THE IDENTIFICATION OF PHENYLALANINE ON PAPER CHROMATOGRAMS

by

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INTRODUCTION

During studies on the sulphur metabolism of mammalian tissues cultivated *in vitro*[1], it was observed that paper chromatograms of the synthetic tissue culture medium[1,2] in which homocysteine had been incorporated formed a characteristic blue color during treatment with mercuric nitrate following ninhydrin development. Investigation of this phenomenon[3] showed that the color reaction of homocysteine with ninhydrin was catalysed by certain ingredients of the inorganic salt solution[4] present in the synthetic medium. It was considered of interest, therefore, to study the possible effect of this inorganic salt solution on the ninhydrin reaction with other amino acids. The results reported in this communication describe a characteristic color test for phenylalanine that permits the identification of as little as 10μg of this amino acid on paper chromatograms even under conditions of incomplete separation and resolution.

MATERIALS AND METHODS

One-dimensional descending paper chromatograms were used, with Whatman No. 1, No. 4, No. 3MM or Schleicher and Schuell No. 597 paper. The *n*-butanol-acetic acid -water solvent system of WOIWOD[5] was used throughout most of these experiments but various other solvent systems containing ethanol, propanol, butanol and water were also found suitable. Color development was effected by spraying the chromatograms with 0.2 or 0.4% ninhydrin in water-saturated *n*-butanol[6] followed by drying at 110° C for 5 minutes. The reheating at 110° C after spraying with ninhydrin was found to be necessary for development of the characteristic phenylalanine color. When this treatment was employed, satisfactory color development was obtained with ninhydrin dissolved in either *n*-butanol or 95% ethanol. Full details of the preparation of samples and the chromatographic procedures have been described previously[3].

The detailed composition of synthetic tissue culture Medium M 150 has been reported previously[1,2]. The inorganic salt solution used as a base in this medium was Hanks' modified Tyrode's solution[4]. All compounds employed in this study were of the highest grade obtainable commercially and were tested individually to establish their relative positions on paper chromatograms. In carrying out these tests, the phenylalanine compounds (10 mg) were dissolved in 5 ml of Medium M 150, 1 drop of 1 *N* NaOH added and chromatograms prepared. This procedure afforded good resolution of all the compounds studied except tyrosine, diiodotyrosine and dihydroxyphenylalanine. With these three compounds, the substitution of 1 drop of 1 *N* HCl for the NaOH resulted in satisfactory resolution on the chromatograms. Under these experimental conditions phenylalanine, and the phenylalanine derivatives and peptides tested, were found to be relatively free from other ninhydrin-positive materials.

Absorption spectra were measured in a Beckman Spectrophotometer (Model DU) employing 1 cm Corex cells. Strips were cut from the developed paper chromatograms and fitted to the inner walls of the cells, which were then filled with *n*-butanol. Readings were taken at appropriate wavelengths over the range from 400 to 750 mμ.

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For quantitative measurements of the phenylalanine color reaction, a Densitometer (Welch Densichron) was employed.

EXPERIMENTAL

Reaction of phenylalanine on paper with ninhydrin and Hanks' solution

Paper chromatograms of Medium M 150 formed typical spots corresponding to the twenty amino acids in the mixture when developed with butanol–acetic acid–water, sprayed with ninhydrin and dried at 110°C. On these chromatograms, phenylalanine was found slightly above the valine position or in the leucine region, depending upon the exact composition of the butanol–acetic acid–water or other alcoholic developing solvents. Partial separation was always obtained in these experiments but a clear resolution of the phenylalanine to a separate and distinct spot was not achieved. However, if these ninhydrin-developed chromatograms were subsequently dipped into Hanks' solution and redried at 110°C, a deep blue color formed in the phenylalanine region. The absorption curves of the phenylalanine-ninhydrin color, before and after treatment with Hanks' solution, are shown in Fig. 1 (Curves A and B). The ninhydrin-phenylalanine control (Curve B) exhibits an absorption peak at 560 mμ. Treatment with Hanks' solution (Curve A) has shifted the absorption maximum to 600 mμ and has increased the intensity of the color.

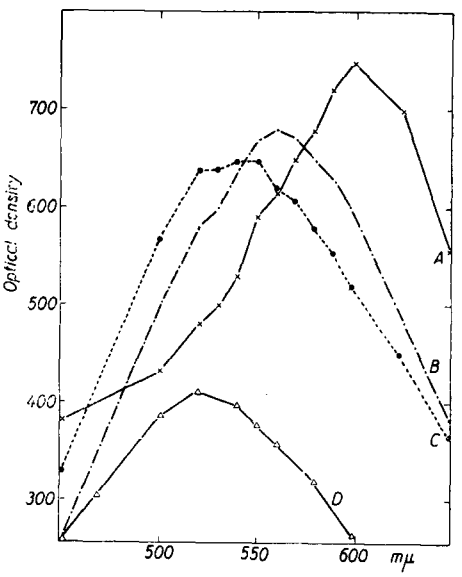

Fig. 1. Stability of phenylalanine-ninhydrin color complex and effect of treatment with Hanks' solution on absorption maximum and color stability. Curve A, characteristic phenylalanine color formed by development with ninhydrin and treatment with Hanks' solution. Curves B, C, D, phenylalanine controls developed with ninhydrin but not treated with Hanks' solution. Curve B, freshly-developed; Curve C, 24 hours standing after development; Curve D, 96 hours standing after development.

Stabilizing effect of Hanks' solution on the phenylalanine-ninhydrin color

During the course of these experiments, it was observed that phenylalanine controls treated with ninhydrin alone formed an unstable color that faded rapidly, Fig. 1, Curves B, C and D. Optical density readings taken 24 hours after development of the chromatogram (Curve C) showed an appreciable decrease in color intensity from that of the freshly-developed chromatogram (Curve B). By 96 hours (Curve D) the color had faded almost completely and the typical ninhydrin absorption maximum at 560 mμ had disapeared.

In contrast to this marked instability of the phenylalanine controls, the blue color formed by treatment with Hanks' solution after ninhydrin development appeared to remain stable indefinitely. Absorption readings taken at time intervals up to 7 days showed no decrease in color intensity from that of the freshly-developed chromato-

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gram (Curve A). Further investigations are now in progress to determine whether this characteristic blue color, by virtue of the stability and altered absorption maximum, can be used for the quantitative determination of phenylalanine on paper chromatograms.

Effect of inorganic salts and pH on the phenylalanine color reaction

Since the characteristic blue color reaction between phenylalanine and ninhydrin was obtained after treatment of the chromatograms with Hanks' solution, the effect of the individual components of this salt solution was next determined. In these experiments, the synthetic tissue culture medium, which contains 50 mg of DL-phenylalanine per liter, was concentrated to dryness *in vacuo* over sulphuric acid and reconstituted in water to a final 25-fold concentration. Portions of this concentrated solution (10 λ, containing 12.5 μg of phenylalanine) were chromatographed, developed with ninhydrin and dried at 110°C, as described previously. The chromatograms were then dipped into solutions of the individual components of Hanks' solution made up at 10 times their normal concentrations, and redried at 110°C. Of the nine components of this salt mixture (NaCl, KCl, $CaCl_2$, $MgSO_4.7H_2O$, $MgCl_2.6H_2O$, Na_2HPO_4, KH_2PO_4, $NaHCO_3$ and glucose), the only substance catalyzing the blue color reaction was found to be sodium bicarbonate. Moreover, the concentration of sodium bicarbonate was not found to be critical, since good color development was obtained with bicarbonate solutions ranging from 0.15 to 10.0%. Further experiments showed that the color formation also occurred when ninhydrin-treated chromatograms were dipped into 0.05*M* tris buffer at pH 9.0 or into 0.1*N* sodium hydroxide solution. From these results, it was concluded that the characteristic blue color formation was dependent on alkaline conditions subsequent to the ninhydrin treatment and could not be attributed to a catalytic effect of the specific inorganic salts present in the Hanks' solution.

Specificity of the phenylalanine color reaction

Various phenylalanine isomers, derivatives and peptides were next tested under these experimental conditions, in order to determine the specificity of the phenylalanine color reaction. The results of these experiments are presented in Table I.

TABLE I

COMPARATIVE ABILITY OF PHENYLALANINE DERIVATIVES AND COMPOUNDS TO EXHIBIT BLUE COLOR FORMATION AFTER DEVELOPMENT WITH NINHYDRIN AND TREATMENT WITH HANKS' SOLUTION

*Compound tested**	*Blue color formation*
L-Phenylalanine	+
D-Phenylalanine	+
DL-Phenylalanine	+
L-Tyrosine	±
Dihydroxyphenylalanine	—
Diiodotyrosine	—
Acetyl-D-phenylalanine**	—
Chloracetyl-DL-phenylalanine**	—
DL-Alanyl-DL-phenylalanine	—
Glycyl-DL-phenylalanine	—
β-Phenyl serine	—

* 200 μg of each compound present on chromatograms. ** Not ninhydrin-positive.

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It is evident (Table I) that formation of the characteristic blue color is specific for phenylalanine but is not dependent upon the optical configuration of this amino acid. Introduction of hydroxy, iodo, acetyl or chloracetyl groups into the phenylalanine molecule results in loss of the chromogenic activity. No activity was observed for phenylalanine combined in peptides with alanine or glycine, nor was activity found with β-phenyl serine.

Tyrosine was the only compound tested that showed any development of the phenylalanine color. However, the color formed with this amino acid was much less intense than in the case of phenylalanine and was unstable, fading rapidly to a grayish blue and eventually to the typical ninhydrin color.

Sensitivity of the phenylalanine color reaction

In most of the present experiments, the phenylalanine color reaction was studied in Medium M 150, which contains a complete supplement of amino acids. The solvent systems employed did not completely separate phenylalanine from valine, although they did provide good separation for the majority of the 20 amino acids present. Under these conditions, 10 μg of phenylalanine was required for positive identification by this color test.

Extensive experiments were carried out with water solutions of phenylalanine alone, employing a densitometer, to determine the limit of sensitivity of the color reaction. The minimal quantity of phenylalanine showing detectable color formation, either with or without alkali treatment, was found to be 5 to 10 μg. This figure is in good agreement with the limit of sensitivity of the conventional ninhydrin method[7,9]. It is apparent that treatment with alkali does not increase the sensitivity of the ninhydrin reaction with phenylalanine, but does convert the conventional non-specific ninhydrin method to a stable and characteristic color test specific for phenylalanine.

DISCUSSION

Although phenylalanine in milligram amounts may be determined in solutions by the Kapeller-Adler method[9] or detected in microgram quantities on paper chromatograms by the conventional ninhydrin procedure[7,8], neither determination is specific in nature. The present experiments have shown that phenylalanine can be detected in biological systems by the stable blue color formed when ninhydrin-treated chromatograms are dipped into Hanks' modified Tyrode's solution or sodium bicarbonate solution.

The purple color formed by the reaction of amino acids in acid solution with ninhydrin is generally attributed to the formation of diketohydrindylidenediketohydrindamine[10,11,12] through intermediate stages of oxidative deamination and condensation. The mechanism of the present test for phenylalanine appears to depend upon initial formation of the colored phenylalanine-ninhydrin complex followed by conversion to a characteristic blue color through the action of dilute alkali on this complex. This action was found to be characterized by a shift in the absorption maximum from 560 to 600 mμ, and was accompanied by greatly increased stability of the resulting new color. This color change was found to be specific for free phenylalanine and was not exhibited by derivatives or peptides of phenylalanine nor by any other free amino acid.

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Although the exact mechanism of this action of alkali on the phenylalanine-ninhydrin complex has not yet been determined, conditions for the color change do not appear to be critical. Comparable color development was demonstrated by the use of mildly alkaline Hanks' solution, 0.05 *M* tris buffer, pH 9.0, sodium bicarbonate solutions (0.15 to 10.0%), or 0.1 *N* sodium hydroxide. However, the use of sodium hydroxide is not recommended if the chromatograms are to be preserved for any length of time. Since the same degree of color formation occurred on chromatograms of synthetic Medium M 150 as on chromatograms of aqueous solutions of phenylalanine alone, interference by other amino acids and by a wide range of physiological compounds, including vitamins, purines, pyrimidines and accessory growth factors, is excluded. The only compound found to react slightly under these conditions was tyrosine, and its position on the paper chromatograms at some distance from the phenylalanine region, together with the instability of the color formed, make the possibility of its confusion or interference negligible.

A particular feature of this method is that it can be used for the detection and identification of phenylalanine in the presence of other amino acids, even under conditions of incomplete resolution in which the phenylalanine region is obscured by the leucines or valine. In addition, this method may be applied to chromatograms previously treated with ninhydrin for other determinations. For these reasons, the method has proved of value in studies on the amino acid metabolism of tissue cells cultivated *in vitro*[13]. The simplicity and sensitivity of this new method suggest that it should prove suitable for application to other biological systems.

SUMMARY

Phenylalanine on paper chromatograms forms a deep blue spot when sprayed with ninhydrin, heated, and subsequently treated with Hanks' modified Tyrode's solution.

Development of the characteristic phenylalanine color is accompanied by a shift in the absorption maximum from 560 to 600 mμ and by a greatly increased color stability.

The color reaction appears to depend upon treatment with alkali subsequent to the ninhydrin color development and may be brought about by Hanks' solution, sodium bicarbonate, tris buffer at pH 9.0, or dilute sodium hydroxide.

The blue color reaction appears specific for free phenylalanine, irrespective of optical rotation, and is not formed by derivatives or peptides of phenylalanine.

The characteristic blue color may be used for the identification of phenylalanine in quantities of 10 μg or greater in the presence of other amino acids or under conditions of poor separation and resolution on the chromatograms.

RÉSUMÉ

La phénylalanine forme, sur les chromatogrammes sur papier, une tache bleue intense après pulvérisation à la ninhydrine, chauffage et traitement ultérieur avec la solution de Tyrode modifiée par Hanks.

L'apparition de la coloration caractéristique de la phénylalanine s'accompagne d'un déplacement du maximum d'absorption, qui passe de 560 à 600 mμ, et d'une stabilité de coloration très accrue.

La réaction colorée semble dépendre d'un traitement alcalin suivant le développement de la coloration à la ninhydrine et peut être provoquée par la solution de Hanks, le bicarbonate de sodium, le tampon tris à pH 9.0 ou la soude diluée.

La coloration bleue est spécifique de la phénylalanine libre, quelque soit sa configuration stérique, et n'est pas donnée par les dérivés ou les peptides de la phénylalanine.

La coloration bleue caractéristique peut servir à identifier des quantités de phénylalanine de l'ordre de 10 μg au moins en présence d'autres aminoacides ou lorsque la séparation et la résolution sont mauvaises sur les chromatogrammes.

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ZUSAMMENFASSUNG

Mit Ninhydrin bestäubtes und nachher mit der Hankschen geänderten Tyrod-Lösung behandeltes erhitztes Phenylalanin bildet auf Papierchromatogrammen einen dunkelblauen Fleck.

Die Entwicklung der charakteristischen Phenylalanin-Farbe wird von der Erhöhung des Absorptionsmaximums von 560 auf 600 mμ, sowie von einer viel grösseren Farbstabilität begleitet.

Die Farbenreaktion scheint von der, auf die Ninhydrinfarbenreaktion folgenden Alkalibehandlung abzuhängen und kann durch die Hanksche Lösung, Natriumbikarbonat, Tris-Puffer mit pH-Wert 9.0, oder verdünntes Natriumhydroxyd herbeigeführt werden.

Die blaue Farbenreaktion scheint spezifisch für freies Phenylalanin zu sein, unabhängig von der optischen Drehung und wird von Phenylalanin-Derivaten oder -Peptiden nicht aufgewiesen.

Die charakteristische blaue Farbe kann zur Identifizierung von 10 μg oder mehr Phenylalanin, in Anwesenheit von anderen Aminosäuren oder bei schlechten Trennungs- und Resolutionsbedingungen des Chromatogrammes benützt werden.

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