Little is known of the upper limits of accuracy of these methods, but a possible disadvantage of all of them is the absence of a barrier to prevent any bacteria contaminating the test samples from spreading over the surface of the plate.

Application to substances other than penicillin. Great caution must be exercised before placing reliance on results obtained by this method when applied to other antibiotics. If the inhibitor is not diffusible, the method is obviously of no use. The composition of the agar may need special attention (e.g. the presence of peptones would presumably interfere with sulphonamides; penicillin B or notatin gives large zones of inhibition in the presence of glucose, but none in its absence). With some substances (e.g. helvolic acid (Chain, Florey, Jennings & Williams, 1943)), the curve relating diameter of inhibition zone to concentration of inhibitor is too flat to be of much use for quantitative work. Results with sparingly soluble inhibitors may need special interpretation. Change in pH of the medium during growth of the bacteria may influence the assay value in a variety of ways. If the potency of the inhibitor depends on the number of bacteria being inhibited, special procedures for ensuring even and reproducible seeding of the plates may be re-

quired. Other potential limitations of the method can be imagined, but the method has been successfully applied to the quantitative assay of gigantic acid (Philpot, 1943) and of substances of plant and fungal origin quite different in character from penicillin. The method has been of use in the semi-quantitative investigation of several other substances.

SUMMARY

1. Full practical details are given of a method of assay of penicillin which is applicable also to certain other antibiotics.

2. The method compares favourably as regards speed and accuracy with most other methods and has been in routine use for nearly 4 years.

3. Less than 1 ml. of solution (which need not be sterile) is required for an assay and the presence of ether or chloroform does not interfere.

4. Quantitative information on limits of accuracy is given.

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Technical Notes on the Partition Chromatography of Acetamino-acids with Silica Gel

By A. H. GORDON, A. J. P. MARTIN AND R. L. M. SYNGE, *Wool Industries Research Association, Torridon, Headingley, Leeds, 6 and The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1*

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We record here some technical aspects of the experience which we have gained in the use of our partition chromatographic method (Gordon, Martin & Syngé, 1943a) for the quantitative analysis of amino-acid mixtures.

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PREPARATION OF SILICA GEL

The characteristics of different batches of silica gel have been found to vary so much as a result of differences in starting material and minor variations in preparative procedure that we have introduced a standard test to which

we subject all our products before employing them in analysis. A mixture of 3 mg. each of acetylphenylalanine and acetyllecine is subjected to analysis on a column (1 cm. diameter) containing 3 g. of the gel specimen under test, with 1-3% butanol-chloroform as developing solvent and methyl orange as indicator.

The sum of the titration values for the two resulting fractions should agree within 2% with the titration value of the mixture before analysis. All our preparations of gel which have been suspended in dilute hydrochloric acid before the final washing with distilled water have conformed to this recovery test.

The recovery of acetylphenylalanine in the first band issuing from the column should be at least 99%. With some batches of gel that we have prepared this figure has been as low as 89%, the total recovery of acetamino-acids from the column remaining quantitative (see Table 1). This effect we attribute to differing adsorptive properties of the silica. Adsorption of the Freundlich type can lead (DeVault, 1943; Weiss, 1943) to 'tailing' of the bands, an effect superposed on, and much greater than, that due to variation of partition coefficient with concentration of solute. Moreover, this effect would be expected to be relatively greater for the aromatic acetylphenylalanine than for acetyllecine, thus further increasing the degree of over-

Numerous preparations of gel (500 g. batches) have been made from I over a period of 1½ years, during which time we have noted no change in its properties. In view of the profound differences between these two specimens of water-glass it is likely that our directions for the preparation of satisfactory silica gels have no universal applicability. We give below the conditions that we have found necessary for the preparation of satisfactory gels from waterglasses I and II. It should be emphasized that these gels are 'satisfactory' for the quantitative analysis of mixtures of acetamino-acids and not necessarily for other purposes.

With waterglass I the presence of methyl orange (MO) during the precipitation (as previously recommended—Gordon *et al.* (1943a)) was found necessary. If MO was omitted, a very 'tailing' gel resulted. 'Ageing' was necessary, as previously stated, to obtain satisfactory indication in gels precipitated in the presence of MO. Precipitating at a lower temperature did not improve the properties of gels precipitated in the absence of MO.

With waterglass II it was not found necessary to have MO present during precipitation, nor was 'ageing' necessary. Lowering the temperature of precipitation definitely improved the quality of the gel. Otherwise the procedure used was as previously described. Some of the experiments on which these conclusions are based are given in Table 1.

Table 1. *Preparation and properties of various silica gels*

Date of preparation	Batch of gel	Waterglass used	MO present during precipitation?	Temp. of precipitation*	'Ageing' (days)	Percentage recovery of acetylphenylalanine in test
iv. 42	D	I	Yes	R	2	100
vii. 43	α	I	No	R	2	96
vii. 43	β	I	No	R	0	98
ix. 43	H	I	Yes	R	1	100
ix. 43	J	I	No	C	0	89
ix. 43	γ	II	No	C	0	99
xi. 43	δ	II	No	R	0	96
xi. 43	ϵ	II	No	C	0	101

* R signifies precipitation at room temperature with reagents at room temperature.

C signifies precipitation in cold room (2°) with reagents previously cooled to that temperature.

lapping. Similarly with mixtures of acetyltryptophan and acetylvaline subjected to the same test the recovery of acetyltryptophan varied from 80% (with our 'worst tailing' gels) to 95% with our 'best'.

In the above experiments the distribution isotherm between mobile and non-mobile phases resultant from adsorption on surfaces and partition between two liquid phases determines the separation of substances in a single column. Wachtel & Cassidy (1943) suggest that when the former process predominates, the name 'chromatography' should be retained, but that when the latter predominates a new name should be used. In our opinion this would be confusing, and we prefer to speak of 'adsorption chromatography' and 'partition chromatography' in cases where the procedures can be differentiated. By chromatography we thus imply the technical procedure of analysis by percolation of fluid through a body of comminuted or porous rigid material irrespective of the nature of the physico-chemical processes that may lead to the separation of substances in the apparatus.

We have experience of the preparation of silica gel from two different specimens (I and II) of waterglass (140° Twaddell) purchased in 1 cwt. drums from Jos. Crosfield Ltd., Warrington, Lancs. II was purchased 18 months after I.

We have found that the 'background' colour of MO used as an indicator in the test described above serves as a rough guide to the quality of the gel. 'Good' gels have a flesh-pink background colour, whereas 'tailing' gels appear yellow to orange-yellow. As expected from the theory of chromatography, with 'tailing' gels the bands have very sharp fronts, whereas with 'good' gels the fronts are somewhat diffuse.

INDICATORS

The primary defect of methyl orange as indicator for acetamino-acids on silica gel partition chromatograms is that it is leached from the columns by the solvent mixtures of higher alcohol content. It has been mentioned (Gordon *et al.* 1943 a, b) that pelargonin chloride is practically free from this defect. We have now compared a number of representative anthocyanins (listed in Table 2) as indicators for this purpose. Comparisons were made in 1 and 17% butanol-chloroform and in 5 and 30% propanol-cyclohexane. Approximately 0.002 millimols each of acetyl-glycine, acetylalanine, acetylvaline and acetyllecine were employed as a test mixture on 1 cm. columns. The indicators compared were in aqueous solution 0.025-0.05% (w/v).

Except for (3) (Table 2), which faded on the columns in a few minutes, all the anthocyanins studied gave a permanent coloration to the columns. The pelargonidin and cyanidin derivatives (1, 2, 4, 5) were more sensitive than any of the delphinidin derivatives (6-9), which were insufficiently sensitive to be useful. Between the former in butanol-chloroform columns there is little to choose, whereas in propanol-cyclohexane (4) and (5) are superior indicators to (1) and (2). None are sufficiently sensitive for ordinary analytical work in 1-3% butanol-chloroform, but in the other solvent mixtures the sensitivity is comparable to that of methyl orange, and the colour change somewhat easier to observe.

salt of the dye, kindly given to us by Dr Rydon. Used in 0.025% aqueous solution, we find that it is leached by solvents no more than the anthocyanins and that it is a good indicator on 1-3% butanol-chloroform columns as well as with the other solvent mixtures, giving blue (acid) bands on a purple to salmon-pink background. We find, however, that it can be used with only some of our gels (α , β , γ , ϵ —Table 1). With others (H , δ) it is quite insensitive, giving a blue or purple background colour.

It is desirable on many grounds that this variability of the silica should be eliminated. The best hope seems to be through standardization of the waterglass from which it is prepared.

Table 2. *Anthocyanins tested as indicators*

No.	Trivial name	Chemical name	Key reference to structure
(1)	Pelargonin chloride	7:4'-Dihydroxy-3:5-di-(β -glucosidoxy)-flavylium chloride	Robinson & Todd (1932c)
(2)	Cyanin chloride	7:3':4'-Trihydroxy-3:5-di-(β -glucosidoxy)-flavylium chloride	Robinson & Todd (1932c)
(3)	—	3:3':4'-Trihydroxy-5:7-di-(β -glucosidoxy)-flavylium chloride	MacDowell, Robinson & Todd (1934)
(4)	Mecocyanin chloride	5:7:3':4'-Tetrahydroxy-3- β -gentiobiosidoxy-flavylium chloride	Grove, Inubuse & Robinson (1934)
(5)	Peonin chloride	7:3'-Dihydroxy-3:5-di- β -glucosidoxy-3-methoxyflavylium chloride	Robinson & Todd (1932c)
(6)	Violanin chloride	?	Karrer & de Meuron (1933)
(7)	Bilberry pigments	?	Bell & Robinson (1934) Willstätter & Zollinger (1916)
(8)	Malvin chloride	7:4'-Dihydroxy-3:5-di- β -glucosidoxy-3':5'-dimethoxyflavylium chloride	Robinson & Todd (1932b)
(9)	Hirsutin chloride	4'-Hydroxy-3:5-di- β -glucosidoxy-7:3':5'-trimethoxyflavylium chloride	Robinson & Todd (1932a)

Sources of the above anthocyanins:

- (1) From petals of scarlet dahlia 'Coltness Gem' according to Willstätter & Mallison (1915).
- (2) From petals of dark red dahlia according to Willstätter & Mallison (1915).
- (3) Synthetic. From Prof. A. R. Todd.
- (4) Synthetic. From Prof. Sir R. Robinson.
- (5) From petals of dark red garden peonies according to Willstätter & Nolan (1915). For a good yield the petals should be dried as rapidly as possible.
- (6) From petals of dark purple pansies according to Willstätter & Weil (1916). The product was not thoroughly purified.
- (7) From berries of *Vaccinium myrtillus* L. Pieces of filter paper were dyed by immersion in the juice of stewed bilberries. They were washed repeatedly with distilled water. The pigment was then eluted from the paper with glacial acetic acid containing a little HCl, and the eluate was evaporated to dryness below 40°.
- (8) From petals of *Malva sylvestris* L. according to Willstätter & Mieg (1915).
- (9) Synthetic. From Prof. A. R. Todd.

None of the anthocyanins studied are sensibly leached on 17% butanol-chloroform columns and all are slightly leached on 30% propanol-cyclohexane columns. Even here, though, leaching is so slow that bands having one-sixth the rate of acetylglycine may readily be observed.

Peonin chloride and pelargonin chloride have so far been used by us for routine analytical work. Solutions of pelargonin chloride seem to keep indefinitely when stored in the cold room, but peonin chloride fades under these conditions, and solutions are best made up immediately before use.

It remains to find an indicator that is sensitive on 1-3% butanol-chloroform columns and not leached when the butanol concentration is raised to 17%. Liddell & Rydon (1944) have made progress in this direction, and have found that the dye formed by coupling diazotized amino-*R* acid with *N*-phenyl- α -naphthylamine possesses the desired properties. We have confirmed this work with the ammonium

EXTRACTION OF ACETAMINO-ACIDS FROM ACETYLATION MIXTURE

In order to bring about quantitative extraction of the less readily extractable acetyl derivatives, we have employed (Gordon *et al.* 1943b) a column procedure, absorbing the acetylation mixture in silica gel. We now find that this procedure may lead to extraction by the organic solvent of free mineral acid, whose presence promotes destruction of acetamino-acids (perhaps by esterification) during the concentration of the extract. Acetyltryptophan is especially readily destroyed. The extent of the destruction seems to depend on the amount of mineral acid present. Since the amount of sulphuric acid added to the acetylation mixture was not rigorously controlled during our experiments with tyrocidine (Gordon *et al.* 1943b), this effect

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. Syngé, 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

BY H. F. LIDDELL AND H. N. RYDON, *A Ministry of Supply Laboratory*

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An indicator suitable for use in partition chromatography (Martin & Syngé, 1941; Gordon, Martin & Syngé, 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 chromatography.

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: