ferent concentrations of the material at different stages in development. If sucrose is capable of affecting development during these stages, as seems evident (whatever the specific effect may be), this might well explain the great variety of anomalies produced. As Weiss (*1*, 483) has pointed out, "Not only may the same type of malformation arise in various ways, but various kinds of malformations may also be caused by the same disturbance." The present results seem to be another illustration of this point.

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Quantitative Microdetermination of Amino Acids after Paper Chromatography[1]

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Paper chromatography (*1*) and microbiological determination are the two most useful modern methods of protein analysis. Paper partition chromatography was initially only a qualitative or semiquantitative method, which several workers soon tried to improve so as to meet quantitative standards. Generally this has been attempted by employing the ninhydrin reaction and measuring the color and area on the paper (*2–4*) or by extracting the amino acids from the paper spots and performing the ninhydrin reaction or a copper amino nitrogen determination (*5–9*). Even with these refinements, however, the estimation of the amino acids is only approximately quantitative, or the process is tedious, especially when it is necessary to extract the amino acid and use special reagents.

We have been using a method which results in the accurate determination of quantities of amino acids of about 10 γ; only rarely (in the case of histidine) is it necessary to use a greater amount of the compound (Table 1) to obtain a proportionality between the color produced and the quantity of amino acid present in the paper spot. Our procedure consists in (1) localizing the spots on the paper and marking lightly their limits with a pencil, under an ultraviolet lamp; (2) cutting the spots, introducing them in a volumetric 10-ml flask and developing a color reaction with ninhydrin; and (3) measuring the color intensity at the end of the reaction. Localization of the spots by ultraviolet fluorescence is possible only after the paper is dried at room temperature for some days or heated at 100°–105° C for about 5 min. We prefer the heating. According to Patton *et al.* (*10*), this fluorescence is essentially due to the paper surface being modified by deposition of the amino acid.

TABLE 1

Quantity of amino acid (in γ)	Optical density (2-cm cell)					
	Glycine	Alanine	Valine	Glutamic acid	Phenylalanine	Histidine monohydrochloride
3	0.13					
5	0.17	0.16	0.14	0.09	0.09	
7	0.20					
10	0.29	0.25	0.21	0.15	0.12	
15		0.34	0.29	0.22	0.16	
20						0.16
30						0.21
40						0.27

The marked spots are cut, introduced into a 10-ml dried volumetric flask, and 0.5 ml of an appropriate ninhydrin solution is added. The volume of ninhydrin solution must be less than 1 ml, otherwise the reaction becomes less sensitive. The flasks are heated for 15 min in a bath of vigorously boiling water, with occasional shaking, and are then immersed in water at room temperature for about 5 min. Distilled water is added, and the color intensity is measured in a colorimeter. Meanwhile, the piece of paper is allowed to remain in the volumetric flask, the error due to it being negligible. For the colorimetry we use a Lumetron colorimeter with a 550-mμ filter and a microcell 2 cm thick.

A blank with a piece of filter paper, of about the same size as that with the spot, and ninhydrin reagent are prepared in the same way. We found that the blanks gave the same values for widely different pieces of paper and therefore may be neglected when comparative results are being obtained—that is, when pure amino acids are used as the standard. Of course, after the use of ammonia in paper chromatography, it is necessary to eliminate this compound; otherwise the blank may be variable.

The ninhydrin solution must be almost neutral, since the optimum pH for the reaction, without the use of reducing agents, is about 7. For the small volume in which the reaction is performed the contribution of the piece of paper to changing the pH may be very important. We noticed that different papers (Whatman No. 1, No. 2, and others), when immersed in 0.5 ml of ninhydrin solution, may more or less strongly modify the pH. This is especially true after developing the chromatogram. The contribution of the amino acid to alteration of the pH is negligible. It is necessary, therefore, to correct the pH of the acid aqueous ninhydrin solution with a buffer to obtain a final pH

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of 6.80–7.0. We used the following ninhydrin solution with good results: A fresh 1% ninhydrin solution in water is mixed with an equal volume of a sodium veronal–HCl buffer of pH 7.0. This pH is obtained by mixing 5.36 volumes of 0.1 *M* sodium veronal with 4.64 volumes of 0.1 *N* HCl.

Table 1 illustrates the results we obtained with pure amino acids.

The sensitivity of the ninhydrin reaction is different depending on the amino acid (*11, 12*), histidine being the least sensitive. The lower quantities of amino acid mentioned in the table give values greater than expected; the reaction is quantitative only above these lower limits. For glycine the Lambert-Beer law is followed only above, and including, 5 γ; for alanine, valine, glutamic acid, and phenylalanine the lower quantitative limit (inclusive) is 10 γ, whereas for histidine this limit is 25 γ. Fig. 1 illustrates this, showing that for the quantities employed, optical density is directly proportional to concentration.

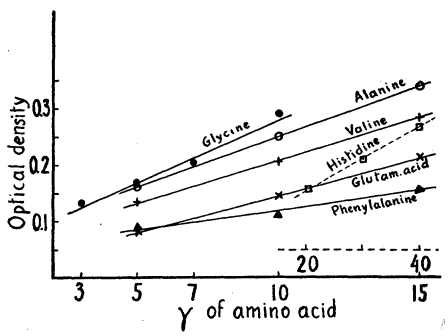

FIG. 1.

In order to determine an amount of amino acid below the lower quantitative limit, a known quantity of amino acid is added to the paper spot with a fine capillary pipette (e.g., 0.005 ml and 10 γ of amino acid), and afterwards the reaction is performed as usual, the piece of paper with the spot having previously been dried. For the quantities of histidine normally found in proteins it is generally necessary to add a small amount of this compound to the respective spot in paper chromatographies corresponding to about 200–400 γ protein, to obtain a quantitative color reaction.

We hope that the procedure will prove to be useful also in the hands of other workers. The reproducibility of the method lies in general within an error of 3–5%, and duplicates usually give errors inferior to this.

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Evidence for the Heparin Nature of the Nonspecific Hyaluronidase Inhibitor in Tissue Extracts and Blood Serum[1]

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The possible role of the nonspecific (nonantibody) hyaluronidase inhibitor of blood serum in the animal body, and its remarkable changes in concentration in infectious diseases, cancer, liver and kidney damage, and many conditions of stress (*1*), led to an earlier study (*2*) of its anatomical origin. A wide variety of tissues from the rabbit was investigated as possible sources of the serum inhibitor, but it could be demonstrated in none of the tissue extracts obtained. This might be explained by the very low concentration of inhibitor in these tissues. It was further found that certain steroids and hemoglobin derivatives were inhibitors *in vitro*, but none of these could be the serum factor in question since the latter is heat-labile and its effect is decreased in the presence of phosphate. It is well known that isolated polysaccharides of the heparin group are potent inhibitors (*3–9*), and, as our work developed, evidence accumulated suggesting that the native heparin-protein complex (*10*) probably present in low concentrations in normal serum (*11*) might exert a hyaluronidase inhibition. It should be mentioned that certain mucoproteins of human serum prepared by the perchloric acid method of Winzler *et al.* (*12*) were devoid both of anticoagulant (*13*) and hyaluronidase inhibitor activities (*14*).

The first suggestion as to the chemical nature of the nonspecific hyaluronidase inhibitor was provided by the finding that most of the serum inhibitor migrated with the albumin in the electrophoretic field (*15*), a finding later confirmed (*16*). It was previously known that purified commercial heparin added to plasma migrated with an electrophoretic mobility intermediate between that of heparin and albumin (*17*). This seems also to be the case for one of the components obtained by electrophoresis of the native heparin complex obtained from ox liver capsule extracts (*18*).

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