Specific Determination of Hydroxy-L-Proline in Biological Materials.* (22395)

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Paper chromatographic studies on the amino acid metabolism of animal tissues *in vitro*(1) have shown that marked changes in amino acid content of synthetic medium M 150(2,3) occur during the cultivation period. Medium M 150 contains 60 ingredients, including 20 amino acids, in a modified Tyrode's solution(4) and its complexity made it difficult to detect changes in amino acids that were present in low concentrations or that did not separate completely on the chromatograms. Particular difficulty was encountered with hydroxyproline, which reacted only slightly on development with ninhydrin(5), isatin(6), vanillin(7) or isatin and *p*-dimethylaminobenzaldehyde(8). For this reason, an effort was made to develop a selective method for hydroxyproline that could be applied in the presence of high concentrations of other amino acids. The present communication reports such a specific method, based on a modification of the conventional ninhydrin procedure. The application of this method to complex synthetic media and to deproteinized chick embryo extract is presented.

Methods. Samples of media for analysis (5.0 ml) were concentrated to dryness *in vacuo* over H_2SO_4, reconstituted in 0.2 ml of deionized water and 10 λ portions used without desalting. One-dimensional descending chromatograms (Schleicher and Schuell No. 597 or Whatman No. 1 paper) were developed for 2 successive 18-hour periods in either *n*-butanol-acetic acid-water or *n*-butanol-ethanol-water. The chromatograms were dried at 110°C for 2 to 3 minutes and sprayed with 0.4% ninhydrin in water-saturated *n*-butanol or 95% ethanol. Full details of the chromatographic procedures have been reported previously(1,9,10). Chick embryo extract was prepared by grinding 11-day-old embryos and centrifuging off the tissue pulp(11). The supernatant extract was adjusted to pH 4.0 with 1 N H_2SO_4, precipitated proteins removed by centrifuging, and the pH readjusted to 7.2. Two volumes of acetone were added, the mixture allowed to stand overnight in the refrigerator, and centrifuged. The final supernatant was concentrated to dryness in the usual manner and used for analysis. Synthetic medium M 150 was prepared as described previously(2,3). All chemicals and reagents were of the highest purity obtainable. Spectrophotometric measurements were made by cutting appropriate areas from the developed chromatograms and fixing them to the inner walls of 1 cm Corex cells. Determinations were then made in a Beckman, Model DU. Ultraviolet examinations were carried out with a laboratory hand lamp (short wave model, 2537 Å).

Results. Development of a specific color with hydroxy-L-proline and ninhydrin. Previous studies on the determination of phenylalanine in synthetic medium M 150(10) showed that dipping ninhydrin-developed chromatograms in 1% sodium bicarbonate produced a stable blue color characteristic of this amino acid. At the same time, this alkali treatment converted the faint yellow hydroxyproline chromogen to a bright pink spot that faded within 5 minutes. Further studies on this reaction have now shown that dipping the chromatograms into 1 N HCl, within 2 minutes of the bicarbonate treatment, intensifies and stabilizes the pink hydroxyproline chromogen, and discharges the characteristic phenylalanine color. The hydroxyproline chromogen was firmly bound to the paper chromatograms and could not be eluted with water. Washing of the chromatograms with water removed the majority of other ninhydrin and amino acid colors and left the hydroxyproline chromogen as a clear and dis-

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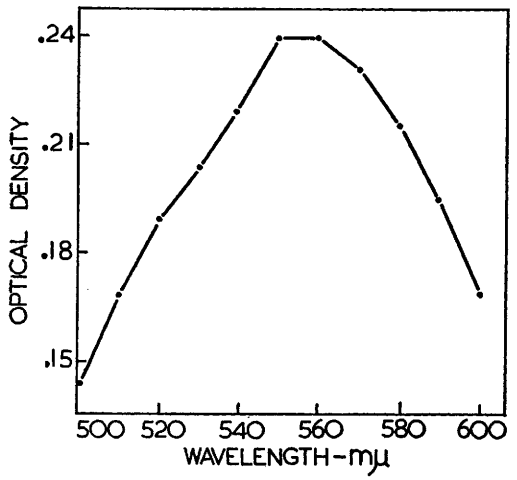

FIG. 1. Absorption curve of new hydroxyproline chromogen. Measurements made in Beckman, Model DU, using 1 cm Corex cells containing area cut from developed chromatogram. 25 μg hydroxy-L-proline.

tinct spot. The absorption curve of this new hydroxyproline color is presented in Fig. 1 and shows an absorption maximum at 550 to 560 mμ. It is evident that the alkali and acid treatment has caused a major shift in the absorption maximum away from the characteristic yellow imino acid and ninhydrin color (absorption maximum at 440 mμ).

Application to synthetic media and deproteinized embryo extract. The application of the method to synthetic medium M 150 containing different levels of hydroxyproline and proline and to deproteinized chick embryo extract is illustrated in Fig. 2. This figure shows 2 identical chromatograms, both developed by the conventional ninhydrin procedure but the second subsequently treated with bicarbonate and hydrochloric acid. By the conventional ninhydrin method (Fig. 2, 1 to 6), the presence of hydroxyproline in these materials is almost impossible to detect, due to the faintness of the yellow color produced and overlapping by more strongly-reactive amino acids. Application of the new procedure (Fig. 2, 1_4 to 6_4) removes the interfering amino acids, and hydroxyproline can now be detected readily by its characteristic color. As little as 5 to 10 μg of hydroxyproline can be demonstrated, either alone or in synthetic medium M 150. At the same time, the failure of proline to react increases the specificity of the method. The chromatogram also shows (Fig. 2, 3_4) that hydroxyproline is one of the major components of deproteinized chick embryo extract.

Fluorescence under ultraviolet light. Examination of the chromatograms under a hand ultraviolet lamp showed that the new hydroxyproline chromogen exhibited a strong pink fluorescence. This property could not be detected after the conventional ninhydrin development nor after subsequent alkali treatment but appeared immediately on acid and water treatment. In Fig. 3 is shown the hydroxyproline region of the previous chromatogram (Fig. 2) photographed under ultraviolet light. The marked fluorescence of the hydroxyproline chromogen is clearly shown in the chromatogram developed by the present procedure while only a faint fluorescence is visible in the areas developed by the conventional ninhydrin method. As little as 5 μg of hydroxyproline exhibits strong fluorescence (Fig. 2, 6_4). In additional experiments 1 to 5 μg were detected readily.

Discussion. Studies on the hydroxyproline content of foods(12) and biological materials generally have been handicapped by the lack of a specific method that can be applied to the analysis of complex mixtures and by the unavailability of a microbiological method for this amino acid(13). The majority of the methods so far devised either fail to distinguish clearly between hydroxyproline and proline or cannot be applied to materials in which the hydroxyproline concentration is low in proportion to that of other amino acids. The present method overcomes these difficulties through the formation of a characteristic chromogen specific for hydroxyproline but not proline. Moreover, the method was found suitable for the analysis of synthetic media in which hydroxyproline represented only 1% of the total amino acids present. Because the specific hydroxyproline chromogen remained fast to the paper chromatograms, it became possible to wash away, with water, overlapping spots and determine this amino acid under conditions of poor separation. In this way, deproteinized chick em-

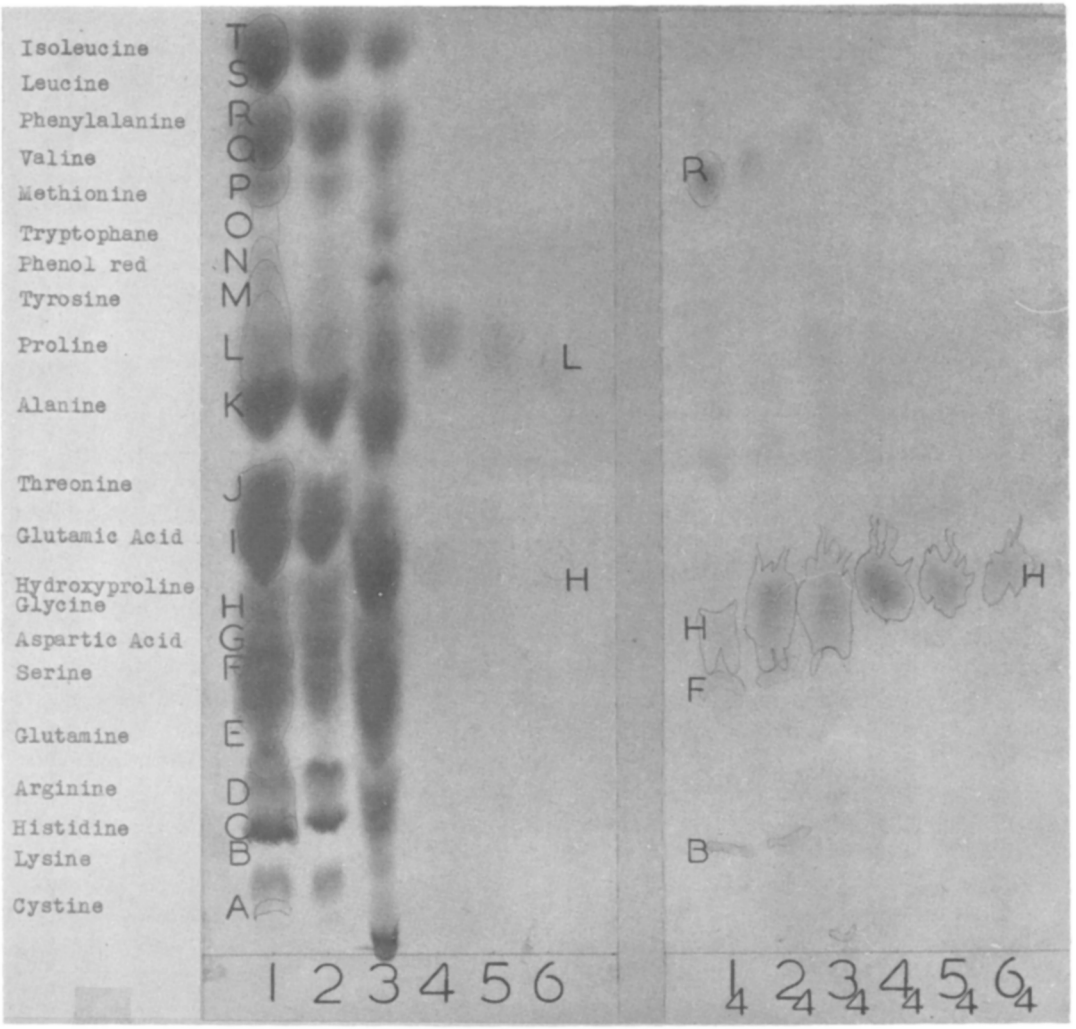

FIG. 2. Duplicate chromatograms, both developed by ninhydrin, but chromatogram on right subsequently treated with 1 N $NaHCO_3$, 1 N HCl and washed with water. 1 and 1_4, synthetic medium M 150, containing 5 μg of hydroxyproline and 20 μg of L-proline; 2 and 2_4, M 150 containing 25 μg of hydroxyproline and proline; 3 and 3_4 deproteinized chick embryo extract; 4 and 4_4, 25 μg of hydroxyproline and proline in water; 5 and 5_4, 10 μg of hydroxyproline and proline in water; 6 and 6_4, 5 μg of hydroxyproline and proline in water.

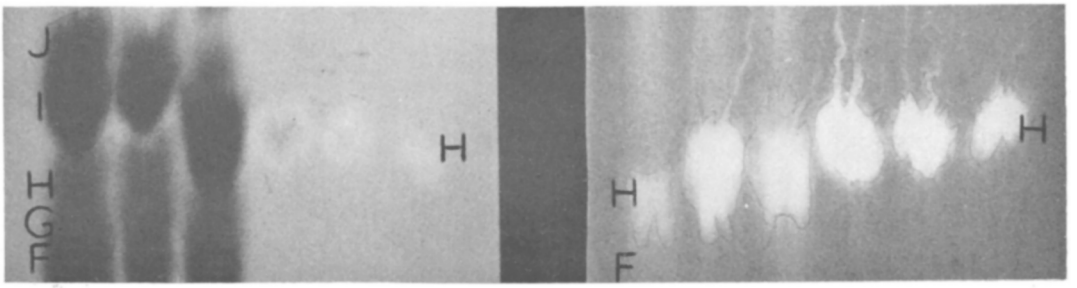

FIG. 3. Hydroxyproline region of Fig. 2 photographed under ultraviolet light.

bryo extract was found to contain a relatively high concentration of hydroxyproline. This observation is in marked contrast to the low level reported by Westfall, Peppers and Earle (14), who used the conventional isatin method.

This procedure for hydroxyproline has been used successfully in studies on the amino acid metabolism of tissues cultivated in synthetic medium M 150(1). This precludes interference by approximately 60 compounds of biological importance, including amino acids, vitamins, purines and pyrimidines, salts and certain accessory growth factors. The only compound observed to form a somewhat similar compound was phenylalanine but this chromogen was discharged by the acid treatment used to stabilize the hydroxyproline color. An unusual feature of the method is that it can be applied to paper chromatograms previously developed with ninhydrin for the measurement of other amino acids. The chemical nature of the hydroxyproline chromogen has not been determined, although there appears to be some resemblance to the benzene-soluble red compound observed by Troll and Cannan(15). It should be noted, however, that the chromogen isolated by these workers was formed by proline as well as by hydroxyproline. The intensity of the specific chromogen and its fluorescence varied with the concentration of hydroxyproline and attempts to develop a quantitative method are now in progress.

Summary. A new, specific method for hydroxyproline is reported, based on the development of a characteristic chromogen through ninhydrin development of paper chromatograms followed by rapid treatment with dilute alkali and dilute acid. The new chromogen has an absorption maximum at 550 to 560 mμ and fluoresces strongly under ultraviolet light. Application of the method to complex biological media showed that 5 to 10 μg of hydroxyproline could be detected. A high level of this amino acid was shown in chick embryo extract for the first time.

1. Pasieka, A. E., Morton, H. J., and Morgan, J. F., *J. Nat. Cancer Inst.*, 1956, v16, 995.

2. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

3. Morgan, J. F., Campbell, M. E., and Morton, H. J., *J. Nat. Cancer Inst.*, 1955, v16, 557.

4. Hanks, J. H., and Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

5. Dent, C. E., *Biochem. J.*, 1948, v43, 169.

6. Acher, R., Fromageot, C., and Jutisz, M., *Biochim. et Biophys. Acta,* 1950, v5, 81.

7. Jepson, J. B., and Smith, I., *Nature,* 1953, v172, 1100.

8. Curzon, G., and Giltrow, J., *ibid.*, 1954, v173, 314.

9. Pasieka, A. E., and Morgan, J. F., *Biochim. et Biophys. Acta,* 1955, v18, 236.

10. ———, *ibid.*, 1956, v19, 356.

11. Morgan, J. F., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 665.

12. Aronson, J. R., and Elvehjem, C. A., *Food Res.*, 1956, v21, 109.

13. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, v156, 703.

14. Westfall, B. B., Peppers, E. V., and Earle, W. R., *J. Nat. Cancer Inst.*, 1954, v15, 433.

15. Troll, W., and Cannan, R. K., *J. Biol. Chem.*, 1953, v200, 803.

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