Specific Determination of Proline in Biological Materials.* (22661)

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Paper chromatographic studies on the amino acid metabolism of animal tissues cultivated *in vitro*(1) have shown that marked changes in the amino acid content of synthetic medium M 150(2,3) occur during the cultivation period. Medium M 150 contains 60 ingredients, including 21 amino acids, in a modified Tyrode's solution(4). Its complexity made it difficult to detect changes in certain amino acids, such as proline, that were present in low concentration or that reacted weakly with ninhydrin. Application of the isatin method for proline(5), or various modifications of this method(6-10), proved unsatisfactory since many other amino acids produced colors similar to that of proline. In earlier studies from this Laboratory(11-13), it was shown that the conventional ninhydrin procedure could be made specific for certain amino acids by treatment of the chromatograms with dilute acid or alkali subsequent to the ninhydrin development. Accordingly, similar procedures were tried with the isatin method and from these experiments, a specific and quantitative method for proline has been developed.

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 paper chromatograms were developed for 18 hours in either *n*-butanol-acetic acid-water or *n*-butanol-ethanol-water solvent systems. The chromatograms were then dried at 110°C for 2 to 3 minutes, dipped in isatin reagent (0.4% isatin dissolved in *n*-butanol containing 4% acetic acid), and reheated at 110°C for 10 to 15 minutes. Subsequent treatment of the chromatograms, with acid, to produce a selective color for proline is discussed in the succeeding paragraph. Full details of the general chromatographic procedures have been reported(1,11-13). Synthetic medium M 150 was prepared as described earlier(2,3). All chemicals and reagents were of the highest purity obtainable. Spectrophotometric measurements were made by cutting appropriate areas from the chromatograms and fixing them to the inner walls of 1 cm Corex cells filled with water. Optical densities were then determined in a Beckmann, Model DU. Direct measurements of the color intensity on the chromatograms were made with a densitometer (Welch Densichron).

Results. Selective development of color with proline and isatin. Paper chromatograms of medium M 150 were prepared, developed as described, and dipped in the isatin reagent. These chromatograms were then treated with various combinations of the acid and alkali procedures previously found suitable for specific color development with other amino acids(11-13). These experiments showed that washing the isatin-treated chromatograms with 1 N HCl, and suspending them at room temperature for 10 to 15 minutes intensified the blue proline chromogen, and at the same time caused almost complete fading of all other colored spots. Further

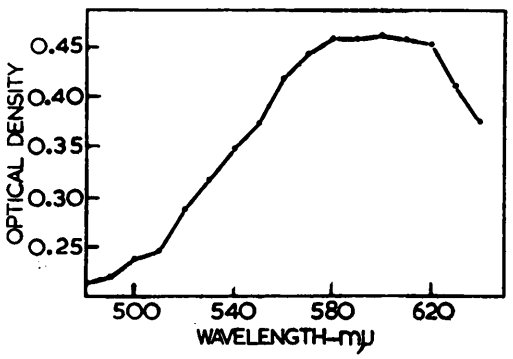

FIG. 1. Absorption curve of proline chromogen. Measurements made in Beckmann, Model DU, using 1 cm Corex cells containing area cut from developed chromatogram. 8 μg proline.

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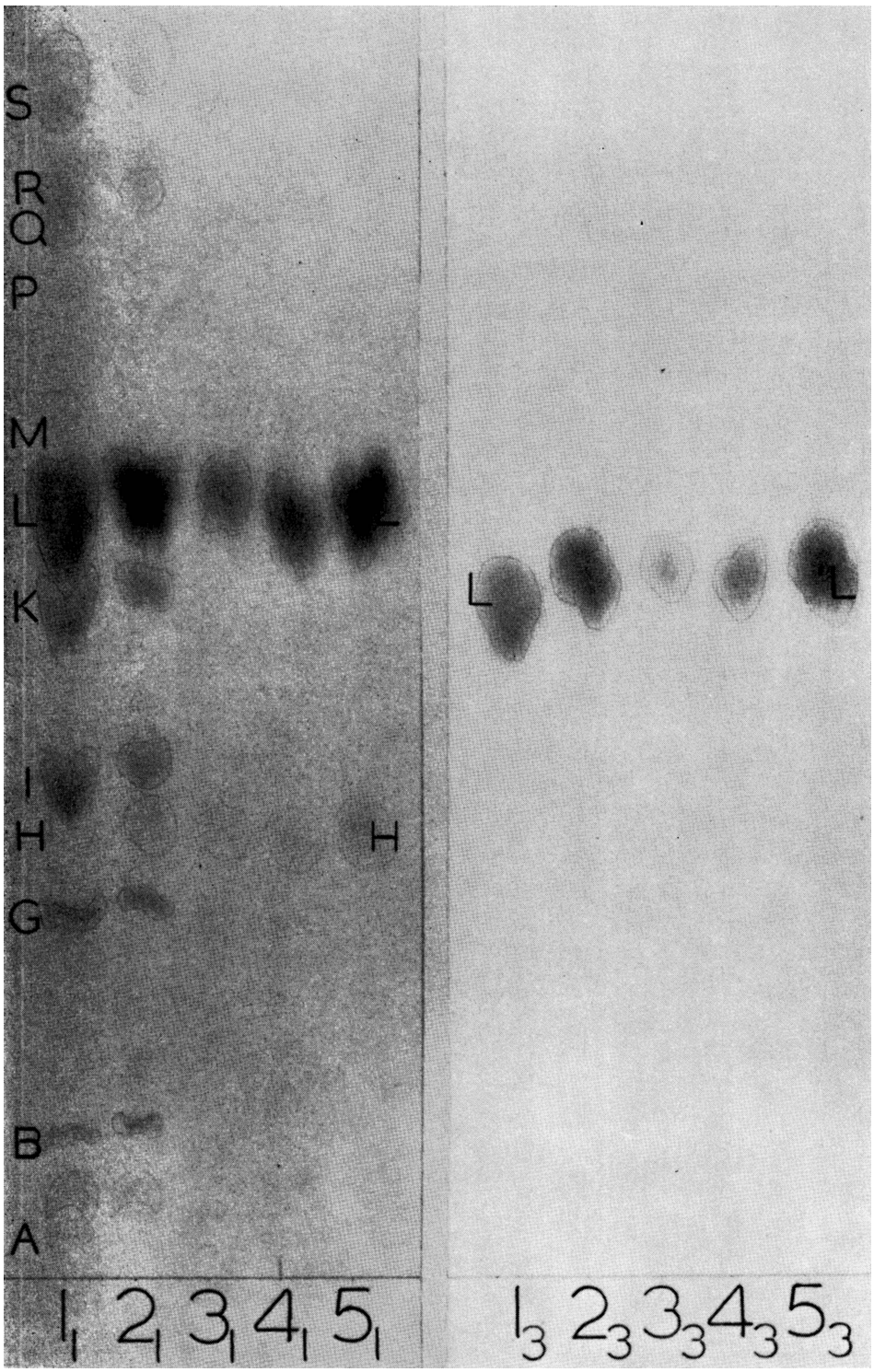

FIG. 2. Duplicate chromatograms, both developed by isatin, but chromatogram on right subsequently dipped into 1 N HCl and washed with water. 1_1 and 1_3, synthetic medium M 150, containing 20 μg of proline; 2_1 and 2_3, M 150 containing 25 μg of proline; 3_1 and 3_3, 5 μg of proline in water; 4_1 and 4_3, 10 μg of proline in water; 5_1 and 5_3, 25 μg of proline in water.

experiments established that this acid treatment bound the proline color so firmly to the paper chromatograms that the excess isatin reagent could be removed without eluting the proline chromogen. This was accomplished by washing with distilled water while the chromatograms were still damp. In this way, clear and distinct blue proline spots were obtained on a perfectly white background, free from any contamination with other amino acids. The absorption curve of this proline color is presented in Fig. 1 and shows a maximum at 600 mμ, with a broad plateau between 580 and 620 mμ. The absorption curve of the proline and isatin chromogen does not appear to have been reported previously.

Application of specific proline method to complex synthetic media. The application of this method to synthetic medium M 150 containing different levels of proline and hydroxyproline is illustrated in Fig. 2, showing 2 identical chromatograms, both developed by the conventional isatin procedure(5,6), but the second subsequently dipped into 1 N HCl and washed with water until the orange-yellow background color of the isatin reagent has been removed. By the conventional procedure (Fig. 2, 1_1 and 2_1), the presence of 20 and 25 μg of proline can be seen (L region), but interpretation of the chromatogram is made difficult by the large number of other amino acids also showing color development with isatin, and by overlapping of the proline spot with alanine (K) and tyrosine (M). Application of the new procedure (Fig. 2, 1_3 and 2_3) removes the interfering amino acids and proline is left as the only spot. The comparative behaviour of proline (L region) and hydroxyproline (H region) is also shown on these chromatograms. By the conventional isatin procedure (Fig. 2, 3_1 to 5_1), 5, 10, and 25 μg of proline show distinct color development, while the same quantities of hydroxyproline show only slight color formation. Application of the new procedure (Fig. 2, 3_3 to 5_3) clarifies the proline color development and, at the same time, completely removes the hydroxyproline color.

Quantitative measurement of proline by the new procedure. The gradation in color intensity with graded concentrations of proline (Fig. 2, 3_3 to 5_3) suggested that this procedure might be made quantitative. Attempts to elute the proline color with water, dilute HCl, dilute NaOH, methanol, ethanol, butanol, propanol, acetone, carbon tetrachloride, or benzene were unavailing, even though the extractions were performed with mechanical shaking until the filter paper was reduced to a pulp. Attempts were then made to determine proline directly on the chromatograms, employing a densitometer to measure peak density of graded levels of this amino acid. Following these measurements, the proline areas were cut from the chromatograms, placed on the inner walls of 1 cm absorption cells containing water, and the color intensity measured in a spectrophotometer at 600 mμ, employing as a blank a cuvette containing untreated filter paper. By careful placement of the proline spot to cover the center of the cuvette face, accurate measurements of peak density were obtained. The standard curves obtained by these two methods, based on the average results from 10 separate chromatograms containing graded amounts of proline, are presented in Fig. 3. By the spectrophotometer method (Fig. 3, Curve A), a linear response is exhibited over the concentration range of 1 to 16 μg. With the densitometer

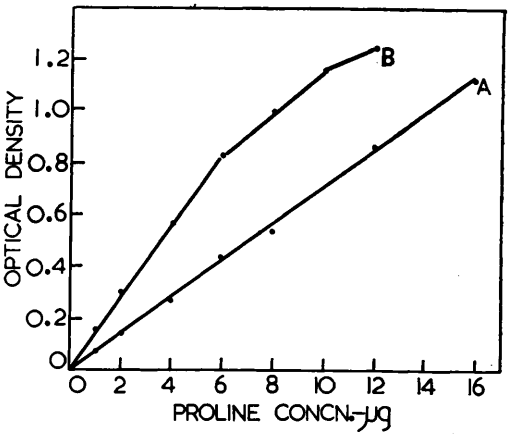

FIG. 3. Standard curves for graded concentrations of proline in M 150. Curve A, measurements made in Beckmann at 600 mμ. Curve B, measurements made on chromatograms with a densitometer. All points represent avg values from 10 separate chromatograms containing total amounts of proline specified.

method (Fig. 3, Curve B), linearity is maintained from 1 to 6 μg, then falls off. Attempts to maintain linearity by measuring both area and peak density were unsuccessful, and, for this reason, the spectrophotometer method is recommended for use.

Discussion. The isatin method has been used for determination of proline in known amino acid mixtures(5-10) but application of this method to complex biological media is difficult, since many other amino acids form colored complexes resembling that of proline. The present experiments have shown that the isatin method can be made specific for proline by subsequent treatment of the chromatograms with dilute acid. The chief advantages of this new procedure are simplicity and degree of specificity achieved. Moreover, use of this method with synthetic medium M 150 precludes interference by approximately 60 substances of biological importance, including amino acids, purines, pyrimidines, vitamins, and accessory growth factors. In addition, this method does not require desalting, even though the concentration of inorganic ions in the synthetic medium is high. Binding of the proline chromogen to the paper and fading of the isatin colors of the other amino acids resulting from the acid treatment makes it possible to apply this method under conditions of poor separation and resolution on the chromatograms. Quantitative studies have established that the intensity of the color is linear over a concentration range of 1 to 16 μg of proline. This range is sufficient to measure the levels of proline normally encountered in natural substances. The application of this method for proline, in conjunction with the specific method for hydroxyproline previously reported(13), makes it possible to determine selectively each of these amino acids in biological materials.

Summary. A specific method for determining proline is reported, based on treatment of isatin-developed paper chromatograms with dilute acid. None of the 20 other amino acids of synthetic medium M 150, including hydroxyproline, reacted by this procedure. The proline chromogen exhibits an absorption maximum at 600 mμ. The color is remarkably stable and can be used for the quantitative determination of proline over the range of 1 to 16 μg.

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