular amino acid to obtain the concentration of the amino acid. The amino acid was identified by its R_F value as defined by Consden, *et al.*[1]. In case there remained doubt as to the identity of a color band on the chromatogram, known amino acids were added to the amino acid mixture and the position of the increase in areas of the light transmission against distance plot noted.

Discussion

The various factors which influence the success of this method and which have been investigated by us are as follows:

Filter Paper.—About 15 brands of filter paper were studied. The suitability of these filter papers varied widely. As a result of this investigation we concluded that the ideal paper must be dense so that the rate of flow of phenol would be slow, otherwise the resolution of the amino acids on the strip will not be sharp. Further, the filter paper must be uniform with a good light transmission. And lastly, the filter paper must be free of impurities which give rise to extraneous colors. The filter paper which was found to fulfill these conditions best is the Carl Schleicher and Schuell quantitative filter paper number 507 cut against the machine direction.

Resolving Solvent.—We have confined our studies entirely to freshly made aqueous phenol solutions using Merck reagent grade. In the beginning part of this research, we employed phenol saturated with water in the butter dish and water saturated with phenol in the bottom jar. This arrangement was abandoned for several reasons but principally because the R_F values of the amino acids were too erratic, and the system was much too sensitive to minor temperature changes. As noted in the experimental part of this paper, 80% aqueous phenol, both in the butter dish and in the bottom of the jar, seemed to yield the best results.

Various phenol concentrations were investigated, using identical concentrations in the butter dish as well as in the bottom of the jar. The R_F values of the various amino acids are shown in Table I as a function of the phenol concentration, the *p*H of the applied amino acid solutions being 5.0.

TABLE I

R_F VALUES AS A FUNCTION OF THE PHENOL CONCENTRATION EXPRESSED IN GRAMS OF PHENOL PER 100 G. OF AQUEOUS SOLUTION

	Phenol concentration					
Amino acid	60	65	70	75	80	85
Serine	0.73	0.33	0.34	0.31	0.30	0.32
Glycine	.73	.38	.40	.35	.36	.38
Methionine	.92	.73	.74	.70	.70	.72
Phenylalanine	.92	.80	.80	.75	.76	.78
Lysine	.63	.37	.30	.25	.23	.19
Arginine	.63	.45	.41	.35	.33	.28
Alanine	.74	.53	.53	.53	.52	.52
Leucine	.94	.77	.78	.75	.74	.73
Glutamic	.63	.36	.35	.31	.32	.23
Theonine	.70	.48	.47	.44	.44	.38
Aspartic	.63	.18	.18	.13	.14	.077
Valine	.88	.72	.70	.69	.69	.67

The amino acids most sensitive to changes in phenol concentration are aspartic, glutamic, lysine and arginine. Indeed, these are the amino acids whose R_F values are the most variable under all conditions.

***p*H.**—The *p*H of the amino acid solutions was adjusted with the addition of sodium hydroxide or with hydrochloric acid, and the R_F values determined using 80% aqueous phenol. These results are shown in Table II.

TABLE II

R_F VALUES AS A FUNCTION OF THE *p*H OF THE AMINO ACID SOLUTIONS APPLIED TO THE FILTER STRIPS

	R_F values at *p*H		
Amino acid	3.30	5.00	8.60
Aspartic	0.17	0.14	0.15
Lysine	.28	.23	.32
Serine	.35	.30	.33
Glutamic	.33	.32	.30
Arginine	.37	.33	.40
Glycine	.40	.36	.38
Threonine	.47	.44	.48
Alanine	.55	.52	.55
Valine	.70	.69	.72
Methionine	.74	.70	.70
Leucine	.76	.74	.75
Phenylalanine	.78	.76	.74

As will be noted from Table II, the R_F values of the amino acids are largely independent of the *p*H of the amino acid solutions. The color developed was also independent of the *p*H from values of 4 to about 7.5. More alkaline *p*H values changed the blue purple color characteristic of all amino acids tested to a red purple, and at *p*H values of about 9 the color became a brick red. At *p*H values acid to about 4, a red color was also obtained.

Developing Solution.—Various developing solutions were studied and the solution which appeared to give the maximum color intensity and which was most reliable in performance had a small amount of phenol added to it; the composition of this solution is given in the experimental part. This solution must be freshly prepared to obtain uniform colors.

Color Development.—It has been our objective to develop the maximum color intensity possible for a given amino acid concentration. When the filter-paper strips are heated at 90° in the oven, they reach a constant level of color intensity in about five minutes, and this intensity remains essentially unchanged for at least thirty-five minutes of heating. However, when the strips are removed from the oven after the constant level of intensity has been attained and the color intensity measured in the densitometer after successive intervals, it is found that the color intensity increases with time at room temperature. This increase in optical density of the filter strips amounted to about 30% after twenty-four hours. The increase in optical density with time was finally recognized as probably being due to moisture uptake by the paper from the atmosphere. It was then found that a five-minute treatment in the steam oven after the dry oven brought the strips to their final color intensity. This final color was stable within experimental error for at least forty-eight hours provided the strips were protected from light.

Color Developed.—Contrary to some reports in the literature, the tint of the color developed with ninhydrin was as near as could be determined exactly the same for all the amino acids investigated, provided the *p*H of the amino acid solution was within the range of 4 to 7.5. Figure 1 shows the per cent. transmission for colors developed by alanine and by aspartic acid on the filter paper as a function of the wave length of the light. The colors developed by a number of other amino acids were investigated, and were found to be practically identical with the two shown in Fig. 1.

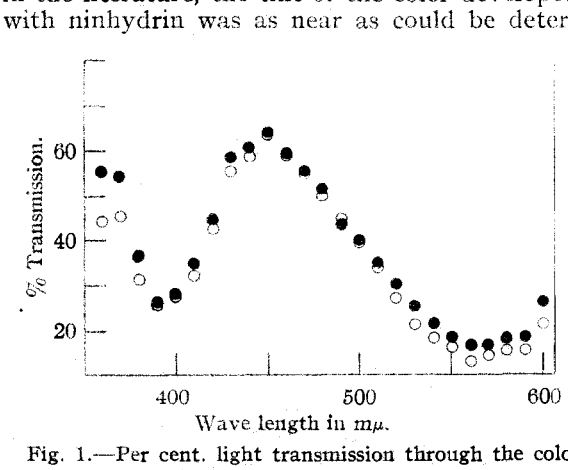

Fig. 1.—Per cent. light transmission through the color bands of alanine (open circles) and of aspartic acid (solid circles) on the filter paper as a function of the wave length of light.

There is a broad minimum in the optical transmission in the neighborhood of 570 mμ and this is the reason for using a light filter of this wave length in the densitometer.

Amino Acid Concentration.—As noted above, the per cent. light transmission was plotted on semi-log graph paper (Keuffel and Esser No. 358-51) against the distance along the filter paper strip allowing the space between each line on the graph paper to equal 2 mm. The areas between the plotted curve and 100% transmission were measured with a planimeter in square inches. These areas were then divided by the amount of amino acid in micromoles and this ratio was plotted against the area in square inches. It was found that the points for arginine, serine, valine, glutamic acid, leucine, threonine, alanine and lysine fell about a line whose least square equation is

$$A/C = 380.4 - 9.10A \qquad (1)$$

where A is the area in square inches and C is the amount of the amino acid in micromoles. The probable per cent. error for 68 determinations extending from 0.67 to 13.5 micrograms of the above acids was ±8.82. The per cent. errors were more or less independent of concentration.

The results for aspartic acid, phenylalanine, methionine and glycine did not follow equation 1. All of these acids developed less color than the first group of amino acids although when A/C was plotted against A, linear relations were obtained. The equations for these amino acids are

Aspartic	$A/C = 340 - 10.0A$	(2)
Phenylalanine	$A/C = 210 - 6.5A$	(3)
Methionine	$A/C = 160 - 0.5A$	(4)
Glycine	$A/C = 275 - 12.3A$	(5)

We did not attempt to evaluate the errors associated with equations 2, 3, 4 and 5. Since these acids did not develop the maximum color, the amount of color which is developed is probably very much dependent on how the reaction is carried out.

The addition of sodium chloride up to 0.1 N had no significant influence on the color developed by alanine, but 0.2 N sodium chloride produced a reddening of the color and reduced the area about 10%. The R_F values for all the amino acids were independent of the amounts of the amino acids present.

Mixtures of Amino Acids.—The greatest utility of paper chromatography is in the study of mixtures of amino acids such as are obtained in protein hydrolysates. Shown in Fig. 2 is a

light transmission curve plotted on a semi-log scale against the distance in cm. along the filter paper strip. The mixture consisted of 3.38 micrograms each of arginine hydrochloride, valine, leucine, threonine, alanine and lysine hydrochloride.

There was too much overlapping of the arginine and the threonine for separate resolutions, but the sum of the micromoles of these two acids was 0.048 and the same calculated by the use of equation 1 is 0.043 micromole. The amounts of the other amino acids in micrograms were lysine hydrochloride 3.78, alanine 2.84, valine 3.51, and leucine 3.32. Where there was overlapping of the amino acid curves as shown in Fig. 2, a vertical line is drawn between the two overlapping amino acids from the peak transmission to the 100% transmission line and the respective areas assigned on the basis of this line. Providing there is not excessive overlapping, this treatment yielded good results.

All resolutions of amino acids on filter paper strips were done at room temperature and no thermostating was attempted. We have been unable to detect any important influence of temperature using 80% aqueous phenol except that the phenol moved faster at higher temperatures, and if this rate became too high the separation of the amino acids was not sharp. The rate we have tried to maintain was about 40 cm. or less in forty-four hours. If temperature gradients exist, the filter paper strips should be arranged so that they face the gradient; otherwise, the amino acid bands will be distorted.

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Summary

1. A new arrangement of the filter paper chromatography of Consden, Gordon and Martin is described.

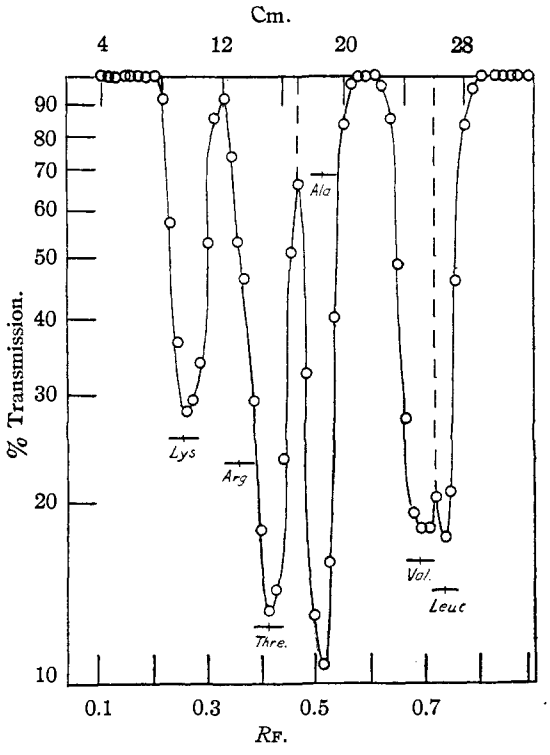

Fig. 2.—Optical transmission curve for a mixture of 3.38 micrograms each of lysine hydrochloride, arginine hydrochloride, threonine, alanine, valine and leucine.

2. The main features of this new approach consist in the use of a superior grade of filter paper, of 80% aqueous phenol, and attention to the details of color development.

3. It appears that arginine, serine, valine, glutamic acid, leucine, threonine, alanine and lysine give the same micromolar color values, and by the technique described it is possible to estimate these acids in the range of 0.67 to 13.5 micrograms with a probable per cent. error of a single determination of ±8.82. Aspartic acid, phenylalanine, methionine and glycine yield lower micromolar color values.

4. The application of this method to amino acid mixtures is described.

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