Increased sensitivity and semiquantitative determination of phenylthiohydantoin derivatives of amino acids with a modified spray reagent

The EDMAN degradation procedure[1] is a universally employed technique for the determination of amino acid sequence of proteins and peptides. The resulting derivatives of the terminal amino acids are readily separated by paper chromatography[2,3]. Quantitative estimates of the phenylthiohydantoin (PTH) derivatives separated by paper chromatography are not completely satisfactory, however, because of the amount of time and material required. We have found that by a simple modification of the spray reagent commonly employed for the localization of the PTH derivatives[2], it is possible to increase the sensitivity of reagent for the detection of the amino acid derivatives and at the same time to obtain a semiquantitative estimate of the amount of the derivative on the chromatogram.

PTH derivatives of the various amino acids were purchased from Mann Research Laboratories, Inc. Solutions of the PTH derivatives were made up in ethanol. Two stock mixtures of PTH derivatives were made up to contain varying concentrations of four derivatives, as indicated in Fig. 1. The highest concentration of any amino acid on the chromatograms shown in Fig. 1 is 0.1 μmoles while the lowest concentration is 0.0008 μmoles. The chromatography was carried out in solvents D and E of EDMAN AND SJÖQVIST[3]. The papers were dipped in 10% formamide and acetone, blotted on filter paper to remove excess solvent, and then dried slightly in air to remove excess acetone. Aliquots of 0.01 ml of each one of the mixtures of amino acids were then applied to these papers, giving a spot approximately 1 cm in diameter. Before chromatography the papers were allowed to equilibrate for approximately 20 min in rectangular glass tanks lined on all four sides with paper dipped in the solvent. The chromatography was run in an ascending manner and the solvent was allowed to rise approximately 20 cm.

The spray reagent used to visualize the PTH derivatives and give a semiquantitative estimate of their concentration was a simple modification of the iodine azide spray reagent. A solution of sodium azide containing 1.5 g per 100 ml was used to dilute a solution of potassium iodide–iodine solution which contained 2.54 g of iodine and 8 g of KI per 100 ml. The azide solution was stable and could be stored for reasonable periods of time. The potassium iodide–iodine solution was made up fresh each day. The actual spray reagent was made by diluting the potassium iodide–iodine reagent with the sodium azide solution in ratios of 1:128, 1:64, 1:32, 1:16 and 1:4. Thus five different concentrations of the potassium iodide–iodine were obtained for the spray reagent.

After development, the chromatograms were sprayed lightly with the reagent on both sides of the paper. The spots were fully developed within 2 min after spraying. The blue color of the background faded on drying when the dilute iodine reagent was employed. After spraying with the most dilute spray reagent, in order to detect the lowest possible concentration of material on the chromatogram, the chromatogram is allowed to dry and immediately sprayed with the next most concentrated spray. This procedure can be repeated with increasing concentrations of the spray. In this manner, the order of magnitude of the material on a chromatogram can be determined.

Fig. 1 shows the results obtained on chromatography of the mixtures of PTH

derivatives. It is apparent that the spray reagent containing the lowest concentration of iodine is the most sensitive. With this spray reagent less than 0.001 μmoles of a PTH derivative could be detected. When the concentration of the iodine in the spray reagent was doubled, the spot containing 0.001 μmoles of the PTH could no longer be detected but 0.004 μmoles of PTH or higher were visible. Table I indicates the limits of detectability of the PTH derivatives with different dilutions of the spray reagent.

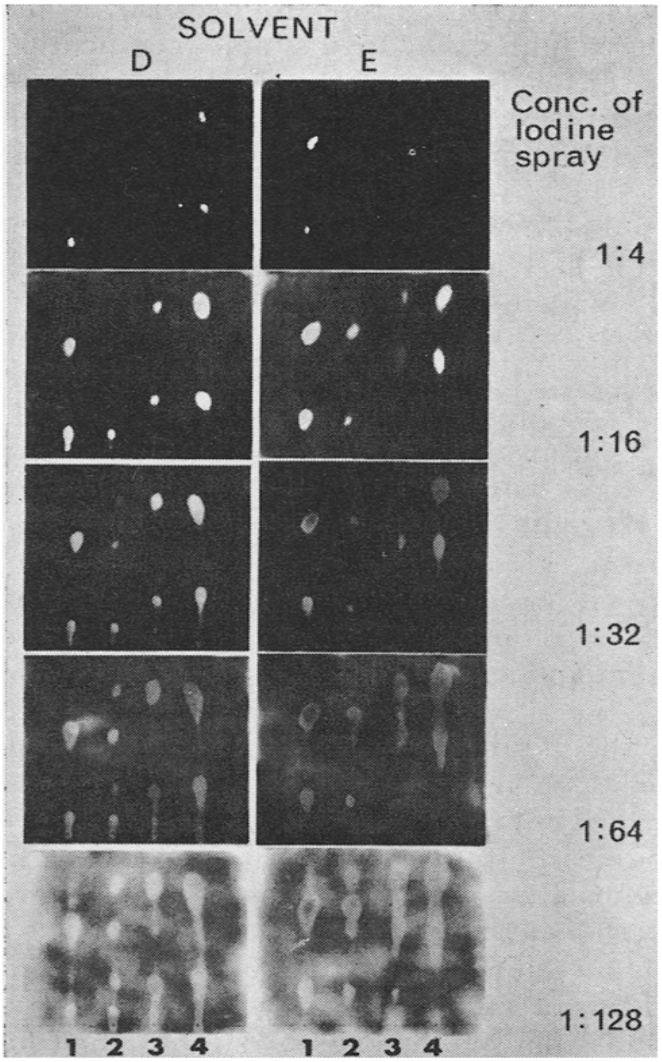

Fig. 1. Use of varying concentrations of iodine azide spray reagent in order to obtain a semi-quantitative analysis of PTH derivatives after chromatographic separation. The following mixtures of four amino acids were used as indicated in the text.

PTH derivatives		*Concentration of PTH derivative (μmoles)*			
Solvent D	*Solvent E*	*1*	*2*	*3*	*4*
Proline	Methionine	0.0008	0.004	0.02	0.1
Valine	Glycine	0.1	0.02	0.004	0.0008
Alanine	Glutamic acid	0.0008	0.004	0.02	0.1
Lysine	Histidine	0.1	0.02	0.004	0.0008

TABLE I

EFFECT OF CONCENTRATION OF IODINE ON LEVELS OF DETECTION

Dilution of iodine reagent	*PTH derivative, detectable μmoles*
1/128	0.001
1/64	0.002
1/32	0.005
1/16	0.01
1/4	0.1

As shown in Fig. 1, the dimensions of the spots also indicate the concentration of the material on each spot. When the derivative is present in higher concentrations, the area of the spot is larger. The ability of the PTH derivatives to decolorize the spray reagent is not the same for all derivatives[2]. For our work, we have always attempted to evaluate the concentration of the particular derivative present by comparison with standards of varying concentrations of the compound run on the same chromatogram.

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Diazotised sulphanilic acid reagent as an aid to the identification of some oxo acid 2,4-dinitrophenylhydrazones

Paper chromatography of the 2,4-dinitrophenylhydrazones of oxo acids of blood and urine may yield useful information in the investigation of biochemical abnormalities in various diseases. The solvent systems currently employed do not resolve faster moving components which may include those of particular metabolic interest, *e.g.* phenylpyruvate and the "keto leucines". In an attempt to assess the relative concentrations of individual components in such composite spots, diazotised sulphanilic acid was used as a dipping reagent and was found to have some general use in the identification of oxo acid hydrazones.