Table 2. Growth and vitamin B_{12} formation with aspartic acid + one of the following amino acids.

Amino acids added	Mycelium max. amount, g dry weight per litre	Vitamin B_{12} max. amount, μg per litre	Ratio $\frac{\mu g \text{ vitamin } B_{12}}{g \text{ mycelium}}$
β-alanine	3.3	60	18
Arginine	2.2	70	32
Glutamic acid	2.9	95	33
Glycine	3.0	100	33
Histidine	2.7	100	37
Leucine	2.9	80	28
Lysine	2.6	100	38
Proline	3.3	100	30
Serine	2.8	130	46
Tryptophane	2.7	95	35
Valine	2.4	100	42
No addition	2.6	60	23

50 %. Column 4, Table 2, shows that the greatest stimulation of the vitamin production was obtained with serine and valine (in the presence of aspartic acid). In the case of valine this is in good agreement with the experiments given in Table 1. Serine, however, when being the sole nitrogen source, was found to give a very low vitamin formation. The reason for this must be, that if serine is the only nitrogen source such a small amount of mycelium is formed that a high vitamin formation is impossible.

Glutamic acid and glycine have, when added in small amounts, a less marked effect on the vitamin B_{12} production than would be expected from Table 1 (if these amino acids constitute the sole nitrogen source they give rise to vigorous growth and vitamin production). The reason for this peculiar behaviour (Table 2) is probably that these amino acids, when added in small amounts, are used for the nitrogen assimilation and accordingly hardly anything remains for the formation of vitamin B_{12} which, as mentioned above, mainly takes place after the cessation of the growth. Serine and valine, on the other hand, are very bad nitrogen sources for growth and are thus not used during the growth phase but are left over to the vitamin forming phase.

Received September 3, 1957.

An Efficient Method for the Separation and Identification of Alkaloids in Biological Material

R. BONNICHSEN, A. C. MAEHLY and S. NORDLANDER

Statens Rättskemiska Laboratorium, Stockholm, Sweden*

The detection of alkaloids in biological material has long been based largely on colorreactions and crystal precipitation methods under the microscope. In recent years, paper chromatography has been employed to an increasing degree, pioneered by Munier *et al.*[1] and used by Jatzkewitz [2,3], Vidic [4] and others.

Buffered paper was employed by Carless and Woodhead [5], Goldbaum and Kazyak [6] and others. Schmall *et al.*[7] refined this technique by buffering zones to different pH values on the same paper. We have modified their technique for toxicological work and extended it to the identification of more than 30 compounds mainly of the alkaloid group. Quantitative estimation by spectrophotometry has also been worked out for many alkaloids. The basic principle of the method is as follows:

The tissue (whenever possible urine is employed, otherwise kidney, liver or blood) is heated with hydrochloric acid for 1 h for hydrolytic cleavage of adducts with glucuronic acid etc., and extracted in the usual way *via* amylacetate, 0.1 N HCl and chloroform.

The chloroform extract is dried, and evaporated to dryness. The residue is dissolved in hot 75 % ethanol and the ethanolic solution applied to a filter paper.

The migration and efficient separation of alkaloids and some other toxicologically important compounds on filter paper is achieved only if the free base is allowed to migrate in the direction of descending pH values and depends on the pH of the buffered zones as well as on the partition coefficients between the buffers and the mobile organic phase. The most efficient way to prepare the filter paper was the subject of extensive experimentation.

* The Government Laboratory for Forensic Chemistry.

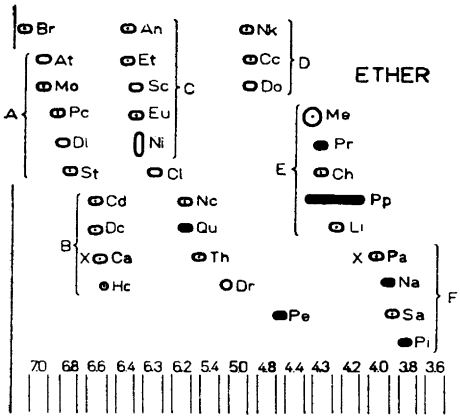

Fig. 1. The R_F or pH values of common alkaloids and other compounds of toxicological importance. The descending technique was used, the solvent (here ether) appears to migrate from left to right. The buffered zones of the paper are labelled with the corresponding pH value. The abbreviations shown stand for the following 33 compounds (if the name does not correspond to that given in the "Merck Index", the "Merck" name is added in brackets).

An Antipyrin (phenazone)
At Atropine
Br Brucine
Ca Coffeine
Cc Cocaine
Cd Codeine
Ch Chloropromethazine
Cl Cliradon (ketobemidone)
Dc Dicodid (dihydrocodeinone)
Dl Dilaudid (dihydromorphinone)
Do Dolantine (meperidine)
Dr Dromorane (3-hydroxy-N-methyl-morphinan)
Et Ethylmorphine
Eu Eucodal (dihydrooxycodeinon)
Hc Hydrocodeinon (synkonin)
Le Lergitin (N-benzyl-N′, N′-dimethyl-N-phenylethylene-diamine)
Me Methadone
Mo Morphine
Na Narcotine
Nc Novocaine
Ni Nicotine
Nk Nikethamide (nicamide)
Pa Phenacetin
Pc Paracodin (dihydrocodeine)
Pe Percaine (dibucaine)
Pi Piperine
Pp Papaverin
Pr Promethazine
Qu Quinine
Sa Santonin
Sc Scopolamine
St Strychnine
Th Thebaine

Compounds that are not clearly resolved from all others are indicated in groups (A—F).

The following symbols are used for the individual spots:

⊙ colored by Dragendorff's reagent and absorbing ultraviolet light.
○ colored by Dragendorff's reagent but not absorbing ultraviolet light.
● colored by Dragendorff's reagent and fluorescing in ultraviolet light.
× ⊙ not colored by Dragendorff's reagent but absorbing ultraviolet light.

The pH values finally adopted for ether and chloroform respectively as the mobile phases are shown in Figs. 1 and 2.

Fig. 1 shows the distribution on the chromatography sheat of 33 standard substances. The positions of spots are averaged from a number of runs on different days and with somewhat varying amounts of material (10—50 μg of each compound). The solvent was *ether* and the descending method was used at room temperature and in the dark. The buffer

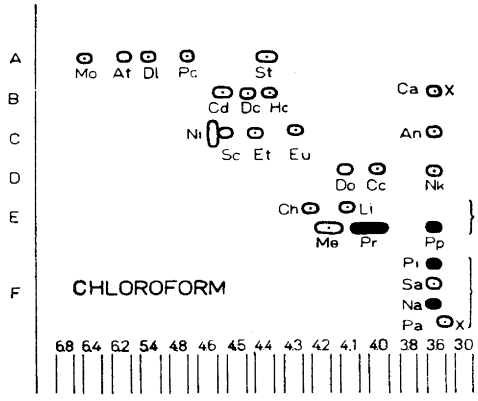

Fig. 2. Those compounds that are not cleary resolved when using ether as the mobile phase are here shown on a chromatogram where chloroform is the moving solvent. Groups A to F of Fig. 1 are each shown on 1 or 2 horizontal lines. The symbols used are the same as in Fig. 1.

Table 1. Buffer solutions employed. The amounts of 0.10 M citric acid and 0.20 M disodium phosphate listed were diluted to a final volume of 100 ml with distilled water.

pH	X ml 0.1 M citric acid	Y ml 0.2 M Na_2HPO_4	pH	X ml 0.1 M citric acid	Y ml 0.2 M Na_2HPO_4
3.0	39.3	10.2	4.8	25.2	24.8
3.6	33.9	16.1	5.0	24.3	25.7
3.8	32.3	17.7	5.4	22.2	27.8
4.0	30.7	19.3	6.2	16.9	33.1
4.1	30.0	20.0	6.3	16.2	33.8
4.2	29.4	20.6	6.4	15.4	34.6
4.3	28.6	21.4	6.6	13.6	36.4
4.4	27.8	22.2	6.8	9.1	40.9
4.5	27.2	22.8	7.0	6.5	43.6
4.6	26.7	23.3	—	—	—

composition as it is presently employed is listed in Table 1. Only disodium phosphate and citric acid are needed. The compounds are quite evenly distributed over the whole paper and some of them show distinct R_F values or "pH values", a term better suited for this technique. However, most of them appear in groups marked A to F in Fig. 1. Fortunately these groups are almost all clearly separated with *chloroform* as the mobile phase (Fig. 2).

Exceptions are chloro-promazin, methadone and phenbenzamine in group E and also all of group F. In the latter case, optical properties help in identification: piperine and narcotine show a blue fluorescence and the former also gives a distinct yellow color with Dragendorff's spraying reagent. Santonine and phenacetine both absorb ultraviolet light (UV-lamp in conjunction with fluorescein treated paper) but only the former reacts with Dragendorff's reagent.

The position of a spot (R_F or pH value) is never regarded as an absolute invariable and standards of known purity and concentration are always run on the same sheet of paper. Qualitative identification was sometimes aided by cutting the spot into a narrow rectangle, wetting it with liquid paraffin oil and measuring its spectrum *vs.* a blank treated in the identical way. If the paper had been sprayed with Dragendorff's reagent, a short treatment with ammonia vapour to restore the free base preceded the oiling.

Semi-quantitative evaluation was usually possible by extracting the spot with dilute ammonia and chloroform, evaporating and dissolving the residue in 75 % ethanol for spectrophotometric analysis. In cases where the spectrum did not show any characteristic bands or where the amount of material was insufficient, known amounts of standard solutions were run simultaneously on the same paper as the unknown, and the amount of the unknown estimated by matching spot sizes.

Transfers of individual "spots" to another paper for re-chromatography proved to be a useful and often indispensible means for separation and purification. If sufficient amounts of material were present, elution and evaporation were used. In other cases, a simple transfer could be achieved by sandwiching the cut-out spot between a new filter paper and a small glassplate with the help of paper clips and running the paper in the usual manner.

Some of the main advantages of the method for the toxicologist are: The need of only very small amounts of material (10 μg for qualitative identification is usually the lower limit), the relative speed of the process and the possibilities for purifying individual compounds from an often complex array of "spots" from tissue extracts.

A detailed report together with application of the method to actual toxicological cases will be published later.

1. Munier, R., Macheboeuf, M. and Cherrier, N. *Bull. soc. chim. biol.* **34** (1952) 204.
2. Jatzkewitz, H. *Z. physiol. Chem. Hoppe-Seyler* **292** (1953) 94.
3. Jatzkewitz, H. *Deut. med. Wochschr.* **79** (1954) 541.
4. Vidic, E. *Arzneimittel-Forsch.* **5** (1955) 291.
5. Carless, J. E. and Woodhead, H. B. *Nature* **168** (1951) 203.
6. Goldbaum, L. R. and Kazyak, L. *Anal. Chem.* **28** (1956) 1289.
7. Schmall, M., Wollish, E. G. and Schafer, E. G. E. *Anal. Chem.* **28** (1956) 1373.

Received August 13, 1957.