Sodium 1:2-naphthoquinone-4-sulfonate as a reagent for identification of amino acids and peptides and for quantitative estimation of proline and hydroxyproline separated on paper chromatograms.

Sodium 1:2-Naphthoquinone-4-sulfonate (0·02% in 5% aqueous sodium carbonate solution) has been suggested by MÜTING[1]) as a spraying reagent for identification of amino acids on filter paper. When testing this reagent on circular paper chromatograms irrigated with n-butanol-acetic acid-water, it was found that the water in the spray caused diffusion and spreading of the bands of amino acids separated on paper with consequent poor definition of the bands. This difficulty was eliminated by dissolving the reagent in aqueous acetone and modifying the procedure for treatment of the chromatogram with the reagents so as to obtain clear and well defined bands of varying colours of visible intensity. Various modifications of the reagent were tried and the following reagents were finally selected, as they were found to yield best results.

Reagent I: 0·100% Sodium 1:2-Naphthoquinone-4-sulfonate (Eastman Kodak Co.), in acetone. This reagent was prepared by dissolving 0·300 gr. of the sulfonate in 10 c.c. of water and making up the volume to 300 c.c. with acetone. The reagent can be kept for about a week in the refrigerator without deterioration.

Reagent II: 95% alcoholic alkali: 2 c.c. of 5N sodium hydroxide made up to 100 c.c. with 95% alcohol.

100 c.c. of the alcoholic alkali and 30 c.c. of the Reagent I were mixed immediately before use.

Procedure: The chromatogram after development was first dipped into the reagent I and heated for about three to five minutes at 80—90° C to promote colour development. The amino acids appear as light rose coloured bands or spots without showing any distinct difference in colours between the various amino acids.

For further development the chromatogram was then dipped in Reagent II (100 c.c. of alcoholic alkali containing 30 c.c. of reagent I). This reagent mixture was prepared immediately before use. The paper was kept immersed in the reagent for about half to one minute until the colours develop in intensity and then dried at room temperature. The reagent turned pink in colour after some time and was rejected after development. Fresh mixtures were always prepared each time.

The colours given by various amino acids and peptides are as follows:

Table 1. *Colours given by amino acids.*

Amino acids	Colour	Amino acids	Colour
Leucine	Greenish blue	Glutamic acid	Green
Iso-Leucine	Light green	Threonine	Green
Nor-Leucine	Light green	Serine	Green
Phenyl Alanine	Light green	Hydroxyproline	Orange
Valine	Bluish green	Glycine	Greenish blue
Methionine	Bluish green	Asparagine	Light blue
Tyrosine	Grey	Aspartic acid	Light blue
Trytophane	Brown	Arginine	Light blue
γ-Aminobutyric acid	Blue	Lysine	Light blue
α-Aminobutyric acid	Blue	Ornithine	Light blue
β-Aminobutyric acid	Brown	Histidine	Dark Violet
Proline	Orange	Cystine	Dark blue
Alanine	Green	Cysteine	Dark blue
β-Alanine	Blue	Glutamine	Greenish blue

Table 2. *Colours given by peptides.*

Peptides	Colour	Peptides	Colour
Alanyl-Glycine	Bluish green	Diglycyl-Glycine	Green
Glycyl-Glycine	Yellowish green	Leucyl-Glycyl-Glycine	Light green
Glycyl-1-Tryptophan	Yellowish green	Glutathione	Brown
Glycyl-Leucine	Yellowish green		

As little as 5 micro grams of the amino acids can be detected by this reagent. One of the typical circular paper chromatograms developed according to the technique described by GIRI and RAO[2]) and treated with the reagents is shown in Fig. 1. Fig. 2 is the comparative circular paper mixed chromatogram showing the amino acid bands as revealed by treatment with ninhydrin reagent (0·1% in acetone) and sodium 1:2-Naphthoquinone-4-sulfonate reagent. It is clearly seen from the figures that the amino acid bands on the chromatogram treated with this reagent appear as distinct and clear bands without any spreading. The colours are stable over long periods compared to the colours obtained by ninhydrin reagent. Most amino acids produce blue or green colours. Proline and hydroxyproline, however, yield orange, tryptophan brown, and tyrosine grey. Histidine develops almost immediately as intense violet spot or band.

The reaction is specially sensitive to proline and hydroxyproline, which appear on the chromatogram as distinct orange coloured bands and may be of value as a confirmatory test for these amino acids. The reagent is also useful for the identification of γ-amino butyric acid which is widely distributed in plant and animal tissues. As γ-aminobutyric acid bands appears between proline and valine bands on the chromatogram irrigated with n-butanol-acetic acid-water as solvent, the presence of this amino acid can be easily detected by the appearance of a green band between valine (bluish green) and proline (orange) bands. The only other amino acid bands which appear between valine and proline bands are tyrosine and tryptophan and the colours produced by these amino acids being grey and brown are distinctly different from the colour produced by γ-amino butyric acid.

In addition to the qualitative differentiation and identification of amino acids by this reagent, our preliminary obser-

vations have shown that the reagent can be used for the quantitative determination of proline and hydroxyproline separated on the chromatogram. After development of the colour with the reagent, the proline and hydroxyproline bands can be cut out and the orange colour can be extracted with 5 c.c. of water and the intensity of the colour of the aqueous extract can be determined in KLETT-SUMMERSON colorimeter using

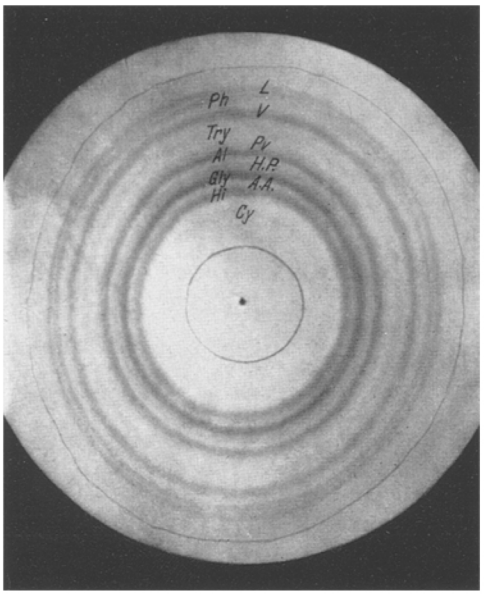

Fig. 1. Circular paper chromatogram showing the separation of aminoacids in the form of concentric circles after treatment with sodium-1:2-Naphthoquinone-4-sulfonate. *L* Leucine; *Ph* Phenylalanine; *V* Valine; *Try* Tryptophan; *P* Proline; *Al* Alanine; *H.P.* Hydroxyproline; *Gly* Glycine; *A.A.* Aspartic acid; *Hi* Histidine; *Cy* Cystine.

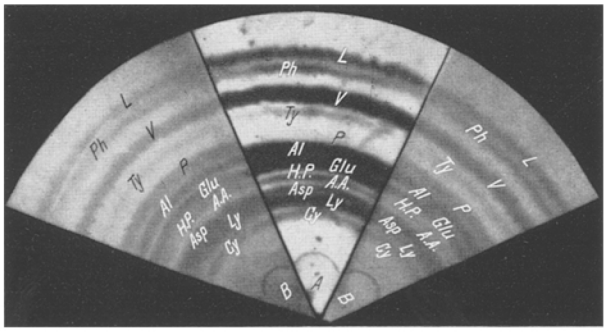

Fig. 2. Circular paper mixed chromatogram showing the amino acid bands separated on paper after treatment with ninhydrin reagent (Sector A) and sodium 1:2 Naphthoquinone-4-sulfonate reagent (Sector B). *L* Leucine; *Ph* Phenylalanine; *V* Valine; *Ty* Tyrosine; *P* Proline; *Al* Alanine; *Glu* Glutamic acid; *H.P.* Hydroxyproline; *A.A.* Aspartic acid; *Asp* Asparagine; *Ly* Lysine; *Cy* Cystine.

green filter No. 540, according to the procedure described by GIRI et al[3]) for the quantitative determination of amino acids separated on the chromatogram treated with ninhydrin reagent. Calibration curves showed direct proportionality between the colour density and the concentration of proline and hydroxyproline between the range 10—100 micro grams. The colour after extraction with water was found to be stable. Using this method the proline and hydroxyproline contents of protein hydrolysates are being investigated and full details of this method will be published elsewhere.

Department of Biochemistry Indian Institute of Science, Bangalore 3.

K. V. GIRI and A. NAGABHUSHANAM.

Eingegangen am 29. September 1952.

[1]) MÜTING, D.: Naturwiss. **39**, 303 (1952).
[2]) GIRI, K. V., and N. A. N. RAO: Nature [London] **169**, 923 (1952). — J. Indian Inst. Sci. **34**, 95 (1952).
[3]) GIRI, K. V., A. N. RADHAKRISHNAN and C. S. VAIDYNATHAN: Analyt. Chem. **1952**.