may account for some of the anomalies in the quantitative results. Our 'separating funnel' extraction procedure (Gordon *et al.* 1943*a*) is not subject to this defect.

Dr G. R. Tristram informs us that he has overcome this trouble by placing a short column of silica gel saturated with water below the gel in which the acetylation mixture is absorbed.

SUMMARY

The following technical aspects of the quantitative analysis of amino-acids by partition chromatography are discussed: the preparation of silica gel; choice of indicators; preparation of material for chromatography.

We are grateful to Prof. Sir R. Robinson, F.R.S., and to Prof. A. R. Todd, F.R.S., for gifts of synthetic anthocyanins; to Mrs L. M. Synge, Chester, for collecting and drying petals; and to Mr W. Wolff for his technical assistance. We wish to thank the Director and Council of the Wool Industries Research Association and the International Wool Secretariat for permission to publish part of this work.

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A New Indicator for Use in Partition Chromatography: 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine

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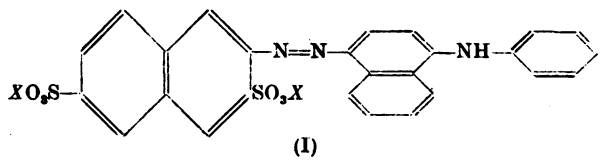
An indicator suitable for use in partition chromatography (Martin & Synge, 1941; Gordon, Martin & Synge, 1943*a*) should possess two main characteristics: it should not be leached from the column by the solvents used in developing the chromatogram and it should give an easily visible colour change. The first of these requirements calls for insolubility in organic solvents; the second is best fulfilled by an indicator which is pale in alkaline solution and which changes to a deeper and contrasting colour in acid solution. In this paper we describe the preparation and use of an indicator which appears to meet these requirements fully.

We prepared azo-dyestuffs by coupling various diazonium salts with *N*-phenyl-α-naphthylamine and studied their behaviour in partition chromatograms. The dyestuff prepared from sulphanilic acid and *N*-phenyl-α-naphthylamine gave a satisfactory colour change (purple bands on a brown column) but was very easily leached from the column by 17% butanol-chloroform. The dyestuffs prepared from amino-*G*-acid (β-naphthylamine-6:8-disulphonic acid) and from amino-*R*-acid (β-naphthylamine-3:6-disulphonic acid) both change from pale pink in alkaline solution to deep blue in acid solution; both are very resistant to leaching but the colour change with the dyestuff derived from amino-*R*-acid was the more satisfactory on the gels we have used and this material (I) is the one we recommend, although the amino-*G*-acid derivative may be useful under some circumstances:



(I)

The preparation of the ammonium ('Indicator *R*-NH_4') (I; $X=NH_4$) and sodium ('Indicator *R*-Na') (I; $X=Na$) salts of 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine is described in detail below; the other indicators mentioned were prepared similarly. The preparation is easy and the starting materials are commercially available, thus making the new indicator much more accessible than the anthocyanins which have been recommended for the same purpose (Gordon, Martin & Synge, 1943 *a*, *b*, 1944).

The two indicators derived from (I) change from pale pink to deep blue in aqueous solution over the following approximate pH ranges:

'Indicator *R*-NH_4' (I; $X=NH_4$) ... pH 5·0–3·8
'Indicator *R*-Na' (I; $X=Na$) ... pH 4·6–3·6

The ammonium salt, '*R*-NH_4', has proved the more useful in partition chromatography, giving pink to lilac backgrounds on satisfactory gels, whereas the sodium salt, '*R*-Na', gave solid blue backgrounds on the same gels. Clearly the surface properties of an indicator may be as important as its pH range in determining the background colour obtained.

We demonstrated the usefulness of our indicator '*R*-NH_4' by carrying out duplicate separations of acetyl-phenylalanine and acetyl-leucine, using 2% butanol-chloroform and a sample of silica gel ('γ') kindly provided by Dr R. L. M. Synge; the indicator was used in 0·025% aqueous solution. A minor disadvantage of the new indicator is its instability; on keeping, the solid tends to lose ammonia, but this is easily remedied by adding a little ammonia and boiling off the excess when making up the indicator solution. In our experiments the total recovery of acetylamino-acid was 99–100%, the recovery of acetyl-phenylalanine being 98% and that of acetyl-leucine 101%. The intense blue bands were very easily seen on the pale pink background and it was very much easier to separate the fractions with this indicator than with methyl orange; in other experiments we have found that the indicator is not appreciably leached even by 17% butanol-chloroform. Gordon *et al.* (1944) describe their experiences with this indicator, which they have found very satisfactory with suitable gels. Dr G. R. Tristram, working in Prof. A. C. Chibnall's laboratory, has also found the new indicator of value in the partition chromatographic separation of acetylamino-acids.

The new indicator, '*R*-NH_4', is recommended for use in the partition chromatographic separation of acetylamino-acids and similar compounds, since it seems to us to be an improvement over the other indicators which have been suggested for this purpose. On suitable gels it gives a colour change which is much more easily seen than that given by methyl orange and, unlike methyl orange, it is not appreciably leached by solvents. Unlike the anthocyanins it is readily available and is sensitive in 1–3% butanol-chloroform. Fewer specimens of silica gel prepared by the method of Gordon *et al.* (1943*a*) are suitable for use with the new indicator than with methyl orange, but this difficulty should be overcome by a closer specification of the method of preparation of the gels.

EXPERIMENTAL

Preparation of the indicator

Amino-*R*-acid (44 g.; a commercial sample, 70% pure) is dissolved in hot water (200 ml.) containing Na_2CO_3 (5·3 g. anhydr.), giving a solution faintly alkaline to litmus. Ice and water are added to the cooled solution to bring it to 600 ml. Concentrated HCl (25 ml.) is added at 0° and the solution diazotized by addition of 10% $NaNO_2$ (69 ml.); at the end-point there should be a slight excess of nitrite and the solution should be acid to Congo red. The diazonium salt is filtered off and made into a paste with water (600 ml.); this is added to a cooled solution of phenyl-α-naphthylamine (22 g.) in ethanol (500 ml.), with good stirring. The coupling is brought about by stirring at 10–20° for 3 hr., during which time water is added, when necessary, to reduce the viscosity of the mixture; the final volume is 1750 ml.

The temperature is finally raised to 65° and the mixture allowed to cool overnight; the acid (I; $X=H$) is thus obtained in an easily filterable form (thin needles). Next day it is filtered off and washed with a little water.

The ammonium salt ('Indicator *R*-NH_4') (I; $X=NH_4$) is obtained by dissolving the acid in boiling water (2500 ml.) containing an excess of ammonia, filtering and evaporating to dryness. Traces of impurity may, if desired, be extracted from the dry salt by extraction with light petroleum, b.p. 40–60°. Yield: 46 g.

Table 1. *Separation of two acetylamino-acids by partition chromatography with 'Indicator R-NH_4'*

		R (band rate*)	Titre (ml. of 0·0114 N-$Ba(OH)_2$ required for fraction)	Recovery (%)
Exp. 1.	Acetyl-phenylalanine fraction	0·85	0·764	98
	Acetyl-leucine fraction	0·54	1·052	101
	Total	—	1·816	99
Exp. 2.	Acetyl-phenylalanine fraction	0·82	0·768	98
	Acetyl-leucine fraction	0·46	1·056	101
	Total	—	1·824	100

* Band rate as defined by Martin & Synge (1941).

The sodium salt ('Indicator *R*-Na') (I; X=Na) is obtained by dissolving the acid in water (1700 ml.), at 60°, containing a slight excess of Na_2CO_3, salting out with NaCl (50 g.) and filtering when cold. Traces of impurity may be removed, if desired, by extraction of the dry salt with petroleum ether, b.p. 80–100°. Yield: 43 g.

Separation of acetyl-phenylalanine and acetyl-leucine

For the trial separations synthetic mixtures of acetyl-phenylalanine (1·84 mg.) and acetyl-leucine (2·06 mg.) were used. Columns were made up, as described by Gordon *et al.* (1943*a*), with 3·0 g. of silica gel, 1·4 ml. of a 0·025% aqueous solution of 'Indicator *R*-NH_4', and 2% butanol-chloroform. The chromatograms were developed with 2% butanol-chloroform and the evaporated fractions titrated with 0·0114N-$Ba(OH)_2$. The titre of the original mixture of acetylamino-acids was 1·828, 1·830 ml. The results obtained are shown in Table 1.

SUMMARY

1. The preparation of a new indicator, the ammonium salt of 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine, is described.

2. This indicator is recommended for use in partition chromatography; its advantages for this purpose are discussed and examples of its use are given.

We are indebted to the Director General of Scientific Research and Development, Ministry of Supply, for permission to publish this paper.

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The Metabolism of 2:4:6-trinitrotoluene (α-T.N.T.)

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During the last war it was realized that prolonged exposure to T.N.T. might, in some cases, have serious effects on the health of workers in shell-filling factories, and much experimental work was therefore carried out on animals in order to throw light on the many problems presented by this observation. On the chemical side the information which resulted was, however, disappointing. Thus Dale (1921) encountered great difficulty in seeking to isolate and identify the metabolic products present in the urine of rabbits which had collectively received 13·2 g. T.N.T. The two crystalline products which he obtained represented no more than 7% of the T.N.T. administered; and while the identity of one, tetranitroazoxytoluene, was established, that of the other, believed to be a dinitroaminotoluene, was left in doubt. We have therefore sought to obtain further information on the difficult problem of the fate of T.N.T. in the body.

While only scanty studies have been made of the metabolic fate even of simple aromatic nitro compounds, it is possible to formulate a series of products which may arise when T.N.T. is administered to animals. Of the reduction processes, the more probable ones are the reduction of a single nitro group to an amino group, possibly through the intermediate stage of a hydroxylamino compound. Thus α-T.N.T. (I) might give rise either to 2:6-dinitro-4-hydroxylaminotoluene (II) or to its isomer, 2:4-dinitro-6-hydroxylaminotoluene (III), each of these compounds then undergoing further reduction to yield 2:6-dinitro-4-aminotoluene (IV) or 2:4-dinitro-6-aminotoluene (V); the dinitro-aminotoluenes might be similarly further reduced to the nitrodiaminotoluenes (VI, VII). Of the oxidation processes, the likely ones are those in which the CH_3 group has been oxidized to CH_2OH or COOH to give trinitrobenzyl alcohol (VIII) and trinitrobenzoic acid (IX), or the formation of trinitro-*m*-cresol (X) by introduction of a phenolic hydroxyl into position 3 of the T.N.T. molecule. Nor can the possibility of simultaneous oxidation and reduction with the production of such compounds as 2:6-dinitro-4-aminobenzyl alcohol (XI) and 2:6-dinitro-4-amino-*m*-cresol (XII) be eliminated.

The excretion of any of the products (I)–(XII) as such, is, however, not the whole story, for some of