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Note

Effect of some organic buffers on the estimation of aspartic acid and resolution in amino acid analysis*

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The use of lithium buffers in ion-exchange column chromatography has improved the analysis of "physiological" samples containing non-protein amino acids (for example, see refs. 1 and 2). During a study of the amino acids of pea-leaf chloroplasts[3], we became aware of difficulties in the estimation of aspartic acid and also its separation from BIA, an amino compound present in peas[4]. The problem was found to be caused by the presence of some organic buffers (described by Good *et al.*[5]) used in the preparation of the chloroplasts. It is apparent that several organic buffers, including tricine, bicine, HEPES and EPPS, interfere with the resolution and estimation of aspartic acid and neighbouring compounds.

EXPERIMENTAL

A Beckman Model 119BL automatic analyser was used with a single column (240 × 9 mm) of W-2 resin. The first buffer contained lithium (citrate) at a concentration of 0.2 *N*, pH 2.83. The starting temperature was 40 °C, with a rise (to 66 °C) beginning at 44 min; this early temperature rise allowed the satisfactory resolution of asparagine, glutamic acid, glutamine and homoserine, although resolution of a few other physiological amino acids (not present in our plant samples) was impaired.

Samples for analysis were prepared from buffer solutions and amino acid standards or leaf extracts, and the pH was checked with a meter. The volume loaded was 0.5 ml.

Physiological amino acid standards were obtained from Hamilton. Amino acids were extracted from pea leaves (*Pisum sativum*) by grinding in water and immediately adding 5-sulfosalicylic acid (50 mg/ml) to precipitate proteins. After centrifugation, the solution was filtered through a Millipore cellulose mixed-ester membrane (type VM, pore size 0.05 μm). Pea-leaf extracts contained the ninhydrin-positive compound BIA, which eluted *ca.* 3 min after aspartic acid.

* Abbreviations used: BIA = β-(isoxazolin-5-on-2-yl)alanine; EPPS = N-2-hydroxyethylpiperazine-N'-3-propane sulphonic acid; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; MES = 2-(N-morpholino)ethanesulphonic acid; bicine = N,N-bis(2-hydroxyethyl)-lycine; tricine = N-tris(hydroxymethyl)methylglycine.

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RESULTS AND DISCUSSION

The effect of a number of organic buffers on resolution of the amino acids emerging in the early part of analysis was investigated. Amino acid samples used (50–100 nmol per amino acid) were aspartic acid alone, physiological standard mixture or pea-leaf extract. Samples were loaded at a range of pH values from 2.1 to 2.5 (2.2 is the recommended value), with the addition of up to 50 μmol of buffer. Some effects on physiological standards are shown in Fig. 1, and a more detailed survey of the effects on aspartic acid is shown in Table I.

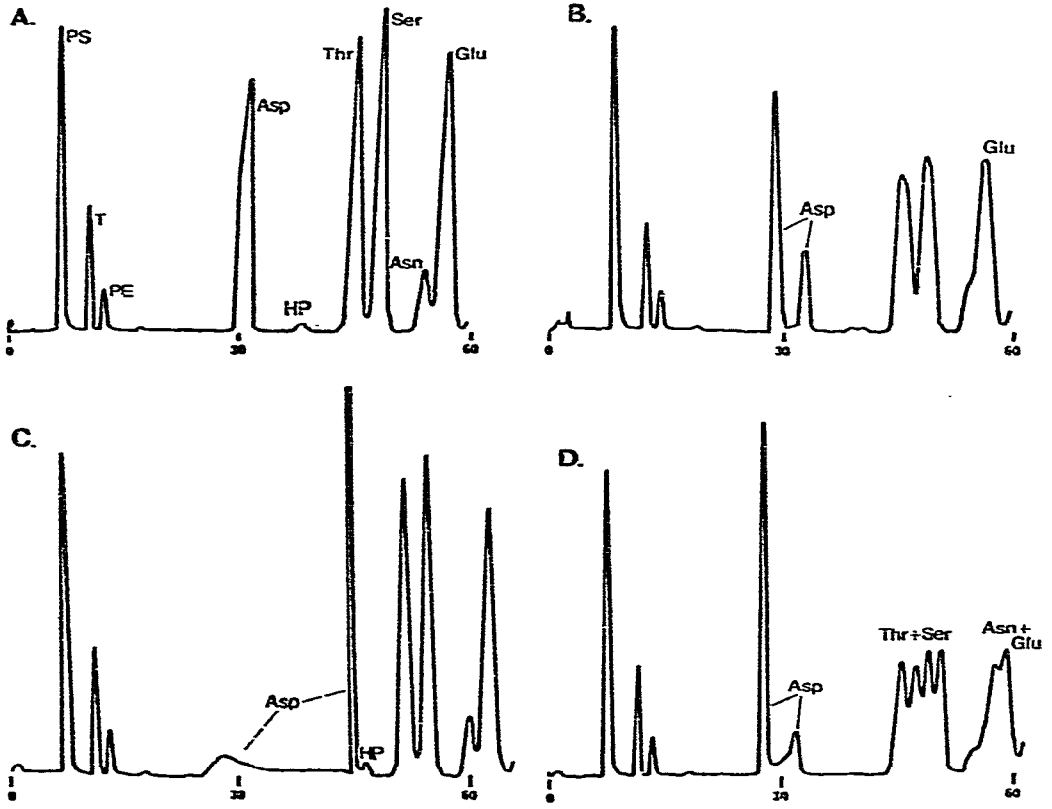

Fig. 1. Effect of organic buffers on elution of amino acids in a physiological calibration standard. The trace represents absorbance of the ninhydrin-reacted eluate, measured at 570 nm. The standard contained 50 nmol of aspartic acid. Time scale in minutes. PS = phosphoserine; T = taurine; PE = phosphoethanolamine; HP = hydroxyproline. A, standard alone; B, standard plus HEPES, (30 μmol) loaded at pH 2.2; C, standard plus tricine (25 μmol) loaded at pH 2.2; D, standard plus HEPES, (30 μmol) loaded at pH 2.5.

HEPES

In the presence of this buffer, aspartic acid emerged as two separate peaks, with an elevated baseline plateau of variable height between the peaks. In plant samples, the aspartic acid region contained three peaks, due to the presence of BIA. At pH 2.5, this effect began to appear with the addition of 12–15 μmol of

TABLE I

BEHAVIOUR OF ASPARTIC ACID IN AMINO ACID ANALYSIS, INFLUENCED BY THE PRESENCE OF ORGANIC BUFFERS IN THE SAMPLE

Aspartic acid (100 nmol) was loaded, with the organic buffer, in a sample volume of 0.5 ml, and a lithium-based analytical system was used. The figures in parenthesis represent proportions of the total aspartic acid recovered in double peaks, each peak expressed as a percentage of the total area (which includes any plateau).

Buffer	*Amount added (μmol)*	*Loading pH*	*Time of elution of aspartic acid peak(s) (min)*	*Notes*
None	—	2.2	32	
HEPES	50	2.15	27 (49%) 33 (51%)	
	25	2.15	29 (48%) 33 (52%)	
	50	2.3	26 (33%) 34 (57%)	Plateau between peaks
	50	2.5	24 (78%) 34 (11%)	Plateau (later compounds: grossly distorted)
	25	2.5	29 (65%) 33 (17%)	Plateau (later compounds: peaks doubled)
	10	2.5	33	
EPPS	50	2.5	27 (63%) 32 (29%)	Later compounds: peaks doubled
Tricine	50	2.2	17 (24%) 45 (54%)	Plateau
	50	2.5	17 (44%) 45 (41%)	Plateau
	25	2.2	24 (20%) 42 (64%)	Plateau
	10	2.2	32 (14%) 40 (74%)	Plateau
	5	2.2	33 (10%) 39 (76%)	Plateau
Bicine	25	2.2	21 (39%) 45 (36%)	Plateau

buffer; at pH 2.15, the threshold was slightly higher. Several peaks immediately following aspartic acid were also affected, becoming first broadened (Fig. 1B), then doubled and progressively more distorted (Fig. 1D), with increasing loading pH and amount of HEPES added. Later peaks (glycine, alanine and those following) and the compounds emerging before aspartic acid (phosphoserine, taurine, phosphoethanolamine) were unaffected.

EPPS

This buffer is a homologue of HEPES, and produced very similar distortions.

Tricine

Severe effects were observed with quite low levels (5 μmol) of tricine, at a range of values of loading pH. Again, aspartic acid emerged as two peaks with an interconnecting plateau, the distance between the peaks varying with the amount of buffer added. The second peak was considerably delayed and caused late elution and some compression of later peaks (Fig. 1C). In plant samples, the compound BIA was not resolved and was completely merged with the second aspartic acid peak.

Bicine

This buffer had an effect similar to that of tricine.

Tris and MES

These buffers had little effect at levels up to 50 μmol, although at the higher concentrations a small leading fore-peak to aspartic acid was sometimes present.

Effect on analysis in sodium buffers

From a limited series of experiments, it is clear that HEPES, EPPS, tricine and bicine also influence the resolution of aspartic acid in a sodium-based analytical system. The effects are similar to those described above, although the appearance of the effects requires several fold higher levels of the organic buffers, compared with the lithium-based system.

CONCLUSIONS

Organic buffers are sometimes present in samples used for amino acid analysis, for example in preparations of purified organelles, or reaction mixtures from enzyme studies. As shown here, buffers of this type can cause serious problems in the resolution of aspartic acid and some other compounds, producing difficulties with interpretation of the chromatographic results. Accurate determination of aspartic acid content may be prevented when part of the compound emerges as a plateau region not recorded by an integrator. With HEPES and EPPS, the effect is intensified as the pH of the sample rises slightly above the recommended loading pH. Inaccurate adjustment of pH may occur with very small sample volumes, and "loading buffers" have in fact very little buffering capacity.

At first, the peak doubling noted for aspartic acid seemed to be so remarkable that the purity of the sample was suspected, but the same effect was consistently seen with a range of samples, including the aspartic acid peak in calibration standard mixtures and in plant extracts. Other workers have reported that the aspartic acid peak may undergo some distortion as the loading pH is varied[6,7], but the effects were quite small compared to the distortions described here. The nature of the buffer–amino acid interaction is not clear; possibly a buffer–aspartic acid complex is formed. Regardless of the explanation, it is clear that caution must be used when buffers such as HEPES, EPPS, tricine and bicine are present in samples that are to undergo amino acid analysis; minimum acceptable concentrations of the buffers should be used, the effect on known standards should be observed, and the pH of samples should be lowered to *ca.* 2.1.

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