Application of Paper Chromatography to the Separation of Non-Volatile Carboxylic Acids

THE separation of several non-volatile carboxylic acids by paper chromatography has been described by Lugg and Overell[1,2]. These workers found that it was impossible to carry out the separation with neutral solvents because the ionization of the acids caused the spots to 'tail'. They overcame the difficulty by swamping both the stationary and mobile phases with formic or acetic acid. Removal of the swamping acid was then a necessary and sometimes tedious operation before the non-volatile acids could be revealed by spraying with an acid-base indicator.

I have now succeeded in separating several non-volatile acids as anions. In a previous communication[3] describing the separation of the volatile fatty-acid anions, it was pointed out that the acids possessing more than one carboxyl group did not move from the starting line when *n*-butyl alcohol saturated with 1·5 *N* ammonium hydroxide was used as the mobile phase. The use of ethyl alcohol – ammonia overcomes this difficulty, and since the water-content of the solvent can be adjusted to any desired level, the flexibility of the method is greatly increased. As expected, the excursions of the acids become greater as the proportion of water in the solvent is increased, so that a pair of acids possessing similar R_F values at low concentrations of water (for example, malic and tartaric) can be completely separated by increasing the proportion of water in the solvent (see table).

R_F VALUES OF NON-VOLATILE CARBOXYLATE IONS IN ETHYL ALCOHOL - AMMONIA ON WHATMAN NO. 1 PAPERS

Ion	EtOH : 15*N* NH_4OH : H_2O = 90 : 5 : 5 v/v		EtOH : 15*N* NH_4OH = 95 : 5 v/v
	Ascending	Descending	Descending
Citrate	0·01	0·07	0·01
Oxalate	0·04	0·15	0·03
Tartrate	0·05	0·18	0·05
Malate	0·07	0·28	0·08
Adipate	0·20	0·44	0·21
Lactate	0·42	0·61	0·44

Development of the papers after drying (5 min. at 95° C.) can be carried out in two ways. Spraying with chlorophenol red (40 mgm. in 100 ml. water adjusted to *p*H 7) reveals the anions as bright yellow spots on a mauve background. Alternatively, by spraying with ammoniacal silver nitrate solution (equal volumes of 0·1 *N* silver nitrate and 5 *N* ammonium hydroxide) and heating at 95° C. for 5 min., the citrate, tartrate, malate and lactate ions are revealed as intense yellow fluorescent spots under an ultra-violet lamp. The adipate and oxalate ions give less intense white fluorescent spots.

Since the acids examined in this work are non-volatile, they can be applied to the paper as such, and recourse to the sodium salts is unnecessary. This is an advantage, because with ethyl alcohol as the mobile phase the sodium ion travels a sufficiently great distance to interfere with the excursions of some of the carboxylate ions. In general, spots containing approximately 10 μgm. of each acid were introduced on the starting line; this concentration gave readily detectable spots on spraying, although amounts so low as 5 μgm. could be detected.

An unexpected feature noticed in this work has been the large differences in the R_F values obtained by the conventional descending chromatographic method[4] and the ascending method of Williams and Kirby[5]. Values are shown in the accompanying table.

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[2] Lugg, J. W. H., and Overell, B. T., *Aust. J. Sci. Research*, A, **1**, 98 (1948).
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[4] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).
[5] Williams, R. J., and Kirby, H., *Science*, **107**, 481 (1948).

Trichloracetic Acid and Feulgen Staining

THE importance of trichloracetic acid as an agent for the removal of nucleic acids from their combination in nucleoproteins has been amply demonstrated by Schneider[1]. The method is now widely used by various authors in different cytochemical procedures. In order to find out whether any differential reactivity of the chromosome parts—namely, euchromatin and heterochromatin—with fuchsin sulphurous acid appears after such treatment, experiments were carried out, mainly on root-tips of *Allium cepa* and *Hordeum vulgare*. A series of different concentrations of trichloracetic acid was prepared and the root-tips treated under different conditions.

It has been noted that heterochromatin, particularly of metaphase chromosomes, becomes sharply stained if the root-tips after fixation in acetic alcohol are treated with 0·25 *M* trichloracetic acid at 60° C. for 40 min., followed by hydrolysis in hydrochloric acid for 20 min. at the same temperature. The heterochromatin, particularly that of the centromeric and telomeric regions, stains sharply in leucofuchsin, the rest of the chromosome remaining unstained. Regions of secondary constrictions, which are also considered to be heterochromatic in nature, show no staining at all at any of the concentrations used. The preparations look similar to those of Levan[2], obtained after mercuric nitrate treatment in his '*Allium* test'.

One interesting feature which has been noticed during these trials, namely, hydrolysis in *N* hydrochloric acid in the Feulgen staining, can safely be substituted by a treatment in 0·25 *M* trichloracetic acid for 50 min. at 65° C., resulting in a sharp staining throughout the chromosome parts. When such treatment is followed by the action of *N* hydrochloric acid at the same temperature for 20 min., complete removal of the nucleic acids takes place. Staining with fuchsin-sulphurous acid without hydrolysis in *N* hydrochloric acid shows clearly that either the splitting of the purine bases according to Feulgen and Rossenbeck[3], or the splitting of the sugar linkages, too, engaged in polymeric bonding of nucleic acid, liberating the aldehyde groups as has been claimed by Overend and Stacey[4], is effected in such a treatment with trichloracetic acid. The effect, moreover, remains similarly pronounced when longer treatment is given with a slightly lower dilution of trichloracetic acid.