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AMINO ACID ANALYSIS BY ION-EXCHANGE CHROMATOGRAPHY USING A LITHIUM ELUTION GRADIENT

INFLUENCE OF METHANOL CONCENTRATION AND SAMPLE pH

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SUMMARY

The separation of amino acids has been achieved on a short column of Chromo-Beads C_2 resin, with a lithium gradient-elution system. The analysis took 8 h. The separation of asparagine and glutamine from glutamic acid was highly dependent on the sample pH and on the methanol concentration in the first buffer of the gradient. The method has been applied to analysis of human plasma and granulocytes for amino acids.

INTRODUCTION

Analysis of physiological fluids and tissue homogenates for amino acids by ion-exchange chromatography with an elution gradient based on sodium citrate buffer solutions does not achieve separation of glutamine from asparagine[1,2]; these amino acids, however, can be separated by using lithium buffers[3]. Since the initial work of Benson *et al.*, several authors have described methods involving use of lithium citrate, with two columns (one for the acidic and neutral amino acids, and the other for the basic ones) and with a discrete elution gradient[3,4]. Few reports describe a continous gradient-elution system on one column for the separation of all the amino acids[5–8].

However, none of these techniques is useful for the separation of glutamine from asparagine under our conditions, *i.e.*, use of Chromo-Beads C_2 resin and a gradient-elution system with lithium citrate. The purpose of this paper is to demonstrate that, by varying the concentration of methanol in the first buffer of the gradient, and the pH of the sample, the separation of all amino acids can be achieved.

EXPERIMENTAL

Chemicals and reagents

The ion-exchange resin used was Chromo-Beads C_2 (Technicon, Tarrytown,

N.Y., U.S.A.). Lithium citrate, lithium chloride, lithium hydroxide, ninhydrin, hydrazine sulphate, methylcellosolve, anhydrous sodium acetate, sulphosalicylic acid and methanol were purchased from E. Merck (Darmstadt, G.F.R.); hydrochloric acid and pure acetic acid from Prolabo; and Brij 35 and norleucine from Technicon. The standard amino acids were obtained from Merck and N.B.C. (Cleveland, Ohio, U.S.A.).

The ninhydrin and hydrazine sulphate solutions were prepared according to Technicon reports[9,10]. The buffered ninhydrin solution contained 13.5 g of ninhydrin, 1 l of methylcellosolve, 65 ml of pure acetic acid, 200 ml of 4 *M* sodium acetate of pH 5.5 and distilled water up to 2 l.

The 20 m*M* hydrazine sulphate solution was prepared in distilled water, and a few drops of concentrated sulfuric acid were added.

The procedure requires four lithium citrate buffers, and a fifth is used to regenerate the resin before injection of the next sample. This buffer is of pH 3.10, and is 0.275 *N* in lithium and 0.07 *N* in citrate; it is prepared from 19.74 g of trilithium citrate tetrahydrate, 2.76 g of lithium chloride, 1 ml of 2,2'-thiodiethanol, and 10 ml of Brij 35 (10%, w/v) solution and is made up to 1 l with distilled water after pH adjustment with 6 *M* hydrochloric acid.

The first three buffers required to establish the elution gradient are of pH 2.75, 2.875 and 3.8. They have the same composition as the buffer of pH 3.10, but the solution of pH 2.75 also contained 70 ml of methanol per litre. 2,2'-Thiodiethanol is added to each solution to prevent oxidation of methionine.

The fourth buffer is of pH 6.5 and is 1.5 *N* in lithium and 0.07 *N* in citrate; it contains 19.74 g of trilithium citrate tetrahydrate, 54.68 g of lithium chloride and 10 ml of the Brij 35 solution per litre.

All the buffers are stored (in closed bottles to prevent contamination by atmospheric ammonia) at 4° for no more than three days; the pH is checked each time before use and is adjusted if necessary.

Materials and apparatus

The apparatus for chromatography comprised a Technicon column (75 cm × 0.63 cm I.D.), connected to a Technicon AutoAnalyzer; measurements were made at 570 nm. The Chromo-Beads resin, type C_2, is 8% cross-linked and has a particle diameter of 13 μm; before use, the resin was converted into the lithium form as described by Perry *et al.*[5]. The resin was washed with acetone, 3 *N* nitric acid and 3 *N* lithium hydroxide solution, respectively, then equilibrated with the buffer of pH 3.10. To fill the column, the resin was divided into five parts, each of which was poured into the column and packed sequentially.

Method

The elution gradient was obtained with a nine-chamber Autograd (see Table I) using a flow-rate of 0.75 ml/min. The temperature was maintained at 39° until the elution of citrulline, then increased to 60°. Complete separation of the amino acids took 8 h. After each analysis, the resin was washed with 0.3 *N* lithium hydroxide for 20 min, then equilibrated with the buffer of pH 3.10. This regeneration and the use of a buffer of pH 2.75 to begin the gradient improved the separation of the threonine–serine group[11]. The column was discarded after twenty runs in order to avoid loss of resolution caused by excessive compaction of the resin.

TABLE I

COMPOSITION OF THE ELUTION GRADIENT

Chamber No.	*Volume (ml) of buffer*			
	pH 2.75	*pH 2.875*	*pH 3.80*	*pH 6.50*
1	40	—	—	—
2	30	10	—	—
3	—	40	—	—
4	—	20	20	—
5	—	—	40	—
6 to 9	—	—	—	40

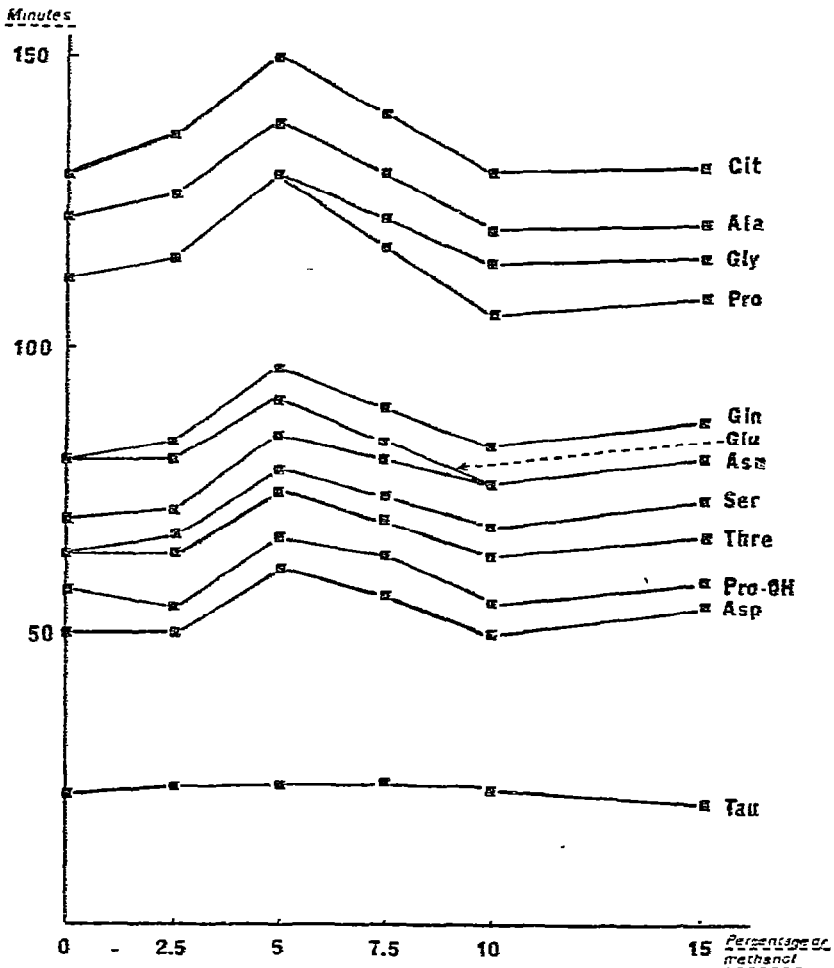

Fig. 1. Effect of the methanol concentration on elution of a prepared mixture of amino acids; 250 nmoles of each amino acid were placed on the column, and the chromatographic conditions were as described in the text.

Sample preparation

Amino acid standards. The amino acids were dissolved in 0.01 *N* hydrochloric acid, the pH of the solution was adjusted to 1.5, and the final concentration for each compound was 2.5 m*M*. The efficiency of the column was checked after every ten runs with 100 µl of this solution, which was stored at −20°. As internal standard, 2.5 m*M* norleucine was used.

Human plasma. Human blood, collected with lithium heparinate by venipuncture, was centrifuged at 3000 *g* for 15 min at 4°; the plasma was deproteinised with sulphosalicylic acid at a final concentration of 3% (w/v), the precipitate was removed by centrifugation for 15 min at 3000 *g* and 4°, and the pH of the supernatant solution was adjusted to 1.5.

Human granulocytes. The granulocytes were isolated according to the method described elsewhere[12]. The cell suspension contained more than 90% of polymorpho-

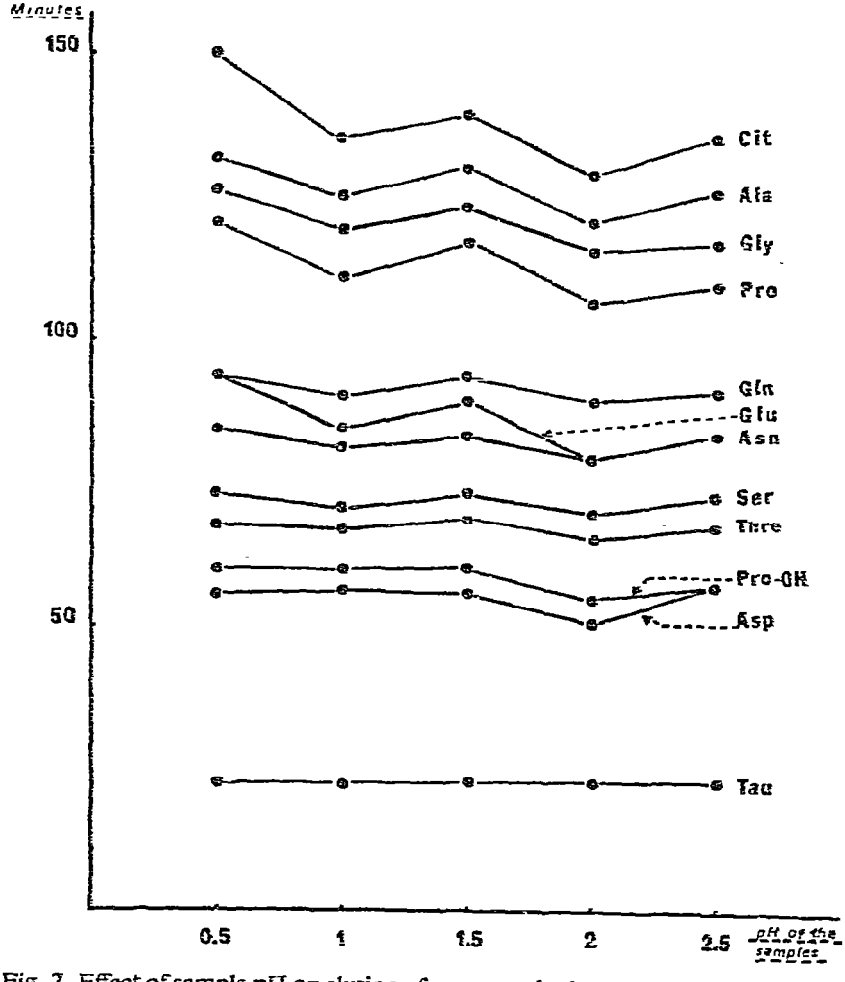

Fig. 2. Effect of sample pH on elution of a prepared mixture of amino acids with a final concentration of 2.5 µmoles/ml and a pH of 1. The pH was adjusted with either HCl or LiOH. Conditions as in Fig. 1.

nuclear leukocytes, with thrombocyte contamination of 1 per leukocyte. The cells were suspended in 1 ml of distilled water containing 250 nmoles of norleucine and submitted to three cycles of freezing and thawing. Then 30 mg of sulphosalicylic acid were added, the suspension centrifuged, and the pH of the clear supernatant solution was adjusted to 1.5.

RESULTS AND DISCUSSION

Ion-exchange chromatography with lithium buffers is the most suitable technique of analysis for glutamine and asparagine. However, the resolution of the acidic amino acids in such systems depends on the pH of the sample[13] and the concentration of methanol in the first buffer of the gradient[14]. Before applying this method to the quantitative study of the free amino acids in human granulocytes, we determined these parameters under our conditions.

Methanol concentration

The concentration (15%, v/v) of methanol used in sodium buffer elution gradients[10] did not separate asparagine from glutamic acid, although all the other compounds were sharply resolved. The effects of various methanol concentrations 0 to 15%, v/v) on the amino acids eluted at 39° (these being the most sensitive to

TABLE II

VARIATION IN PEAK DIMENSIONS WITH SAMPLE pH FOR SOME AMINO ACIDS

The peak height (H) is expressed as absorbance, and the width (W) in cm (see ref. 13); the width is measured at half the maximum height. Where no values are shown for asparagine, glutamic acid and glutamine, this is because, at these pH values, there is no resolution between asparagine and glutamic acid or between glutamic acid and glutamine.

Amino acid	*Peak-parameter*	*Sample pH*				
		0.5	*1*	*1.5*	*2*	*2.5*
Aspartic acid	H	0.382	0.329	0.328	0.314	0.308
	W	0.40	0.40	0.40	0.40	0.4
	$H \times W$	0.153	0.131	0.131	0.126	0.123
Threonine	H	0.378	0.368	0.318	0.320	0.244
	W	0.40	0.40	0.40	0.40	0.50
	$H \times W$	0.151	0.147	0.127	0.128	0.122
Serine	H	0.378	0.366	0.320	0.309	0.272
	W	0.35	0.35	0.40	0.40	0.45
	$H \times W$	0.132	0.128	0.128	0.124	0.122
Asparagine	H	0.145	0.145	0.145		
	W	0.40	0.40	0.40		
	$H \times W$	0.058	0.058	0.058		
Glutamic acid	H		0.434	0.379		
	W		0.45	0.50		
	$H \times W$		0.195	0.189		
Glutamine	H		0.301	0.295	0.265	
	W		0.40	0.45	0.60	
	$H \times W$		0.20	0.118	0.119	

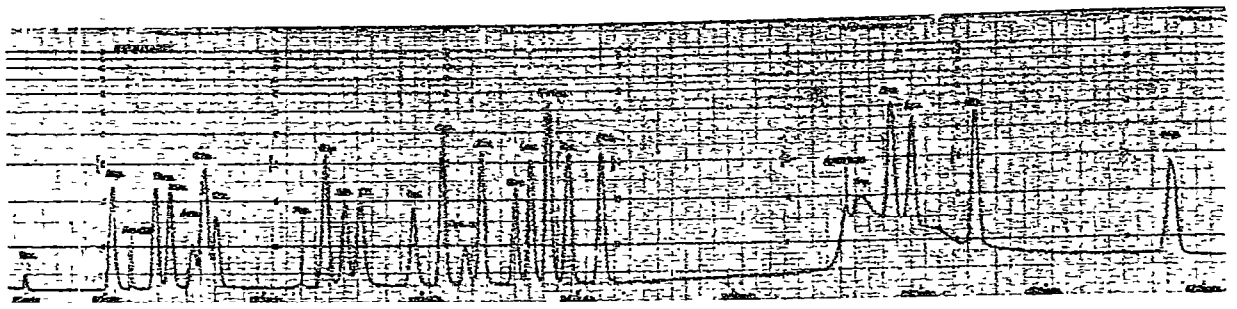

Fig. 3. Chromatogram of a prepared mixture of amino acids.

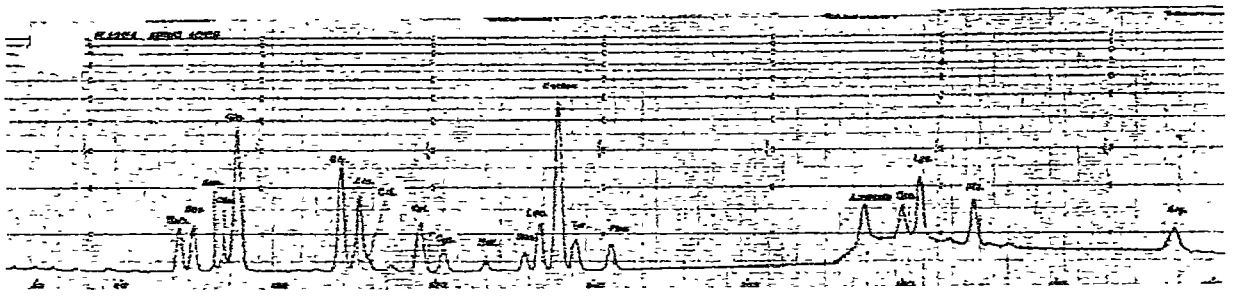

Fig. 4. Chromatogram of 500 μl of pooled deproteinised plasma from fasting humans.

variation) are shown in Fig. 1. A methanol concentration of 5% (v/v) causes a slight increase in elution times; at 10% (v/v) of methanol, no such effect was observed, but glutamic acid and asparagine were not separated. Without methanol, threonine and serine, glutamine and glutamic acid, and proline and glycine, were not separated from each other. Finally, 7% (v/v) of methanol was chosen for use, the separation of glutamic acid being taken as the limiting factor. Another organic solvent, such as *n*-propanol, has been reported to improve the separation of glutamine and asparagine from glutamic acid[4], but the modifications reported were greater than those we obtained with methanol. In some assays, we observed no differences between the two solvents.

Sample pH

It has been reported that, when the sample pH was varied, there were some variations in elution time and in peak area for the acidic amino acids, especially aspartic acid[15]. Fig. 2 shows the variations in elution time of the acidic compounds when the sample pH was varied from 0.5 to 2.5; glutamic acid was the most sensitive to these variations. Indeed, at low pH (0.5), glutamic acid was eluted with glutamine, whereas at pH 2.0 it was eluted with asparagine. Consequently, the pH of all samples and test solutions was adjusted to 1.5. Table II shows the variations in height, width and area of the peaks for aspartic acid, threonine, serine, asparagine, glutamic acid and glutamine. An increase in sample pH led to decreased heights for all these compounds except asparagine. This effect was marked for glutamine, whereas for the other amino acids, it appeared only at a sample pH of 2.5. Thus, increased sample pH causes marked variation in peak area, and, as the calculation of concentration is based

TABLE III

REPRODUCIBILITY OF THE TECHNIQUE

A human-plasma pool was divided into five parts, each of which was deproteinised with sulphosalicylic acid: the supernatant solutions were stored at −20° before analysis, and 500 µl of each were used. Taurine, aspartic acid and asparagine were present in concentrations too low for determination.

Amino acid	*Range of concn. (µM)*	*Mean ± S.D. (µM)*	*Coefficient of variation*
Threonine	101.6–120.8	112.0 ± 7.5	6.6
Serine	95.8–126.2	115.8 ± 12.5	10.8
Glutamic acid	46.6– 54.8	49.2 ± 2.1	4.2
Glutamine	663.5–779.0	681.3 ± 30.9	4.5
Proline	203.6–226.8	215.8 ± 11.5	5.3
Glycine	245.1–286.2	271.0 ± 8.2	3.0
Alanine	251.1–289.8	272.5 ± 9.1	3.3
Citrulline	16.4– 23.7	19.1 ± 2.8	4.6
Valine	181.2–212.4	199.9 ± 8.7	4.3
½ Cystine	37.3– 47.3	43.1 ± 2.0	2.4
Methionine	20.3– 27.4	23.9 ± 1.3	5.4
Isoleucine	28.9– 52.6	40.7 ± 4.1	10.0
Leucine	70.5– 97.9	81.2 ± 5.5	6.7
Tyrosine	41.0– 53.5	48.0 ± 2.8	5.8
Phenylalanine	51.3– 60.6	56.2 ± 1.8	3.2
Ornithine	83.0–118.9	96.6 ± 7.6	7.8
Lysine	178.0–217.6	198.0 ± 8.6	4.3
Histidine	90.3–120.3	101.9 ± 6.0	5.8
Arginine	45.2– 51.3	48.2 ± 2.2	4.6

on peak area, it is important to ensure that the test amino acids and the sample pH are at the same value.

Applications

Fig. 3 shows a typical standard chromatogram of a prepared mixture of 26 amino acids, and Fig. 4, that of human plasma amino acids, the resolution of which is equivalent to that obtained with the standard solution. It was not possible completely to eliminate the small shift in base line that occurred with elution of the ammonia, but this did not affect the resolution and the quantitative determination

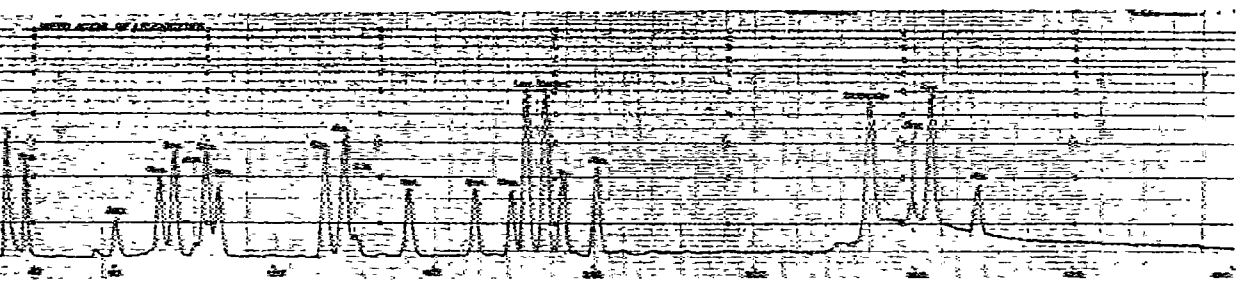

Fig. 5. Chromatogram of fasting human polymorphonuclear leukocytes. The initial suspension contained 10^8 cells.

of the basic amino acids. Results showing the reproductibility of the technique are listed in Table III; the values obtained are similar to those published by other investigators[15,16].

The method was applied successfully to analysis for human granulocytic amino acids (see Fig. 5). The main differences between plasma and granulocytic pools were the high concentrations of taurine, aspartic acid and glutamic acid in the latter samples[17]. The variations of the granulocytic amino acids may reflect those of the whole pool; indeed, it has been shown that the amino-acid-pool composition would be the same in all tissues of an organism[18], and the amino acids due to inherited[19,20] or nutritional[21] and metabolic disorders are also present in polymorphonuclear leukocytes.

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REFERENCES

1 W. H. Stein and S. Moore, *J. Biol. Chem.*, 211 (1954) 915.
2 K. A. Piez and L. Morris, *Anal. Biochem.*, 1 (1960) 187.
3 J. V. Benson, M. J. Gordon and J. A. Patterson, *Anal. Biochem.*, 18 (1967) 228.
4 G. E. Atkin and W. Ferdinand, *Anal. Biochem.*, 38 (1970) 313.
5 T. L. Perry, D. Stedman and S. Hansen, *J. Chromatogr.*, 38 (1968) 460.
6 A. Vega and P. B. Nunn, *Anal. Biochem.*, 32 (1969) 446.
7 C. P. Kedenburg, *Anal. Biochem.*, 40 (1971) 35.
8 J.-F. Bouhours, *J. Chromatogr.*, 94 (1974) 307.
9 *VIth Colloquium on Amino Acid Analysis*, Technicon Monograph No. 3, Geneva, 1968.
10 *Techniques in Amino Acid Analysis*, Technicon Monograph No. 1, Chertsey, 1966, p. 62.
11 M. Bonnafe, *J. Pharm. Belg.*, No. 5 (1965) 193.
12 M. Lahrichi, P. Savigny, Y. Houpert, F. Schiele and G. Siest, in G. Siest (Editor), *Organisation des Laboratoires, Biologie Prospective*, Expansion Scientifique Française, Paris, 1972, p. 609.
13 A. Mondino, G. Bongiovanni and S. Fumero, *J. Chromatogr.*, 71 (1972) 363.
14 A. R. Thomson and B. J. Miles, *Nature (London)*, 203 (1964) 483.
15 M. L. Efron, in *Automation in Analytical Chemistry*, Mediad, New York, 1965, p. 637.
16 M. D. Armstrong and U. Stave, *Metabolism*, 22 (1973) 561.
17 P. Soupart, in J. T. Holden (Editor), *Amino Acid Pools*, Elsevier, Amsterdam, 1962, p. 220.
18 J. Dancis, J. Hutzler and M. Levitz, *Pediatrics*, 32 (1963) 234.
19 J. A. Schneider, K. Bradley and J. E. Seegmiller, *Science*, 157 (1967) 1321.
20 M. Gupta and K. N. Agarwal, *Br. J. Nutr.*, 29 (1973) 151.
21 H. H. Tallan, S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 927.