with eluents (1) and (3) acquired the same rose red colour; in the case of eluent (2) the dye spots retained their original shade.

It can be seen from Table I that the R_F values for the α- and β-naphthol derivatives differ considerably in all three systems. In a separate experiment, one drop of a solution containing both α- and β-naphthol was placed alongside the two drops of the α-naphthol and β-naphthol solutions. After coupling all three spots separately with diazotised Amino-J acid, the paper was dried and developed with isoamyl alcohol–ethyl alcohol–ammonia solution. A typical chromatogram is shown in Fig. 1. It can be seen that on development the spot of the mixture of the two naphthols is separated into two spots, which move parallel to those of α- and β-naphthol respectively.

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Quantitative determination of proline by paper chromatography

Since the ninhydrin reagents are not very sensitive to proline, the isatin method for the detection of this amino acid has frequently been used, even for quantitative purposes. PASIEKA AND MORGAN[1] used the isatin reagent according to the method of ACHER *et al.*[2]. As these authors were not able to elute the coloured product obtained from proline with water or with other mineral or organic solvents from paper, they evaluated the spots—after washing out other spots and the background—by measuring the density of the proline areas in the cuvettes of a spectrophotometer. Since it is well known that densitometric methods *in situ* are subject to inaccuracies, it is to be expected that higher precision could be obtained if it were possible to elute the dye and measure its density in solution.

For the purpose of detecting proline on chromatograms the reagent according to ACHER *et al.*[2] and the reagent according to BARROLLIER *et al.*[3], containing Zn acetate and acetic acid, were compared. The latter is more sensitive and therefore it was used in further work. The coloured reaction product of proline can be eluted from

the chromatogram—after washing out the background with water and drying—with pyridine or with alcoholic or aqueous phenol. The best results as regards intensity and stability of the eluate were obtained by using water-saturated phenol for elution. On measuring the spectra of this eluate with the spectrophotometer SF 4, maximum absorption was obtained at 610 mμ (Fig. 1).

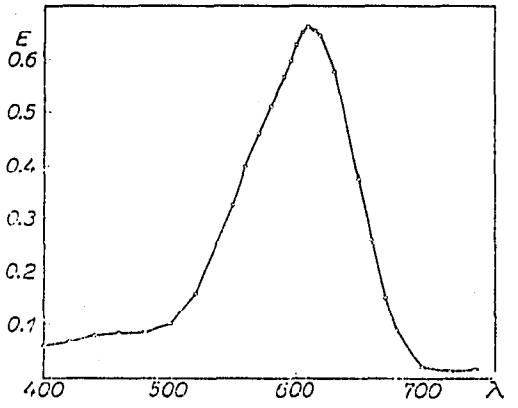

Fig. 1. Absorption curve of the coloured reaction product of proline (28.75 μg) in water-saturated phenol. Measurements made with the spectrophotometer SF 4.

A study was then made of the relation between the extinction and the amount of proline, to find out in which range it is in accordance with Lambert-Beer's law. Varying quantities of proline (ranging from 10–50 μl of 0.005 *M* proline solution) were applied to Whatman No. 1 paper and developed three times with the solvent system *n*-butanol–acetic acid–water (4:1:5) for a total period of 108 hours, which is approximately the time needed for a good separation of proline in an extract from biological material. The proline was then detected and the spots were eluted according to the

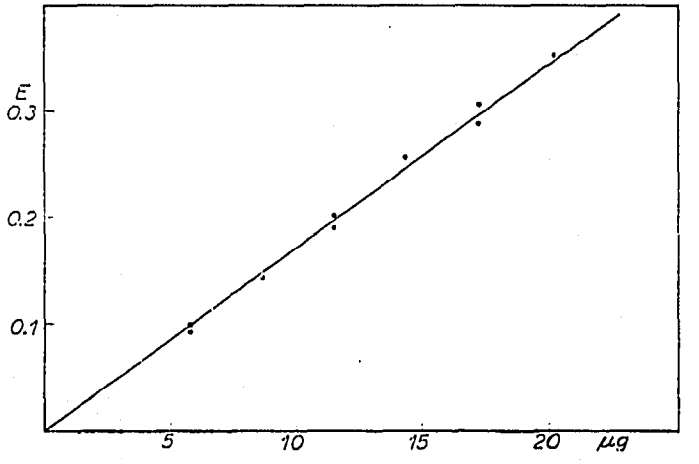

Fig. 2. Standard curve for proline. Measurements made with the Lange photocolorimeter.

method described below. The eluates were measured in the Lange photocolorimeter with the Schott OG 2 filter. The standard curve obtained (Fig. 2) showed that linearity is maintained over the concentration range up to 20 μg of proline.

The stability of the dye in water-saturated phenol in the light and in the dark was studied. The results (Table I) indicated that the eluates are stable for 1 hour in the dark but normal daylight causes a rapid destruction of the coloured proline product.

On the basis of the results obtained in these experiments, the following method for the quantitative estimation of proline by paper chromatography can be recommended.

For paper chromatography amounts of extracts of biological material containing from 5 to 20 μg of proline should be used. It is also necessary to apply at least two concentrations of the standard solution of proline to every chromatogram. After good separation of proline by a suitable solvent system, the spots on the chromatogram are detected by dipping it in the isatin reagent prepared according to BARROLLIER *et al.*[3]

TABLE I

STABILITY OF THE COLOURED REACTION PRODUCT OF PROLINE (23 μg) IN WATER-SATURATED PHENOL

Measurements made with the Lange photocolorimeter.

Time in min	*Extinction*			
	when exposed to light		*when kept in the dark*	
15	0.433	0.435	0.462	0.455
30	0.429	0.433	0.470	0.456
45	0.423	0.427	0.468	0.460
60	0.383	0.385	0.462	0.453
90	0.340	0.333	0.450	0.443
120	0.322	0.326	0.448	0.438
180	0.268	0.249	0.425	0.418
300	0.219	0.221	0.391	0.387

(1 g isatin, 1.5 g Zn acetate, 1 ml acetic acid, 95 ml isopropyl alcohol, 5 ml water) and heating for 30 min at 80–85°. The excess of isatin is removed by washing the chromatogram with warm water (about 30°), so that the background is white or pale yellow. The blank value in both cases is insignificant. After cutting the spots into small pieces, 5 ml of water-saturated phenol is added and the dye is eluted for 15 min in the dark with occasional shaking. Measurement of the density is performed at 610 mμ without unnecessarily exposing the samples to light. The accuracy of this method is $\pm$ 2.5 %.

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