THE DETECTION OF HOMOCYSTEINE IN BIOLOGICAL SYSTEMS

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[2,3] in which homocysteine had been incorporated developed a characteristic color under certain conditions. Since color reactions for homocysteine appear to be lacking in the literature, it was considered of interest to investigate the nature and specificity of this reaction in further detail. The results reported in this communication have shown a specific color test for homocysteine in biological systems.

MATERIALS AND METHODS

The detailed composition of synthetic tissue culture medium M 150 has been described previously[2,3]. The inorganic salt base of this medium consisted of Hanks' modified Tyrode's solution[4].

One-dimensional descending paper chromatograms were prepared in rectangular glass troughs, employing Whatman No. 1 or Schleicher and Schuell No. 597, paper. These chromatograms were developed for 16 hours at room temperature with the butanol-acetic acid-water solvent system of WOIWOD[5]. In later experiments, it was found that Whatman No. 3 mm or No. 4 paper could be used and the development period shortened to 4 or 5 hours in the same solvent system. At the end of the development period, ninhydrin-positive materials were detected by spraying the papers with 0.2% or 0.4% ninhydrin in water-saturated *n*-butanol[6].

Samples of medium M 150, containing the sulphur compounds under test at 0.5 mg per ml, were evaporated to dryness *in vacuo* over sulphuric acid and reconstituted in de-ionized water to approximately a 25-fold concentration. Five to 10 λ quantities of this concentrated material were found to be sufficient for effective separation on the chromatograms. After development in the butanol-acetic acid-water solvent, the chromatograms were sprayed with ninhydrin and dried at 110° C. Following this treatment, they were dipped into saturated mercuric nitrate solution and again dried at 110° C.

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 prepared as described above. These tests also indicated the extent of contamination with ninhydrin-positive materials. Under these conditions, homocysteine showed two ninhydrin-positive spots in addition to the major homocysteine spot and the positions of these contaminants corresponded closely with those of homocystine and homocysteine thiolactone. The unavailability of highly-purified homocysteine for this study was not found to be a serious drawback since homocystine and homocysteine thiolactone, which appeared to be present as contaminants, did not exhibit the characteristic color reaction for homocysteine.

EXPERIMENTAL

Reaction of homocysteine on paper with ninhydrin and mercuric nitrate

Paper chromatograms of medium M 150 containing homocysteine formed typical spots corresponding to the known amino acids in the mixture when sprayed with nin-

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hydrin and dried at 110° C. Subsequent treatment of such chromatograms with saturated mercuric nitrate solution resulted in the formation of a cherry red spot surrounded by a deep blue color in the area of the chromatograms occupied by homocysteine. These colors appeared immediately, in some cases while the paper was still immersed in the mercuric nitrate solution, and remained stable after the papers were dried. During the mercuric nitrate treatment and while the homocysteine color was developing, the other ninhydrin-positive spots turned pink and faded considerably. Chromatograms of medium M 150, which contained no homocysteine, did not form the characteristic cherry red-blue spot when subjected to the combined ninhydrin and mercuric nitrate treatment. Methionine formed a bluish color with ninhydrin, but this color was not increased in intensity on subsequent treatment with mercuric nitrate. Moreover, the position of methionine on the paper chromatograms at some considerable distance from the homocysteine position avoided any confusion with the homocysteine reaction.

Specificity of the homocysteine color reaction

The behaviour of various other sulphur compounds of biological significance was next investigated, employing the chromatographic techniques described previously. The results of these experiments are recorded in Table I.

TABLE I

REACTION OF SULPHUR COMPOUNDS ON PAPER CHROMATOGRAMS WHEN TREATED WITH NINHYDRIN AND MERCURIC NITRATE

*Compound tested**	*Cherry red-blue color formation*
DL-Homocysteine (free base)	+
L-Homocysteine thiolactone HCl	—
L-Cysteine (free base)	—
L-Cysteine HCl	—
D-Cysteine HCl	—
DL-Homocystine	—
L-Cystine	—
D-Cystine	—
*meso*Cystine	—
Dibenzoyl-L-Cystine***	—
L-Methionine**	±
DL-Cystathionine	—
Cysteineamine (β-Mercaptoethylamine)	—
L-Cysteic acid	—
Taurine	—
DL-Lanthionine	—
Glutathione (reduced)	—
Glutathione (oxidized)	—
S-Acetyl glutathione	—
Ergothioneine***	—
Thiolhistidine	—
2,3-Dimercaptopropanol	—
Thiomalic acid	—
Djenkolic acid	—
Antabuse***	—
Benzyl penicillin	—
β, β′-Diaminoethyldisulphide	—
Sodium diethyldithiocarbamate	—

* All sulphur compounds (5.0 mg per ml) dissolved in 25 × concentrated Hanks' solution.
** Methionine forms a blue color with ninhydrin which is not intensified by treatment with mercuric nitrate. *** Not ninhydrin-positive.

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It is apparent (Table I) that formation of the cherry red-intense blue color is specific for homocysteine, since none of the other compounds tested gave this reaction. The specificity of the reaction appears to be quite sharp, since homocysteine thiolactone and homocystine, which normally occur as contaminants or oxidation products of homocysteine, did not give the color reaction. It is of interest, also, that cystathionine and cysteine, which are involved in the metabolic pathway of methionine synthesis and degradation, do not form the blue color with ninhydrin and mercuric nitrate.

Since homocysteine can be detected by this method either in Hanks' salt solution or in medium M 150, interference by the components of this medium is excluded. The detailed composition of M 150 has already been published[2,3] and includes 19 amino acids, 6 purines and pyrimidines, 17 vitamins and certain accessory growth factors. When the components of M 150 are added to the sulphur compounds listed in Table I, it is apparent that nearly 80 compounds of physiological importance have been shown to give a negative reaction by the homocysteine color test.

Effect of inorganic salts on the homocysteine color reaction

During preliminary experiments, it was observed that paper chromatograms of homocysteine in water solutions did not form the characteristic color reaction shown by homocysteine in medium M 150. It was also noted, in chromatograms on Whatman No. 3 mm or No. 4 paper, that the homocysteine color was intensified by spraying with Hanks' solution after treatment with ninhydrin and before dipping in mercuric nitrate. These observations suggested that the reaction was catalysed by the inorganic salts of the Hanks' solution[4], and this effect was investigated in some detail. In these experiments, 5.0 mg quantities of homocysteine were dissolved in 25-fold concentrations of the individual components of Hanks' solution, paper chromatograms prepared and tested in the usual way. The results of these experiments are recorded in Table II.

TABLE II

EFFECT OF INORGANIC IONS ON THE COLOR REACTION OF HOMOCYSTEINE WITH NINHYDRIN AND MERCURIC NITRATE

*Component tested**	*Cherry red-blue color formation*
Water	—
Hanks' solution	+
NaCl	+
KCl	+
$CaCl_2$	+
$MgSO_4 \cdot 7H_2O$	—
$MgCl_2 \cdot 6H_2O$	+
Na_2HPO_4	+
KH_2PO_4	+
$NaHCO_3$	+
Glucose	—

* Homocysteine (5.0 mg per ml) dissolved in solution of each component containing 25 % the normal concentration in Hanks' solution.

It is apparent (Table II) that the homocysteine color reaction is catalysed by a variety of inorganic salts. The effect appears to be due mainly to the anions, with the chloride ion particularly effective, the phosphate and bicarbonate ions also effective but

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the sulphate ion inactive. Since the effective ions are ordinarily present in considerable quantities in biological materials, it was not considered of value to study the salt effect in further detail at this time.

Sensitivity and stability of the homocysteine color test

Experiments were carried out to determine the minimal concentrations of homocysteine required for formation of a detectable color when reacted on paper with ninhydrin and mercuric nitrate. It was found that approximately 100 μg was necessary for a positive test since quantities below this level proved difficult to distinguish from the color of the underlying ninhydrin reaction.

Solutions of homocysteine in medium M 150 were chromatographed after varying periods of storage at 37° C and tested for color formation with ninhydrin and mercuric nitrate. It was found that good color reactions were obtained for at least 2 days but beyond this time the intensity of the blue color formed diminished rapidly. These observations suggest that oxidation to homocystine has occurred, since homocystine does not undergo the characteristic color reaction (Table I). From these results, it would appear that detection of homocysteine by the color reaction with ninhydrin and mercuric nitrate is not reliable in solutions older than 2 days. However, the presence of reducing materials in any appreciable quantity might protect the homocysteine against oxidation and prolong the effective period of this test.

Reaction of homocysteine with ninhydrin at room temperature

Attempts were made to adapt the homocysteine color reaction on paper to a quantitative colorimetric procedure in test tubes. These attempts proved unsuccessful since the blue color formed with ninhydrin and mercuric nitrate precipitated almost immediately. Variations in the ninhydrin reagent and tests with various mercury salts at different concentrations did not overcome the precipitate formation.

During the course of these experiments, it was observed that the presence of Hanks' solution catalysed the reaction between ninhydrin and homocysteine at room temperature. Under these conditions, homocysteine reacted immediately with ninhydrin to form a pink color, whereas the other sulphur compounds tested formed their ninhydrin colors only after 15 to 30 minutes' standing at room temperature. In the case of the amino acids, the true ninhydrin colors did not appear until the papers were heated to 110° C. Attempts are now being made to adapt this observation to a rapid spot test for homocysteine on paper.

DISCUSSION

Although many recent tests for methionine[7,8] and cystine[9] have been reported, the lack of a specific test for homocysteine has made it somewhat difficult to follow the pathway of methionine metabolism in complete detail. The present experiments have shown that homocysteine can be detected in biological systems by a cherry red spot surrounded by a deep blue area formed when paper chromatograms are first sprayed with ninhydrin, heated, and then treated with mercuric nitrate solution. This color reaction is distinctly different from the mercuric nitrate and potassium iodide method used by DENT AND ROSE[10] for the detection of certain sulphur compounds on paper.

The mechanism of this homocysteine color reaction has not yet been determined,

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although the color formation has been found to be catalysed by the inorganic salts of Hanks' solution. The catalytic effect appears to be due to the presence of the anions rather than the cations in this mixture and to be relatively non-specific. The red-blue color formation itself, however, appears to be extremely specific since a wide variety of other sulphur compounds, including homocysteine thiolactone, homocystine and cysteine, failed to react. Of approximately 80 compounds of physiological interest tested, including amino acids, purines and pyrimidines, vitamins and accessory growth factors, no red-blue color formation was detected, except in the case of methionine. With this amino acid, however, its position on the paper chromatograms at some distance from the homocysteine position made it relatively easy to distinguish from homocysteine.

Although attempts to make this reaction quantitative were not successful, the method appears to be of value as a qualitative test for determining the presence of homocysteine in biological materials. The presence of inorganic salts, especially chlorides and phosphates, in biological media and buffers would serve to catalyse the detection of homocysteine by its reaction with ninhydrin and mercuric nitrate.

SUMMARY

Homocysteine on paper chromatograms forms a cherry red spot surrounded by a deep blue area when sprayed with ninhydrin, heated and subsequently treated with mercuric nitrate.

The color reaction appears specific for homocysteine and is not formed by homocystine or homocysteine thiolactone. Nearly 80 compounds of physiological importance, including 28 sulphur compounds, did not respond in this test.

The homocysteine color reaction is catalysed by inorganic salts, including chloride, phosphate and bicarbonate ions.

RÉSUMÉ

Après chromatographie sur papier, pulvérisation à la ninhydrine, chauffage et traitement ultérieur au nitrate mercurique, l'homocystéine donne une tache rouge cerise entourée d'une zone bleu foncé.

Cette réaction colorée semble spécifique de l'homocystéine et n'est donnée ni par l'homocystine ni par l'homocystéine thiolactone. Près de 80 substances d'intérêt physiologique, notamment 28 substances soufrées, ne réagissent pas.

La réaction colorée de l'homocystéine est catalysée par des sels minéraux, en particulier par les ions chlorure, phosphate et bicarbonate.

ZUSAMMENFASSUNG

Nach Ninhydrinbehandlung, Erhitzung und nachfolgender Behandlung mit Quecksilbernitrat, bildet Homocystein einen von einer dunkelblauen Zone umgebenen, kirschroten Fleck auf Papierchromatogrammen.

Diese Farbreaktion scheint spezifisch für Homocystein zu sein; Homocystin und Thiolakton, von Homocystein führen zu einem negativen Ergebnis. Fast 80 physiologisch bedeutende Substanzen, einschliesslich 28 Schwefelverbindungen, erwiesen sich unter diesen Bedingungen als unreaktiv.

Die Farbreaktion mit Homocystein wird von anorganischen Salzen, einschliesslich Chlorid-Phosphat- und Bikarbonationen, katalysiert.

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