ACCELERATED AMINO ACID ANALYSIS WITH LITHIUM CITRATE BUFFERS

THE EFFECT OF RESIN CROSS-LINKAGE ON ELUTION PATTERNS AND THE AVOIDANCE OF MICROBIAL CONTAMINATION OF COLUMNS

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SUMMARY

The elution pattern of acidic and neutral amino acids has been studied using a number of samples of Aminex A5 resin. The degree of cross-linking of the resins was assessed by the tungstate density method and a correlation between elution pattern and density was found. A range of resin densities was defined within which optimal resolution can be expected in the system of ATKIN AND FERDINAND[1].

When lithium citrate buffers are used without preservatives, microbial contamination of columns can occur, resulting in grossly deformed amino acid peaks. The addition of lithium fluoride, caprylic acid, and pentachlorophenol protects against microbial contamination.

INTRODUCTION

In defining the optimal conditions for amino acid analysis with lithium citrate buffers[1] we used a single batch of Aminex A5 resin (batch No. 5028). No preservatives were added to the buffers in the belief that microbial growth would not occur in the presence of lithium salts at the low pH values employed.

Subsequently severe microbial contamination of the resin columns occurred and new resin was needed to replace the affected material. The properties of several new batches of resin were found to be different from that of the original to such an extent that the elution patterns obtained with them were either unsatisfactory or sufficiently different to make it inadvisable to mix the batches.

The studies reported here were designed to overcome these problems in the use of lithium citrate buffers. An effective combination of antimicrobial agents has been tested, and methods for selecting satisfactory batches of resin are described.

The physical properties of spherical ion-exchange resins that affect the elution pattern of amino acids have been defined by HAMILTON[2,3] as the bead diameter and range of diameters, the degree and range of cross-linkage of the resin, and the ion-exchange capacity. These properties can be measured. Other factors which may affect resin performance are such hidden variables as the proportions of *m*- and *p*-divinyl-

benzenes in the divinylbenzene (DVB) used for cross-linking. It is known that *m*-DVB produces a co-polymer with the cross-links spaced evenly through the bead[4], resulting in a greater swelling than the corresponding *p*-DVB co-polymer, and a rate of sulphonation faster than the *p*-DVB co-polymer[5]. *p*-DVB produces a tightly cross-linked nucleus of DVB units to which long chains of polystyrene are attached and in which more pure polystyrene is embedded. Since the commercial DVB's used in resin preparation are likely to contain variable proportions of the two isomers it is evident that different resin types will result. These factors cannot be assessed easily in the resin and we have ignored them. Of the measurable resin properties bead size and range and ion-exchange capacity can be measured easily but the degree of cross-linking is less directly accessible. The wet density[6], specific water regain[7], and sodium tungstate density[8,9] have all been proposed as measures of cross-linkage. We have tested all three methods and come down in favour of the use of tungstate densities. Wet density can be used with relative ease for large resin particles but when dealing with particles of about 15 μm diameter the problem of surface water becomes unsurmountable in routine laboratory practice. The measurement of water regain involves a colorimetric assay of water uptake by the dry resin from a dye solution. We have found that in many cases drying the resin generates soluble yellow materials that enter the dye solution, causing errors in the estimate of water regain. Unless large samples of resin can be spared, it is also difficult to recover sufficient volume of dye solution with a measurable change in extinction. The use of sodium tungstate solutions on the other hand requires only a few milligrams of resin, is relatively simple, cheap and reproducible and has not been found to suffer from any practical disadvantages. What structural property is measured by this method is not altogether clear, although it is related to the degree of cross-linking[9]. Nevertheless we have found that the tungstate density of a resin is a valuable property that can be related to the performance of the resin in amino acid analysis.

EXPERIMENTAL

Ion-exchange capacity

Ion-exchange capacity was determined by the titration method of MILLAR[10]. After air-drying resins at 110° about 2 % of the original water content still remained and this was removed over P_2O_5 under vacuum[11]. Titration was carried out with a Radiometer auto-titrator (TTTIa) to an end point of pH 7.0.

Bead size

Bead size was measured using a microscope equipped with an ocular micrometer that had been calibrated against a graticule. A drop of a dilute suspension of resin in distilled water was placed on a slide. Observation of the beads while settling through the drop of water is a useful preliminary as it readily shows up broken beads which tend to come to rest on the slide with the broken face down, thus masking the fact that they are broken. After settling, a cover slip was placed over the drop and the diameters of at least 100 beads were measured. Each batch of resin examined (see Table I) had bead diameters in the Li+ form ranging from 11 to 16 μm. The mean diameter in every case lay between 13.5 and 14.5 μm with a S.D. of $\pm$ 1.5 μm.

TABLE I

PROPERTIES OF EIGHT BATCHES OF AMINEX A5 ION-EXCHANGE RESIN (BIO-RAD LABORATORIES, RICHMOND, CALIF.)

Resin batch No.	Tungstate density (g/ml)	Range of tungstate density	Mid-range tungstate density	Ion-exchange capacity (mequiv./g)	Mean elution time of alanine[b]	
					min ± S.D.	No. of determinations
5028	1.322–1.326	0.004	1.324	5.23	113.2 ± 1.0	10
3915	1.326–1.330	0.004	1.328	5.04	121.0 ± 0.3	2
7208	1.326–1.332	0.006	1.329	5.12	118.3	1
9474	1.332	0	1.332	5.21	117.8 ± 2.0	10
7066/7127[a]	1.332–1.336	0.004	1.334	5.10	114.1 ± 3.6	12
9081	1.336	0	1.336	5.20	117.1	1
6204	1.346–1.348	0.002	1.347	5.19	123.6 ± 2.3	30

a These two batches were indistinguishable by all the tests applied and were combined.

b The times given refer to the time taken for alanine to leave the bottom of a column of 24 cm (running length) × 11 mm, when pumped at a buffer flow rate of 80 ml/h as described previously[1].

TABLE II

CONCENTRATIONS OF SODIUM TUNGSTATE IN THE FIVE "PRIMARY" SOLUTIONS USED IN MEASURING THE TUNGSTATE DENSITIES OF RESINS

Tungstate density (g/ml)	Sodium tungstate concentration (g/l)
1.310	363.7
1.320	375.5
1.330	387.5
1.340	399.4
1.350	411.5

Tungstate density

The relationship between the density and concentration of sodium tungstate solutions was first determined. Grade B glassware was not accurate enough so all volumetric glassware was calibrated using distilled water at 25°. Sodium tungstate dihydrate (British Drug Houses, Analar grade) was converted to the anhydrous form by drying overnight in an air-oven at 110° and stored in a desiccator. Twelve solutions (1 l) were made up in the range 370–420 g/l using weighed and calibrated glassware, allowed to stand in a water bath at 25.0 ± 0.1° for at least 1 h, and made up to the mark. The flasks were then weighed and the density of the solution calculated. From a linear regression analysis of the results the relationship between the concentration, C g/l, and the density, D g/ml in this range was found to be:

$$C = 1195(D - 1) - 6.81 \tag{1}$$

with a correlation coefficient of 0.9997. Table II gives the concentrations of anhydrous sodium tungstate required to make up solutions of densities in the range 1.310 to

1.350 in intervals of 0.01 g/ml, as calculated from eqn. 1. Solutions of density intermediate between the primary steps were prepared by mixing the required volumes of these "primary" solutions. In this way solutions in density steps of 0.002 g/ml were prepared. A range of sodium tungstate solutions (5 ml of each) is placed in a series of test-tubes and 5 mg of resin added to each one. The tubes are sealed with Parafilm and allowed to stand in a water bath at 25.0° ± 0.1°. Disturbance of the tubes must be avoided during the next 48 h while the resin is reaching hydrostatic equilibrium with the solution. After this the resin can be classed as denser than, lighter than, or isopycnic with each of the solutions in the range.

Amino acid analysis

Amino acid analysis was carried out as described previously[1] with the exception that 2 × 0.4 ml aliquots of buffer No. 2 were used to wash samples into the columns for A/N analyses instead of 3 × 0.2 ml. This minor change results in improved resolution of aspartic acid, threonine and serine.

Microbial contamination of columns

This first became apparent in one analyser over a period of two or three weeks by the gradual broadening of the amino acid peaks emerging after the buffer change in the hydrolysate A/N analysis. No effect on amino acids eluting before the buffer change was observed, nor was the column impaired for use in the basic analysis of physiological fluids. Finally the top 1–2 cm of resin was seen to be contaminated by its darker colour and by its reddish purple colour during regeneration with lithium hydroxide. At this stage all the peaks after the buffer changes were double, as shown in Fig. 1. The contamination subsequently spread to a second analyser with identical results. Removal of the contaminated resin and the use of fresh buffers temporarily solved the problem but contamination recurred within a week. The addition of 5 mM lithium fluoride to the lithium citrate buffers and the replacement of affected resin again restored the situation to normal, but one month later a sudden deterioration in the elution pattern after the buffer change occurred again. Finally the lithium citrate buffers were made up to contain lithium fluoride (5 mM), n-octanoic acid (n-caprylic acid, 0.1 ml/l) and pentachlorophenol (0.5 mg/l). No further problem has been encountered.

Although this problem had all the characteristics of a microbial, probably fungal, contamination we have not been able to elucidate the nature of the contaminant. The buffers in use at the time slowly developed small floating clumps of greyish material resembling cotton wool under the microscope. Attempts to culture a fungus from these buffers demonstrated the presence of a penicillium, which grew readily on potato, dextrose, Agar (PDA) plates containing the lithium citrate buffers (pH 2.58 or 3.65). No growth was observed at other pH values or on normal PDA plates. Penicillium species are such common laboratory contaminants that we cannot be sure that this was the cause of column contamination. Attempts to culture small samples of the contaminated resin on a variety of differently supplemented media were unsuccessful, probably because the resin had been automatically regenerated with lithium hydroxide. The aqueous BRIJ 35 solution used in making up the buffers was seen to contain a white veil-like material. By phase-contrast microscopy this could be seen to have the typical mycelial structure of a septate fungus. All attempts
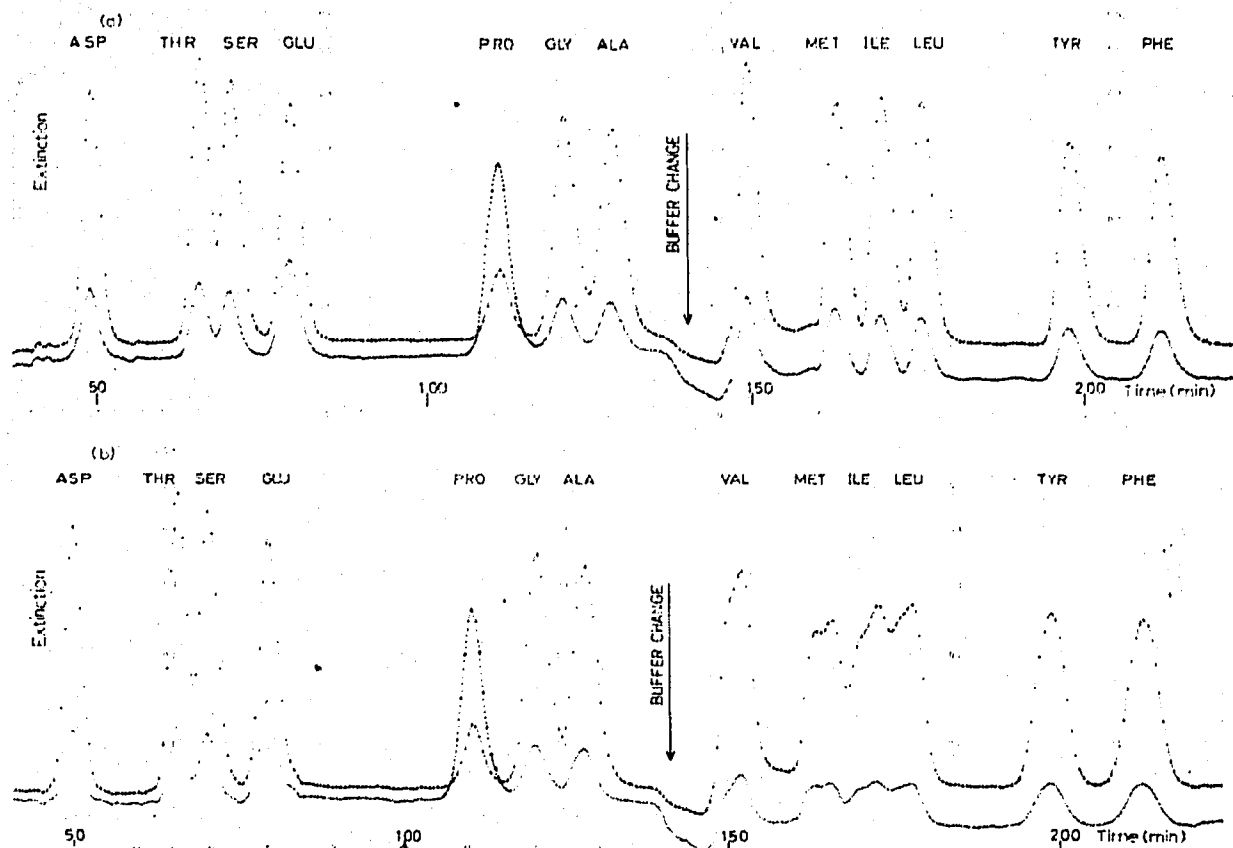
Fig. 1. A comparison of chromatograms from normal and contaminated amino acid analyser columns. (a) Normal acidic and neutral amino acid analysis on a 24 × 1.1 cm column of Aminex A5 resin (batch No. 5028). Elution times have not been corrected for a reaction coil time of 15 min. Elution conditions are as described previously[1]. (b) The same analysis on the same column after contamination. Conditions as in (a).

to induce this fungus to sporulate were unsuccessful; it grew only extremely slowly and only in BRIJ 35 solutions (50 g/100 ml). Subsequently solid BRIJ 35 has been used in preparing buffers rather than using a stored aqueous solution.

Buffer line filters

Originally buffers were passed through a 1-cm pre-column of Aminex A5 resin immediately before entering the main column[1]. In order to avoid contamination the pre-columns have now been replaced by Millipore filters. Two Solvinert filters (URWP01300 and UGWP01300, Millipore Corporation, Bedford, Mass. 01730) separated by a disc of 8 μm stainless-steel wire mesh[1] and supported by another similar mesh are mounted in a stainless-steel holder.

Rejuvenation of contaminated resin

Attempts to rejuvenate contaminated resin were made because of the heavy losses of resin caused by the contamination. Soaking in 3 N sodium hypochlorite solution bleached the contaminating colour but left the resin in a "sticky" form which gave high running pressures in the columns. Refluxing in 6 N HCl for 1 h overcame

the latter problem, but the resin properties were found to be altered so as to give a changed amino acid elution pattern. In a similar way suspending the resin in 2 N HNO$_3$ and raising to the boil, then allowing to cool, removed the contamination but again altered the resin properties as described below.

Elution patterns of various batches of Aminex A5 resin

Eight batches of resin were studied in an attempt to find one that gave an amino acid elution pattern like that of the original batch No. 5028. The batch numbers and properties are listed in Table I. In all cases the resolution of amino acids that elute in buffer 2 (pH 3.65) was satisfactory as judged by a valley to peak ratio of less than 30 % on a 24-cm column. In all cases the ratio was much less than 30 %. The resolution of amino acids eluting in buffer No. 1 (pH 2.59) was also satisfactory except in the cases of the threonine/serine and glycine/alanine pairs. Some resins gave good resolution, others gave sharp peaks too close together, and others gave peaks that were too broad for satisfactory resolution of these pairs. The performance of all the resins tested was satisfactory in the analysis of basic amino acids with the exception of batch No. 9204 which did not resolve lysine and 1-methyl-histidine in the physiological fluid analysis.
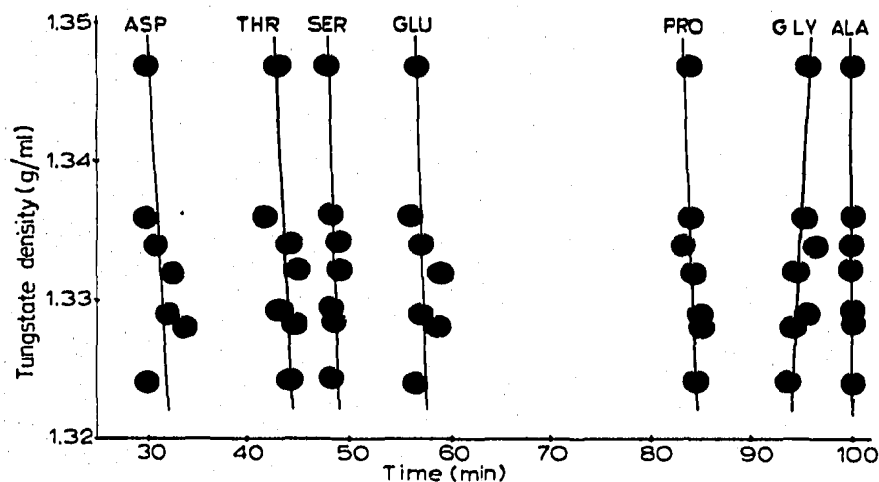

Fig. 2. The correlation between the tungstate density of a resin and the elution pattern obtained under the conditions of Fig. 1. The elution times given for each amino acid on each resin are the times at which the amino acid leaves the bottom of the column, normalised in each case to an alanine time of 100 min. The lines are the lines of least squares fit. In all cases the slope is negative except for glycine (+ 14.7). The slopes for threonine and serine are — 12.8 and — 19.6, respectively indicating that they converge as tungstate density decreases. The radius of the points gives an indication of the standard deviation in the horizontal dimension, ± 1 to 2 min for most amino acids.

The elution times of acidic and neutral amino acids emerging in buffer No. 1 are given in Table I and Fig. 2. Most batches of resin contained a range of tungstate densities. In Fig. 2 the density at the middle of the range is plotted against the elution times for different resins. The mid-range tungstate density is not necessarily the density of the majority of the resin beads as there is no simple way of assessing the quantitative distribution of resin through the range of densities observed. Nevertheless Fig. 2 shows a clear relationship between tungstate density and elution

pattern. As the density increases from 1.324 to 1.347 the amino acids emerge earlier relative to alanine, with the exception of glycine, which emerges later. The resolution of glycine and alanine is thus poorer at high densities. The reverse is true of threonine and serine, though the tendency is not so marked. All the resins of tungstate density greater than 1.332 were unsatisfactory in their resolution of glycine and alanine. Resins of density lying between 1.324 and 1.332, inclusive of both figures, were satisfactory in all respects provided the range of densities was not greater than 0.004. In the case of batch No. 7298, although its mid-range density was 1.329, there was a spread of 0.006 in the densities of its beads and the peaks were too broad for glycine and alanine to be resolved properly. The wide spread of densities is indicative of a wide spread of cross-linkages in the resin, and this will be a dispersive factor leading to broad peaks and poor resolution[2]. An nth order regression analysis of HAMILTON'S[9] data relating the degree of cross-linkage, Y, to the tungstate density, D, gave a good fit with $n = 3$ or 4. The third order equation derived is

$$Y = -4581.9 + 10783.37D - 8507.83D^2 + 2251.488D^3 \qquad (2)$$

From this equation the values of Y at values of D in the range of interest are given in Table III. We give these correlations for interest's sake but consider that the use of per cent cross-linkage is of no practical value in classifying ion-exchange resins for amino acid analysis and prefer to use tungstate density.

TABLE III

RELATIONSHIP BETWEEN TUNGSTATE DENSITY AND CROSS-LINKAGE OF SULPHONATED POLYSTYRENE RESINS

Taken from Hamilton's[9] data.

Tungstate density	Cross-linkage (%)
1.320	6.4
1.322	6.6
1.324	6.8
1.326	7.0
1.328	7.2
1.330	7.4
1.332	7.6
1.334	7.8
1.336	8.0
1.338	8.2
1.340	8.5
1.342	8.7
1.344	8.9
1.346	9.1
1.348	9.4
1.350	9.6

The resins of lower tungstate density gave higher pressures during elution than those of higher density. This is to be expected since low cross-linkage gives rise to a weaker and more compressible structure for the resin beads.

All the resins tested had ion-exchange capacities in the range 5.14 ± 0.10 mequiv./g. Within this range there seems to be little effect of capacity on elution pattern.

TABLE IV

EFFECT OF OXIDATIVE TREATMENTS UPON THE PROPERTIES OF AMINEX A5 ION-EXCHANGE RESINS

Treatment I: suspension in 2 N HNO$_3$ and raising to the boil, then allowing to cool. Treatment II: suspension in 3 N NaOCl overnight, washing, then refluxing for 1 h in 6 N HCl.

Resin batch No.	Treatment	Tungstate density (g/ml)	Range of density	Mean[a] elution time of alanine (min)	Amino acid elution times relative to alanine = 100 min					
					Asp	Thr	Ser	Glu	Pro	Gly
5028	None	1.322–1.326	0.004	113.1	29.9	44.3	48.6	56.4	84.5	93.5
	I	---	---	109.5	30.8	45.6	49.0	57.5	85.3	93.1
	Repeat I	---		103.9	31.6	46.1	49.7	57.2	86.8	92.5
	II	1.310–1.320	0.010	99.4	32.0	47.0	49.6	57.7	87.8	92.0
7066/7127[a]	None	1.332–1.336	0.004	114.1	30.7	44.0	48.9	56.8	83.3	96.0
	II	---	---	112.4	30.6	44.5	49.6	57.1	84.1	96.0
	Repeat II	---	---	109.7	31.0	44.9	49.6	56.6	83.5	95.9
	Repeat II	1.310–1.320	0.010	113.6	31.0	45.1	49.0	56.6	86.3	94.7

[a] See Table I.

Effect of mild oxidation of resins on their properties

The conclusions reached above from a study of different batches of resin are confirmed by the effects of oxidative treatment of two batches of resin which had become contaminated as described above. Table IV shows the elution times of amino acids after a series of oxidative treatments, together with the tungstate densities before and after treatment. In both cases the density decreased, the range of densities became wider, the peaks became broader, glycine and alanine moved apart and threonine converged upon serine, while the running pressure increased.

CONCLUSIONS

In view of the trouble and expense caused by a serious microbial contamination of resin columns we recommend the following alterations to our earlier procedures[1]. Lithium fluoride (5 mM), n-octanoic acid (0.1 ml/l) and pentachlorophenol (0.5 mg/l) should be added to both the lithium citrate buffers. Solid BRIJ 35 should be weighed out instead of using a stored solution, when making up buffers, and more rigorous filtration of buffers entering the columns should be employed. The normal Millipore or Oxoid membrane filters are unsuitable for use in the amino acid analyser but the solvent- and alkali-resistant Solvinert filters can be used.

The problem of a reliable source of ion-exchange resins is an ever-present one, even assuming no catastrophic loss of resin, because of slow wastage and the necessity of "topping-up" columns occasionally. The variability of batches of commercial resin has been reported before[9,12–14], and we do not believe that Aminex A5 resin is any worse in this respect than other available resins. In fact it appears that the use of lithium citrate buffers may demand a more stringent control of resin density than is the case with sodium citrate buffers because of a tendency for glycine and alanine to elute together at high densities.

Provided that a batch of resin contains a range of tungstate densities of less than 0.004, and the mid-range density lies between 1.324 and 1.332 g/ml our evidence

suggests that it will be suitable for use. Resins of lower density are likely to generate high running pressures and to fail to resolve threonine and serine, while resins of higher density are likely to fail to resolve glycine and alanine.

ACKNOWLEDGEMENTS

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