moreover, is greatest when the inhibitor content of the specimen is especially high. Evidently the impurities in crude trypsin solutions prevent effective contact or combination of a considerable proportion of the antitryptic substances with the active trypsin molecules.

Despite these limitations, antitrypsin titrations with crude trypsin extracts have revealed data of apparent significance in support of the autodigestion theory of allergy.[13,17] For critical studies, however, only tests with crystalline trypsin can now be recommended.

*Comment.* It is important to realize that all of the procedures outlined above require meticulous attention to detail, and can only be carried out successfully by a conscientious technician, since the slightest deviation from the required technic shows up inexorably. Nevertheless, the tests are readily adaptable to routine use and an average of 20 separate specimens a day may be titrated by a single, practiced worker.

The method makes it feasible to add to the data ordinarily obtained in experimental and clinical studies of allergy precise information on the protease-inhibiting power of the sera of patients and experimental animals, or of the reagents used. The titrations should aid materially in appraising the significance of the previously-neglected antitryptic effect *in vivo* or *in vitro* of the materials and manipulations employed in any experiment.

The basic film-gelatin-digestion technic should prove readily adaptable also for the titration of proteases other than trypsin, for experiments on the kinetics of these enzyme reactions and for the study of important physiologic and pathologic phenomena, other than hypersensitivity, in which natural proteases, their activators and inhibitors, appear to play so vital a part.

*Summary.* A technically simple, rapid method for the quantitative titration of trypsin or similar proteolytic enzymes, and of protease inhibitors, suitable for critical tests on a large scale is described. The procedure utilizes technics familiar to serologists, rather than to skilled biochemists only, and gives definitive, reproducible results that can be expressed in simple figures. A feature is the provision for the making of a permanent record of each titration. Since the reagents are stable, and readily standardized, comparable findings can be obtained in separate experiments conducted in the same or in different laboratories at any time.

The assistance of Mrs. David A. Cook with records and statistics is gratefully acknowledged.

[17] Burdon, K. L., and Mudd, R. P., PROC. SOC. EXP. BIOL. AND MED., 1949, in press.

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## Quantitative Paper Chromatography: A Simplified Procedure. (17425)

RICHARD J. BLOCK

*From the Department of Biochemistry, New York Medical College, Flower and Fifth Avenue Hospitals, New York City.*

The now classical paper chromatographic method of Consden, Gordon and Martin[1] has been applied to the separation of many types of organic and inorganic substances. Although the method as originally described was only qualitative or roughly quantitative (± 50%), a number of quantitative adaptations have been published. These adaptations may be classified into several general categories: (A) the paper chromatogram is cut so that each substance separated may be extracted with the appropriate solvent and then the concentration of the material in the extract is determined by conventional colorimetry or other means (*cf.*[2]); (B) the materials on the chromatogram are revealed by their own color,

[1] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

by reaction with specific reagents to produce a colored compound, by their microbiological effects when the paper is placed on a suitable test medium, or by radioactivity. In such instances, it has been found by Fisher, Parsons and Morrison[3] that the area on the paper occupied by each substance is proportional to the logarithm of its concentration. This procedure is very satisfactory when the boundaries of the spots are sharply delineated, but cannot be applied with accuracy unless this is so or when the substances are not distinctly separated from each other. (C) In order to overcome these disadvantages, a method has been described[4,5] in which the color density is determined continuously or in overlapping increments with an electronic densitometer along the entire length of a one dimensional chromatogram strip. The color densities, so obtained, are plotted on graph paper, a series of peaks are obtained, and the concentration of each substance or substances is calculated from the area under each density peak. This procedure is very satisfactory for the determination of substances which are well or partly separated from each other, but is exceedingly tedious unless automatic equipment is available. Furthermore, as there is no known single solvent which permits satisfactory separation of all or almost all the amino acids present in a protein hydrolysate, preliminary separation by adsorption, ion exchange or paper chromatography must be resorted to in many instances.[4,6] (D) One of the advantages of the paper chromatogram technic of Consden, Gordon and Martin[1] over the older capillary analysis method of Schoenbein and Goppelsroeder (*cf.*[2]) is the two-dimensional chromatogram. The method often gives even greater resolution than can be obtained by preliminary separation by adsorption, ion exchange or large scale paper chromatography followed by one-dimensional strip chromatography and is much easier to conduct. It has been reported[4] that the concentration of a colored substance on a two-dimensional paper chromatogram can be estimated from the product of the greatest color density times the area of the spot.

The data to be given in this paper show that the concentration of colored substances separated by paper chromatography may be estimated directly on the chromatograms, with reasonable accuracy, either from the product of the area times the color density or more simply from the color density alone. The examples given are for the estimation of amino acids. However, equal success has been achieved with non-volatile amines and it appears that the same principle would apply to other colored substances on paper chromatograms.

1.) Determination of Histidine and Tyrosine in Protein Hydrolysates (*cf.*[7]) *Experimental.* Histidine and tyrosine are separated by paper chromatography employing S. & S. 598 paper with a solvent mixture consisting of N-butanol 100 parts, glacial acetic acid 10 parts, saturated with water. The chromatograms are run in air tight glass chambers (*cf.*[8]) for 3 hours. The paper is then removed, dried in air, and sprayed with freshly diazotized sulfanilamide dissolved in N-butanol. The chromatogram is again dried in air for exactly 5 minutes and then it is sprayed with saturated $Na_2CO_3$ solution. The paper is dried in air and the quantities of histidine and tyrosine are determined from the product of the area of the spot times its maximum color density.[4]

*Results.* A few typical results on standard solutions are given in Table I. In order to ascertain the value of this procedure on protein hydrolysates, histidine and tyrosine analyses were carried out on 3 proteins, the histi-

2 Schoenbein, C. F., *Verh. d. Naturforsch. Ges. Basel*, 1861, **3**, 249; Rheinboldt, H. in Houben, *J. Die Methoden der Organischen Chemie*, G. Thieme, Leipsig, 1925, **1**, 291.

3 Fisher, R. B., Parsons, D. S., and Morrison, G. A., *Nature*, 1948, **161**, 764.

4 Block, R. J., *Science*, 1948, **108**, 608.

5 Bull, H. B., Hahn, J. W., and Baptist, V. H., *J. Am. Chem. Soc.*, 1949, **71**, 550.

6 Block, R. J., and Bolling, D., The Amino Acid Composition of Proteins and Foods, C. C. Thomas, Springfield, Ill., 2nd ed., 1950.

7 Bolling, D., Sober, A. H., and Block, R. J., *Fed. Proc.*, 1949, **8**, 185.

8 Nachod, F. C., Ion Exchange: Theory and Application, Academic Press, New York, 1949, p. 308.

TABLE I.
Standard Curves for Tyrosine and Histidine.

| | mg | A | D | A × D | Factor |
|---|---|---|---|---|---|
| Tyrosine | .0090 | 18.5 | .22 | 4.07 | .0022 |
| | .0090 | 26.5 | .14 | 3.71 | .0024 |
| | .0090 | 16.5 | .24 | 3.96 | .0023 |
| | .0180 | 27.0 | .28 | 7.56 | .0024 |
| | .0180 | 40.5 | .20 | 8.10 | .0022 |
| | .0180 | 24.5 | .30 | 7.35 | .0024 |
| | .0180 | 37.5 | .22 | 8.25 | .0022 |
| | .0180 | 29.5 | .25 | 7.38 | .0024 |
| | .0270 | 53.2 | .20 | 10.64 | .0025 |
| | .0270 | 50.0 | .24 | 12.00 | .0023 |
| | .0270 | 28.0 | .38 | 10.64 | .0025 |
| | | | | | .0024 |
| Histidine | .0078 | 12.5 | .32 | 4.00 | .0020 |
| | .0078 | 12.0 | .34 | 4.08 | .0019 |
| | .0156 | 18.5 | .43 | 7.96 | .0020 |
| | .0156 | 16.5 | .41 | 6.77 | .0023 |
| | .0156 | 20.5 | .40 | 8.20 | .0019 |
| | .0156 | 22.0 | .38 | 8.36 | .0019 |
| | .0156 | 16.5 | .43 | 7.10 | .0022 |
| | .0156 | 26.5 | .26 | 6.89 | .0023 |
| | .0234 | 30.0 | .35 | 10.50 | .0022 |
| | .0234 | 21.3 | .56 | 11.93 | .0020 |
| | | | | | .0021 |

A—Area. D—Density. Factor—mg/A × D.

TABLE II.
Tyrosine and Histidine in Proteins.
(Calculated to 16.0 g of nitrogen).

| Protein | mg used | Tyrosine, g | Histidine, g |
|---|---|---|---|
| Hemoglobin | .04-.32 | 3.0 | 8.2 |
| Casein | .10-.60 | 5.9 | 2.9 |
| Zein | .15-.90 | 5.1 | 1.8 |

dine and tyrosine ratios of which differ widely. These results are summarized in Table II. The values obtained agree with the best in the literature (*cf.*[6]).

2.) Estimation of Amino Acids on Two-Dimensional Paper Chromatograms. During investigations on one-dimensional chromatograms,[4] it was observed that the areas obtained from curves made from the maximum and minimum densities were almost as satisfactory as those prepared from scanning the entire curve. This led to an investigation of the relationship between the maximum color density of the spots on a two-dimensional chromatogram and the concentrations. The following procedure can be used to estimate 14 amino acids on approximately 0.3 mg of hydrolysate N of a protein with the usual type of amino acid pattern, with an average error of less than 10%. Only 5 hours total working time are required. The elimination of the need for determining the area of the spot[4] is an obvious advantage.

*Experimental.* The protein or peptide is hydrolyzed with HCl or $H_2SO_4$ and the excess mineral acid is removed in the usual fashion. The solution of amino acids is then diluted so that it contains approximately 1.5 to 10.0 millimols of each amino acid per ml. Isopropanol (to 10%) is used as the preservative. 0.005 or 0.01 ml aliquots of the hydrolysate are applied to the paper and the amino acids are then separated by two-dimensional chromatography on S. & S. No. 596, Whatman No. 4, or other suitable paper. Water-saturated phenol (in an atmosphere of 0.3% of $NH_3$, moistened NaCN and coal gas) is the first solvent. The chromatograms are then dried in the hood with a blast of warm air until most of the solvent has been removed. Then the sheets are dried before an electric fan over night, 2.5 cm of the leading edge of the phenol run are cut off the paper in order to eliminate the discolored front. The chrom-

TABLE III.
Color Ratios and Amino Acids in Casein.

| Amino acid | Standard color ratio | Corrected experimental color ratios (molecular ratio) | Amino acids in casein millimols per 1.60 mg of N | |
|---|---|---|---|---|
| | | | Found | Calculated[6] |
| Arginine | .92 | 6.4 | 2.4 | 2.4 |
| Histidine | 1.12 | 5.1 | 1.9 | 2.1 |
| Lysine | .45 | 14.1 | 5.4 | 5.8 |
| Tyrosine | .98 | 10.0 | 3.8 | 3.8 |
| Phenylalanine | .99 | 9.7 | 3.6 | 3.1 |
| Methionine | 1.13 | 6.1 | 2.3 | 2.3 |
| Serine | .90 | 13.2 | 5.0 | 5.6* |
| Threonine | 1.02 | 10.2 | 3.8 | 4.0 |
| Leucines | 1.08/2 | 35.8 | 13.5 | 12.8 |
| Valine | 1.02 | 15.1 | 5.7 | 6.3 |
| Glycine | .89 | 8.3 | 3.1 | 2.8-3.7 |
| Alanine | 1.26 | 9.8 | 3.7 | 3.7 |
| Glutamic acid | .72 | 40.4 | 15.3 | 16.1 |
| Aspartic acid | 1.33 | 11.4 | 4.3 | 5.6 |
| Proline | | | ++ | |
| Hydroxyproline | | | — | |

* Uncorrected for hydrolytic losses.

atogram is then developed at right angles to the phenol run with 2,-6 lutedine 55 parts, 95% ethanol 20 parts and water 25 parts by volume in an atmosphere of diethylamine and moistened NaCN. At the completion of the run, the chromatograms are dried in the hood by a blast of warm air and the amino acids are revealed by spraying with 0.1 or 0.2% of ninhydrin. The color is brought out by heating the chromatograms. After the color has developed, the maximum color density of each spot is measured with an electronic densitometer (*cf.*[4,5]) using a 5 x 5 mm or a 15.6 $mm^2$ aperture disc.

*Calculation.* Mixtures of all amino acids in a protein hydrolysate, with the exception of cystine, tryptophan, proline and hydroxyproline, are prepared so as to contain the following quantities of *each* amino acid per ml. : 1.25, 2.50, 5.00 and 10.00 millimols. Twenty-five or more chromatograms are carried out on each of the above standards. The maximum color density of each amino acid on the chromatograms is then determined. The "mean color density", that is the arithmetic mean of the maximum color densities of *all* the spots on each of the 4 standard concentrations, is then calculated. Similarly, the arithmetic means of the color densities given by each individual amino acid on the 4 standard chromatograms is computed. The latter divided by the former value is the "standard color ratio" of the individual amino acids. "Color ratios" then are calculated in the same manner for all amino acids which are seen on two dimensional chromatograms of acid hydrolysates. These values are called "experimental color ratios." The "standard color ratios" vary less than 10% from run to run over a period of several months and over a range of 1.25 to 10.00 millimols of amino acid per ml. The average "standard color ratios" on S. & S. No. 596 paper are given in Table III.

The "experimental color ratios" of amino acids in the unknown (acid hydrolysate) are determined as above and the values found for each amino acid are then divided by the appropriate "standard color ratio." This gives the "corrected experimental color ratio" of each amino acid on the chromatograms (Table III, 3rd column). The proportion of the "corrected experimental color ratios" to each other gives the molar ratios of each amino acid to the other on the chromatograms. The approximate quantities* of the amino acids on the chromatograms are then readily determined if the concentration of one, or better 2 or 3, of the amino acids are known from independent chromatographic or other methods.

In those cases where one or more amino

acids are absent or present in comparatively small amounts in the hydrolysate under analysis, the addition of known quantities of this amino acid to the hydrolysate will serve as an internal standard.

When 25 replicate chromatograms are run, the average per cent error for a mixture of 14 amino acids in proportions simulating β-lactoglobulin, was −2% in one series and −4% in a second. The maximum variations in individual amino acids ranged from −12 to + 9%. This error, however, can be appreciably reduced by increasing the number of chromatograms.

Cystine is largely or entirely destroyed on two-dimensional chromatograms using S. & S. No. 596 or Whatman No. 4 paper under the conditions described above. It is best determined by a combination of the platinic iodide[9] and Fisher[3] procedures. Proline, hydroxyproline and tryptophan are likewise best estimated by special methods, the first two by the isatin method of Fromageot,[10] the last by a combination of the Voisenet-Rhode p-dimethylaminobenzaldehyde reaction (*cf.*[6]) as applied to paper chromatograms and the area[3] or area-density[4] methods.

Methionine,† histidine and tyrosine, which are reasonably accurately estimated by specific chromatographic procedures (*cf.* above) have proven to be valuable for the quantitative estimation of amino acids by the ninhydrin method on two-dimensional chromatograms.

*Results.* The values given in Table III show the color ratios and approximate quantities of arginine, histidine, lysine, tyrosine, phenylalanine, methionine, serine, threonine, leucines, valine, glycine, alanine, glutamic acid and aspartic acid obtained on less than 0.3 mg of casein N. The figures given in the adjoining column (Table III) are those calculated from casein by conventional chemical and microbiological methods.[6]

*Summary.* Two methods are described which permit the estimation of colored substances on one-dimensional and two-dimensional paper chromatograms with a minimum expenditure of time and material.

* Experiments which are underway indicate that the concentration of each amino acid on the chromatograms may be estimated within approximately 15% by the maximum color density method, when the color value is read off the appropriate "standard curve" prepared from two dimensional chromatograms which are run *simultaneously* with the unknown solution. As in all colorimetry, it is important to have the color density of the standard as nearly equal as possible to that of the unknown.

9 Winegard, H. M., Toennies, G., and Block, R. J., *Science*, 1948, **108**, 506.

10 Fromageot, C., private communication, May 1949.

† Cystine and methionine have been determined in casein, lactalbumin and human hair hydrolysates by the area method[3] using platinic iodide[9] as the color reagent and water saturated n-butanol-acetic acid as the developing solvent. The following amounts of cystine and methionine, in grams of amino acid per 16.0 g of nitrogen, were found: casein, 0.3; 3.4, lactalbumin 3.0; 2.6, hair 16.0; 0.6.

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## Prophylactic and Therapeutic Effect of Para-Aminobenzoic Acid and Sodium Salicylate on Experimental Allergic Encephalomyelitis.* (17426)

ROBERT A. GOOD,† BERRY CAMPBELL, AND THOMAS A. GOOD

*From the Departments of Pediatrics and Anatomy, University of Minnesota Medical School, Minneapolis, Minn.*

That encephalomyelitis in experimental animals can be produced by the injection of homologous brain tissue was shown by Rivers, Sprunt, and Berry[1] and confirmed by Ferraro

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† Helen Hay Whitney Foundation, fellow in rheumatic fever.

1 Rivers, T. M., Sprunt, D. H., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.