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GENERAL PROBLEMS ASSOCIATED WITH THE ANALYSIS OF AMINO ACIDS BY AUTOMATED ION-EXCHANGE CHROMATOGRAPHY

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1 INTRODUCTION

It is now almost 30 years since the first automated procedure for the analysis of amino acids by ion-exchange chromatography was published[1]. Although gas–liquid chromatography and high-performance liquid chromatography (HPLC) using microparticulate stationary phases have attempted to rival this system, it still remains the most popular[2,3]. The main reason for this is that the original method has been much modified with dramatic improvements in speed and sensitivity of the analysis due to improved ion-exchange resins. Further improvements have resulted from the development of automatic sample loading, function timing and data processing. The present review does not provide an account of the historical development of the technique, since this has been described many times, most recently by Tristram and Rattenbury[4]. Nor does it review the many modifications to the original procedure, since these have been ably reviewed by others[5]. Furthermore, many of the modifications have involved changes to the buffer systems to solve problems of resolution specific to particular instruments, many of them no longer in production. Similarly, many problems are caused by instrument failure, and although important, are again specific to particular instruments and outside the scope of this review.

The present review is intended to highlight the problems that still exist, many

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of which are independent of the instrument and in some cases the technique used. Some of these problems are more obvious than others; for example, it is generally considered that the hydrolysis of proteins[2,3,5,6] and the deproteinization of physiological fluids[2,3,7] are still major problems in amino acid analysis. Other problems are not quite so obvious and may be overlooked, either because they were in the older literature or because they were not a major concern of the report in which they appeared.

2 SAMPLE PREPARATION AND STORAGE

2.1. Previous treatment of the subject

Problems in this category are usually associated with the analysis of the urine and blood of human subjects, either those with suspected inborn errors of metabolism or those receiving antibiotics as medication for urinary infections Some antibiotics can pass unchanged through the body, appear in the urine or plasma, react with ninhydrin and interfere with the resolution of amino acids. Often ninhydrin-positive degradation products may be formed, either in the body or during the collection of urine into an acid preservative, or while in the very acidic initial buffers of amino acid analysers.

This type of problem was first encountered by Perry *et al.*[8] who found that a sample of urine oxidized with hydrogen peroxide gave an unusual peak eluting near cysteic acid and homocystine on an amino acid analyser. They concluded that the peak was D-penicillamine sulphonic acid, formed by oxidation of the antibiotic D-penicillamine, which had been given to the patient from whom the urine had been collected. In another study[9] in which cystinuric patients had been given D-penicillamine orally, it was found that non-oxidized urine and plasma contained D-penicillamine disulphide (PSSP) and L-cysteine-D-penicillamine disulphide (CSSP). Both of these compounds were detected on an amino acid analyser and were eluted close to each other and to cystine and methionine. Two different analysers were used, one with gradient elution and one with stepwise elution; the former gave the best resolution.

Holmgren *et al.*[10] found two large additional peaks in the arginine region of the chromatogram of urine of patients given ampicillin (α-aminobenzylpenicillin), but they made no comment other than to state that the problem was under investigation. However, Stegink *et al.*[11] found considerable interference from several peaks when the urine and plasma of patients given ampicillin were analysed. In the urine, a small peak was found in the cystathionine position, a larger peak overlapping isoleucine, a number of peaks between β-amino isobutyric acid and ethanolamine, and two peaks between histidine and arginine possibly analogous to those reported by Holmgren *et al.*[10]. Similar smaller peaks were found in the plasma which, like those in the urine, disappeared as soon as ampicillin treatment was stopped. Unsuccessful attempts were made in this study to identify the peaks, but Nyhan *et al*[12] showed that one peak, eluted after β-aminoisobutyric acid, was ampicillin itself. The most detailed study of the problem arising from ampicillin was reported by Perrett[13]. Analysis of a fresh solution of ampicillin gave a major peak in the region of β-aminoisobutyric acid and another in the basic region of the chromatogram, together with a number of minor peaks. With an accelerated system using a short column, only one peak, corresponding to ampicillin, was observed. When this system was

used to analyse fresh samples of urine and plasma from patients receiving ampicillin, the major component detected was again ampicillin, with only trace amounts of degradation products. However, incubation at 60°C of ampicillin in the starting buffer (pH 2 88) of the amino acid analyser resulted in the degradation of ampicillin and the appearance of the degradation products on the amino acid analyser. Such degradation would probably also occur during the collection of urine into an acid preservative

Similar observations have been made with calves given ampicillin by intramuscular injection at a level of 7 mg/kg body weight (Williams and Cockburn, unpublished results). Additional peaks were observed between histidine and arginine, before ammonia and near methionine in blood samples taken 3 h after dosage. In samples taken 8 h after dosage there was no trace of the additional peaks, which agrees with the manufacturers' information (Beecham Animal Health, Brentford, U.K.) that ampicillin is normally excreted within 6–8 h after injection.

More recent studies by Roesel *et al.*[14] have shown that administration of another antibiotic, metheneamine mandelate, caused a marked increase in urinary histidine on a Beckman 120C amino acid analyser. Analysis of the pure antibotic and of its parent compound methenamine gave two large and identical peaks in the position normally occupied by ammonia and histidine. When methenamine was added to a standard amino acid mixture, these peaks were co-eluted with ammonia and histidine and increased their peak sizes by 54 and 476%, respectively. When run on another amino acid analyser, the Jeol 6AH, marked increases were observed for the ammonia and ornithine peaks.

Clearly care is needed, particularly in studies of inborn errors of amino acid metabolism, to ensure that the subject is not receiving antibiotics when urine or plasma is collected for analysis. Similar care is needed in studies of the nutrient requirements of farm animals if blood amino acids are being measured[15]. If such medication is unavoidable then sufficient time should be allowed to elapse for the antibiotic to be excreted before blood sampling.

Another problem involving the prior treatment of the subject providing the sample has been described by Bech-Andersen[16]. Chromic oxide is often added to the diets of animals as a quantitative marker of digesta flow in digestibility trials. Acid hydrolysis of feed, digesta or faeces, prior to amino acid analysis, converts the chromic ions into chromium(III) cations, which bind to the ion-exchange resin and cause impaired resolution of the amino acids. Bech-Andersen[16] suggested using a special "flushing buffer" of low pH (2.3) and high sodium ion concentration (1.1 *M*) after the second buffer in his gradient elution system and the acidic and neutral amino acids had eluted. This procedure overcame the loss of resolution due to the chromic ions, but two additional buffers were required for the basic amino acids and the analysis time increased by 45 min. The addition of EDTA to the sodium hydroxide regeneration buffer can also alleviate this problem.

2.2. Non-protein components of the sample

It has long been evident[17] that it is desirable to remove non-protein substances from foods and feeds prior to their hydrolysis, since lipids, carbohydrates and nucleic acids can affect the accuracy of the amino acid analysis. Effective extraction procedures are lengthy, but there are alternative procedures for minimizing amino acid

destruction during hydrolysis in the presence of carbohydrate. For example Robel[18] studied the effect of the volume of 6 *M* hydrochloric acid used for hydrolysis of corn and found that an acid:carbohydrate ratio of 2800:1 was necessary for optimum amino acid recoveries. More recently, Fong and O'Dor[19] showed that hydrolysis of bovine serum albumin in the presence of D-mannitol, a sugar alcohol, not only caused losses of glutamic acid, proline, glycine and alanine, but resulted in the formation of new compounds which appeared on the chromatogram. These compounds, formed by the reaction of D-mannitol with these amino acids, gave peaks that interfered with the resolution of aspartic acid, threonine and serine. Fong and O'Dor[19] were interested in the effects of D-mannitol on the amino acid composition of marine algae and considered that previously published results should be treated with caution, although they gave no advice on the removal of this interference. In this case, the non-protein constituent, mannitol, was actually a natural component of the sample. However, D-mannitol is used extensively in the food industry (*e.g.* as a flavouring agent and sweetener) and clinically as a diagnostic aid for kidney function, and consequently may occur unexpectedly in certain samples.

Paddock *et al.*[20] showed that hydrolysis of protein-free mixtures of nucleotides, nucleosides and nucleic acids resulted in the formation of amino acids, with glycine being the main amino acid detected and accounting for between 47% and 97% of the amino acids produced. This finding explained the results of an earlier study by Wilson *et al.*[21], in which a sub-fraction of the dialysable portion of extracts of human leucocytes had been found to contain a substantial amount of glycine. The authors concluded[21] that this was formed during acid hydrolysis of hypoxanthine, a major component of the human leucocyte extracts. Paddock *et al.*[20] suggested that nucleic acids and their components should be removed before hydrolysis although no suitable procedure was recommended. Block[17] reported that nucleic acids could be removed from lipid-free tissues by digestion with 10% sodium chloride at 85°C for 6 h: the sodium chloride could be removed by washing with water and the residue dried with hot acetone. Since nucleic acids are widely distributed in such diverse samples as physiological fluids[20], rumen bacteria and ruminant digesta[22], care is needed when carrying out amino acid analysis on such samples: a larger than normal glycine content suggests the possibility of interference from nucleic acids.

2.3. Purification of the sample

The separation of protein from non-protein components is often desirable, but there are also situations where it is necessary to separate mixtures of proteins and polypeptides prior to amino acid analysis. Examples are for the determination of molecular weight and the study of protein patterns in pathological conditions. One of the most widely used methods of achieving such separations is polyacrylamide gel electrophoresis. However, it has been shown[23] that the polyacrylamide gels can contribute to the apparent amino acid composition of the proteins eluted from them. Thus, the eluate from blank gel slices contained small, but significant, amounts of most common amino acids which were particularly important at low protein·gel ratios. The source of these amino acids could not be found in any of the reagents used, although it was shown that incorrectly prepared dialysis tubing could also contribute to the background. Fortunately the amounts of amino acids in the gels were reproducible and linearly related to the volume of gel eluted, so that it was possible to

apply a correction based on the analysis of eluted blank gels when the contamination contributed more than 10% to the amino acid content. It was stressed that the contamination level should be determined for each mixture of proteins examined.

One possible source of contamination investigated in this study[23] was the cleanliness of glassware, no doubt prompted by reports[24,25] of contamination from contact with the analysts' fingers. A broad spectrum of free amino acids was found when the surfaces of human fingertips were soaked in pH 2.2 citrate buffer[25]. The relative abundance of the free amino acids showed little variation but the amounts increased after acid hydrolysis and many unidentified ninhydrin-positive peaks were found[24]. Traces of free serine, glycine, urea and ammonia were found on extraction of laboratory dust with citrate buffer and a larger number of amino acids occurred in hydrolysates of these extracts[25]. Later Hamilton and Myoda[26] published a procedure for the cleaning of glassware which is very time-consuming and probably not carried out in most laboratories. Certainly contamination from sweat and dust is much more serious now than in 1965 because of the marked increase in the sensitivity of amino acid analysers. Other sources of contamination, for example in the reagents used for amino acid analysis will be discussed in Section 3.

2.4. Storage

Perry and Hansen[27] studied the effects of various factors in the preparation of blood plasma on the accuracy of amino acid analysis. They found that storage of deproteinized plasma for 3 months at −20°C led to reductions in the levels of glutamine and tryptophan similar to those observed by Dickinson *et al.*[28]. Since glutamic acid concentrations increased, it appeared that the glutamine had been slowly hydrolysed and it was therefore recommended[27,28] that plasma samples should be stored at −68°C or lower if there is likely to be a long delay before analysis. Olek *et al.*[29] also reported that deproteinized serum could be stored at −30°C for up to one month without change in the concentration of most amino acids, except for aspartic acid and glutamic acid which increased markedly, presumably owing to hydrolysis of aspargine and glutamine. Similar observations were made for free amino acids extracted from maize leaves by Wünsch[30], who carried out analyses at intervals for up to 450 days on samples stored at −30°C.

Blood can also be stored without deproteinization although this is clearly unwise. Perry and Hansen[27] found that storage of blood or plasma at −20°C for 7 days before deproteinization led to marked reductions in the concentrations of the disulphide amino acids. Smaller reductions were also observed on storage at room temperature for several hours. Thornber *et al.*[31] found that concentrations of glutamine and cysteine decreased markedly in plasma stored frozen before deproteinization for up to 72 days. In particular, plasma-free cysteine is extensively bound to plasma proteins (mainly albumin) if deproteinization is not carried out rapidly after blood samples have been obtained. Malloy *et al.*[32] have reported a method for the measurement of free and bound cysteine in plasma. The plasma-free cysteine is estimated after immediate removal of the blood cells followed by rapid deproteinization by sulphosalicylic acid (SSA). The protein-bound cysteine is determined on the protein precipitate after reduction with dithiothreitol.

Although Olek *et al.*[29] found no change in cysteine levels in whole blood stored at room temperature before deproteinization, most other amino acids showed

marked increases or decreases in concentration. Differences were observed for serum stored under similar conditions, with changes in cysteine, aspartic acid, glutamic acid and serine concentrations. Liappis *et al.*[33] reported marked but variable changes in amino acid concentrations in blood plasma stored under blood bank conditions, *i.e.* after sterilization, at 4°C, with anticoagulant [acid citrate dextrose (ACD)] added and analysed, after deproteinization, at regular intervals up to 30 days. The concentrations of most amino acids increased, except those of arginine, methionine and glutamine which decreased. If the anticoagulant was omitted the amino acid concentrations were 2–5 times higher.

Although ACD is used as an anticoagulant in blood bank samples, it is more common to use either heparin or ethylenediaminetetraacetic acid (EDTA). Perry and Hansen[27] found that one batch of tetrasodium EDTA resulted in the appearance of two major ninhydrin-positive compounds on the chromatogram when the plasma was analysed. One compound was eluted with taurine and the other between methionine and isoleucine. Other batches of EDTA did not produce the same effect, but recently Parvy *et al.*[34] reported the appearance of an unusual peak that eluted in the same position as methionine sulphoxide when they collected blood samples in sterile Vacutainer tubes (Becton Dickinson, Oxford, U.K) containing tripotassium EDTA. Further studies using distilled water instead of blood in the tubes revealed the same peak but it was absent from tripotassium and disodium EDTA unless their saturated solutions were heated at 175°C for 2 h in sealed tubes. This led to the appearance on the chromatogram of large amounts of the unidentified compound plus some additional peaks. A possible explanation was the formation of α-amino compounds during the thermal decomposition of EDTA. Whilst recommending care in the use of EDTA as an anticoagulant, Perry and Hansen[27] did not encounter similar problems with heparin and it is possible to purchase Vacutainer tubes containing heparin. Although there is some evidence[35] to suggest that lysine and arginine may be weakly bound to heparin, this is unlikely to affect seriously the amino acid analysis of blood plasma.

2.5. Deproteinization

In the one collaborative trial on the determination of free amino acids in blood plasma[36] the precision reported was much poorer than for protein hydrolysates. It was concluded that this was due mainly to the deproteinization procedure. The most widely used method of deproteinization is precipitation with SSA followed by centrifugation to remove the precipitated protein. After adjustment of pH, which must be close to that of the standard calibration mixture[37], the sample is ready for analysis. The major problem is that resolution in the region of threonine and serine[36,38] and in other areas[39] can be adversely affected, particularly if the amount of SSA added to the column exceeds 200 mg. Comparison of the SSA with other acid precipitants has yielded contradictory results. Picric acid is sometimes preferred[38,40,41], though others[42–44] have found little difference between the use of SSA, picric acid or trichloroacetic acid (TCA). Acid precipitation procedures lead to over-estimates of free tryptophan owing to the release of protein-bound tryptophan from the plasma albumin. Heparin interferes with the binding of tryptophan to albumin and EDTA may be a better anticoagulant[45]. Other methods of deproteinization exist, *e.g.* ultrafiltration[46] and high speed centrifugation[40], these avoid acid precipitation but do

not lead to complete removal of protein[31] and have not been widely adopted.

Thornber *et al.*[31] suggested the use of equilibrium dialysis against citrate buffer as a rapid and simple method of deproteinization. The method was evaluated with synthetic mixtures of known amounts of amino acids, and losses of free amino acids were low, in the range 0.0–8.5% (mean 4.6%). The dialysate could be stored at 0°C for up to 10 weeks with the loss of only tryptophan and could be applied directly to an amino acid analyser. Although promising, this method does not appear to have been widely adopted. If the dialysate is analysed immediately to prevent losses the method provides a useful alternative to acid precipitation procedures

Deproteinization of tissues as well as physiological fluids is often necessary. Manchester[47] has shown the importance of the technique used for the extraction of free amino acids from heart muscle and subsequent deproteinization. Comparison was made of two techniques, one involving extraction of the frozen tissue with water at 100°C for 5 min followed by deproteinization with 5% (w/v) TCA at 0°C, and in the other the pulverized tissue was extracted and deproteinized directly with 5% (w/v) TCA at 0°C. The latter method resulted in markedly lower values for free valine, leucine, isoleucine, methionine and phenylalanine. Manchester[47] concluded that it was impossible to say which set of values represented the true content of free amino acids in tissues and that further studies were needed to re-examine methods of extraction. The same conclusion could be made regarding methods for extracting free amino acids from physiological fluids.

2.6. Hydrolysis

A great deal has been written on this topic in the past and it is not intended to add extensively to it in this review. In an excellent summary of the current situation Gehrke *et al.*[6] state that hydrolysis is the major limiting parameter for both the precision and accuracy with which the amino acid composition of proteins can be determined. Much the same conclusion was reached by Williams[2,3] in revieweing the many collaborative trials carried out on the amino acid analysis of proteins. The so-called "standard procedure" of heating the protein in 6 *M* hydrochloric acid at 110°C for 24 h is a compromise, since no one method can give satisfactory values for all the amino acids. For example, preliminary oxidation is required for the sulphur amino acids, tryptophan needs alkaline hydrolysis, and acid hydrolysis for varying periods, 24, 48 and 72 h, is required for isoleucine and valine (calculated to infinite time) and threonine and serine (calculated to zero time). All these factors need to be considered in relation to the composition of the sample and the analysis of foods and feeds since the presence of non-protein constituents, particularly carbohydrates, can have a major effect on the recoveries of amino acids (Section 2.2).

Many attempts have been and are still being made to solve these problems[6]. The reduction in analysis time achieved in recent years has enabled extensive studies, such as those of Mason *et al.*[48], to be attempted. The hydrolysis procedure recommended[48] was developed from an investigation published in a series of eight papers on the effect of such variables as the acid:nitrogen ratio, the use of sealed tubes *versus* open reflux, hydrolysis time and the stability of amino acids to oxidation with performic acid (with or without phenol) on the recovery of amino acids from feed proteins. Essentially this method consists of oxidation of the sample (10 mg N) with performic acid–hydrogen peroxide (5 mg phenol ml^{-1}) for 16 h at 0°C After removal

of excess oxidizing agents with sodium pyrosulphite and formic acid by rotary evaporation, hydrolysis was carried out by refluxing with 50 ml of 6 *M* hydrochloric acid (1 mg phenol ml^{-1}) at 110°C for 23 h with argon bubbling gently through the hydrolysate After cooling the pH of the solution was adjusted to pH 2.2 with sodium hydroxide and diluted with pH 2.2 sodium citrate buffer thus saving time and reducing losses of amino acids by avoiding rotary evaporation. Tryptophan would still have to be analysed separately and losses of tyrosine still occurred in spite of the addition of phenol as a halogen scavenger. It should be recognized that the method was developed as a rapid procedure for the analysis of feedstuffs where an accurate value for tyrosine might not be important. For other proteins this may not be true. The method was developed for adoption as a standard hydrolysis procedure for the EEC, and was the subject of a collaborative study[49] involving 30 laboratories. The results, with mean coefficients of variation between laboratories in the range 2.47–5.09% for repeatability and 5.09–10.56% for reproducibility, have been regarded as both successful[6] and unsuccessful[50], the latter because of the rejection of 25% of the data as outliers. A major problem with collaborative trials or the adoption of a standard method is to persuade laboratories to flow the procedure precisely without modification. This problem is illustrated by an attempt[50] to modify the procedure of Mason *et al.*[48] by reducing the high sodium content of the final solution, a factor that can cause resolution problems with some resins[50,51]. Hydrochloric acid was used instead of sodium pyrosulphite to remove the excess oxidizing agent, but this modification resulted in substantial losses of phenylalanine as well as tyrosine, losses which were probably due to the amount of chlorine produced being greater than the phenol could scavenge

Complete loss of tyrosine can also occur inadvertently through oxidation, as found by Walker *et al.*[52] when proteins, which had been stored in phosphate-buffered saline containing 0.1% (w/v) sodium azide as a preservative, were hydrolysed in 6 *M* hydrochloric acid. In this investigation phenylalanine was completely, and histidine largely destroyed and the resolution of other amino acids was impaired by the production of artefacts. It was shown that the sodium azide could be removed by dialysis against distilled water. This report highlights another source of problems that may arise when an analysis is being carried out for other scientists, *i.e.* the previous treatment of the sample.

A detailed study of hydrolysis conditions was carried out by Gehrke *et al.*[6]. They compared the use of 6 *M* hydrochloric acid in sealed glass tubes with Teflon-lined screw caps as hydrolysis vessels at 110°C for 24 h. The sealed tubes were also used at a higher temperature (145°C) for a shorter time (4 h). Results for a variety of feeds and pure proteins showed good agreement between the methods. The latter method also gave higher isoleucine and valine, but lower threonine and serine values, but was simpler to use which the authors considered an advantage in terms of improved precision, a conclusion supported by a small-scale inter-laboratory evaluation. The sulphur amino acids still required performic acid oxidation prior to hydrolysis and it was recommended that hydrolysis should be carried at two different times for threonine, serine, valine and isoleucine.

Several attempts have been made to develop a single hydrolytic procedure for all of the common amino acids in proteins including tryptophan and the sulphur amino acids. These include the use of 3 *N* *p*-toluenesulphonic acid[53], methanesul-

phonic acid[54,55], mercaptoethanesulphonic acid[56] and, more recently, the addition of pyridine borane to hydrochloric acid[57]. These methods have been developed for the hydrolysis of pure proteins and have not been successfully applied to foods or feeds where the presence of carbohydrate causes losses of tryptophan. Other problems have been observed and care needs to be taken, even when used for pure proteins. Gardner[58] found that hydrolysis with mercaptoethanesulphonic acid can result in elevated proline values due to the production of a ninhydrin positive compound, presumed to be cysteine, which was eluted with proline. No solution was offered to this problem which, as shown by Gardner[58], can also occur with hydrolysis in hydrochloric acid (particularly in the presence of mercaptoethanol or thioglycollic acid) if the conditions recommended by Moore and Stein[59] (*i.e.* the reconstitution of hydrolysates at pH 6.5 in air before adjusting the pH to 2.2) are not followed. Pyridine borane reduces tryptophan to dihydrotryptophan, which can be measured on an amino acid analyser with the other amino acids. On one amino acid analyser (Technicon) used by Wong *et al.*[57], the dihydrotryptophan was eluted as a single peak, well resolved from phenylalanine and lysine, but when a Dionex D-500 analyser was used, twin peaks were observed between lysine and histidine. When the final column temperature was reduced from 67.2°C to 64°C one of the dihydrotryptophan peaks co-eluted with lysine. This type of problem will be discussed further in Section 4.1.

One advantage of some of the newer hydrolysis procedures[48] is that the removal of the hydrolytic reagent after hydrolysis can be avoided. Normally this is carried out by rotary evaporation, which can result in losses ranging from 0.16% (arginine) to 80% (threonine)[60], although it seems likely that such losses may be due to adsorption of amino acids onto the inside of the hydrolysis tubes, a problem that might be overcome either by eliminating the evaporation stage by adjusting the pH to 1.2 with pH 2.2 sodium citrate buffer or by washing the hydrolysis tubes with 6 *M* hydrochloric acid after transferring the hydrolysates for rotary evaporation[60]. Much greater losses of amino acids due to rotary evaporation *e.g.* over 50% of ornithine, lysine and methionine, were reported by Dawson and Mopper[61]. These authors considered this to be due partly to incomplete redissolution of the sample residue from the walls of the glass flask or to wall-induced condensation reactions. The addition of a small volume of glycerine to coat the walls of the flask before evaporation was recommended to solve this problem[61].

Another alternative procedure to rotary evaporation is to dry the hydrolysates *in vacuo* over sodium hydroxide and phosphorus pentoxide. This was a very popular method until Ikawa and Snell[62] reported that losses of glutamic acid and serine occurred during this procedure due to the formation of O-(γ-glutamyl-serine). However, the method is still used by other workers[58,63] without such problems.

3 CONTAMINATION OF REAGENTS

3.1. Buffer reagents

The modern amino acid analyser is so much more sensitive than its predecessors that contamination has become a much more serious problem. A major source of contamination is the water used to prepare the buffer solutions. Most analysts use deionized water yet it has been known for some time that this can be contaminated by amino acids from the ion-exchange resins used. Steven and Tris-

tram[64] reported trace amounts of several amino acids in deionized water and in solutions of non-protein components of their samples undergoing desalting by ion-exchange. They considered that such contamination would have a minimal effect on the results of analyses of protein hydrolysates, but they were using paper chromatography, a technique much less sensitive than modern methods. Another possible source of amino acid contamination is microbial synthesis. Hamilton and Myoda[26], using an amino acid analyser, found that samples of distilled, conductivity, and bottled sterile water were contaminated with considerable amounts of both free and bound amino acids. This was attributed to microbial contamination, probably by *Pseudomonas*, and they described a system for preparing water free from such microbial contamination[26]. The equipment used may not be available in all countries, and has probably been superseded by the newer system of reverse osmosis, which is claimed to remove 99.9% of particles, bacteria, colloids and organics. Certainly reverse osmosis should be preferred to deionization, since Briddon and Hunt[65] found that the use of deionized water in their buffers led to losses of basic amino acids, particularly arginine, in samples of physiological fluids. Further investigations showed this to be due to the presence of (i) *Pseudomonas*, which exhibits di-hydrolase activity for arginine and decarboxylase activity for ornithine and lysine, and (ii) *Bacillus licheniformis* which decarboxylates arginine. This problem was overcome by autoclaving the distilled water before deionization, and adding sodium azide as a preservative to the sodium hydroxide used for regeneration of the ion-exchange resin. It seems strange that the autoclaving stage should have proved effective since it took place before deionization which has been shown to be a source of contamination[26,64]. It is also surprising that *Pseudomonas* should be a source of some amino acids[26] and yet cause losses of others[65].

Microbial contamination of reagents can cause problems of another sort. Atkin and Ferdinand[66] found a gradual loss of resolution in the valine to leucine region of the chromatogram when using lithium citrate buffers. Examination of the buffers revealed small clumps of material tentatively identified as a species of penicillium, and traces of a fungal-type material were found in the aqueous BRIJ35 added to the buffers as a wetting agent. The problem was cured by using solid BRIJ35 in the preparation of buffers, by the addition of caprylic acid and pentachlorophenol to the buffers as preservatives, and by the introduction of pre-column filters. Modern amino acid analysers frequently have refrigerated cabinets for the storage of buffers which can also reduce microbial growth. Atkin and Ferdinand[66] described a method for the rejuvenation of the contaminated resin: the buffer bottles, buffer tubing and sample tubing should be thoroughly washed with 3% (w/v) sodium hypochlorite solution ensuring that this does not come into contact with the resin.

The chemicals used for the preparation of buffer solutions can also be sources of amino acids. According to one of the main suppliers (Pierce, Rockford, IL, U.S.A.) the main source of such contamination is citric acid. No evidence is given to support this view but it is certainly very possible. Hamilton and Myoda[26] found total amino acid concentrations in the range 2.5–73 μg/l of hydrochloric acid from several different commercial sources. Aspartic acid, threonine, serine, glutamic acid, glycine, alanine, isoleucine and leucine were all found. Samples of ammonium hydroxide were also found to be contaminated although this reagent is rarely used in amino acid analysis. It was recommended that the hydrochloric acid should be mixed with an

equal volume of water and distilled slowly through a column packed with Raschig rings to reduce the contamination to less than 1 $\mu g/l$[26].

3.2. Amino acid standards

A major concern in amino acid analysis is the reliability of the commercially-prepared standard solutions of amino acids used for calibration. If the concentrations of amino acids in these standards are incorrect, owing either to the presence of impurities, or to incorrect preparation or storage, then the results obtained for the samples will be in error. It is possible, of course, to prepare one's own standards and many laboratories do so, but Bolinder[67] showed that even amino acids labelled as chromatographically pure contained impurities, usually other amino acids. Microbiological assay was used to examine 266 amino acids from eight well-known suppliers. Of these only about 100 were not contaminated, 70 had low levels of contamination (less than 0.01%), 68 had medium levels (less than 0.10%) and 25 had high levels (more than 0.10%) Particularly bad were the samples of lysine containing 5.5% histidine, arginine containing 0.68% lysine, leucine containing 0.56% methionine and phenylalanine containing 0.36% tyrosine. The situation may possibly have improved since 1970, with improvements in the manufacture of amino acids. However, there is little published information on this topic, and many analysts are concerned about the reliability of their calibration mixtures. Ambler[63] compared three commercial mixtures with one prepared by a colleague and concluded that one commercial sample differed markedly from the others both in average concentration and in consistency between individual amino acids. He suggested the use of calibration standards from several sources for maximum accuracy

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4.1. Resolution of amino acids

Since the introduction of automated amino acid analysers in 1958, the time taken for analysis has fallen from 24 h to 1 h or less[3]. The chromatogram is thus greatly compressed and probably none of the accelerated systems resolves all the common amino acids quite as well as the first automated system with consequent reductions in accuracy[63,68]. Even if only the common amino acids are present in the sample, there are likely to be problems of resolution owing to variations in resins requiring the manipulation of buffer pH and column temperature.

The problem becomes more severe with more complex samples as Cole and Libadia[69] have recently demonstrated with urine and serum samples when changing from sodium to lithium citrate buffers using the same analyser. Although the lithium system adequately resolved all 39 amino acids in a commercial calibration standard when applied to physiological samples much poorer resolution was obtained. In particular glycerophosphoethanolamine and taurine, homocitrulline and methionine, and penicillamine and proline, were poorly resolved, and cysteine–penicillamine mixed disulphide and penicillamine disulphide were not resolved from cystine. The detection and treatment of metabolic disorders was made more difficult by this problem. The occurrence of unusual amino acids or related compounds is also becoming much more common. These can remain undetected in accelerated systems and cause inaccuracies by adding to the value of the amino acid from which they are inadequately resolved.

Resolution can also be affected by the formation of additional peaks during the analysis. This can occur for a variety of reasons, not least because of incomplete washing of the sample tubes during the loading of the sample. This can give rise to artificial or ghost peaks for each amino acid, a problem that was common[70,71] before the advent of automatic sample loading. An early example of the formation of multiple peaks during chromatography is the conversion of methionine into methionine sulphone and the two isomeric methionine sulphoxides, if the antioxidant thiodiglycol is not added to the initial eluting buffer[72].

Similarly, Cockburn and Williams[73] reported that 2-aminoethylphosphonic acid was eluted as two incompletely resolved peaks, probably owing to the formation of a degradation product or isomer on the ion-exchange column. The resin, pH of the buffer and column temperature were all contributory factors. At low pH (2.14–2.50) and at a column temperature of 65°C the two peaks were eluted together.

The formation of new compounds can also occur before the chromatography stage. Smyth *et al.*[74] carried out a study on the use of N-ethylmaleimide (NEM) for the chemical modification of proteins, and showed that its condensation product with the thiol group of cysteine (cysteine–NEM) appeared as two peaks on an amino acid analyser. It was concluded that this was due to the formation of diastereoisomeric forms, a phenomenon also observed by Bowie *et al.*[75] in a study on the intracellular cystine content of human fibroblasts.

The formation of isomers can also occur while the sample is awaiting analysis in an automatic sample loader. Samples may therefore stand for 24 h or longer in pH 2.2 citrate buffer. Such conditions have been shown[76] to result in the formation of three isomeric forms of (2*S*,3*R*,4*R*)-4-hydroxyisoleucine, the principal free amino acid of fenugreek seed. Three peaks appeared on quite different parts of the chromatogram, but only one peak was observed when the sample was loaded in deionized water at pH 5.5. Clearly another solution to this problem would be to analyse such samples without delay. Cusworth and Westall[77] observed a similar problem of storage in acid conditions which they turned to advantage. In weak acid solution, arginosuccinic acid, an amino acid excreted in the urine of mentally defective children, is converted into two anhydrides owing to cyclization. Amino acid analysis showed that these anhydrides and the unreacted acid appeared as three well-resolved peaks. Unfortunately the amount of the anhydrides formed was variable. To overcome this problem, samples of urine or deproteinized plasma were adjusted to pH 2 and allowed to stand at room temperature for 48 h before analysis. Arginosuccinic acid itself disappeared and a correction factor was applied to compensate for the 75% recovery of the acid as the anhydrides.

Kasai and Sakamura[78] found that a peptide, γ-L-glutamyl-L-aspartic acid, found in certain seeds, was eluted as two peaks on an amino acid analyser. Manual loading of the column appears to have been used since the sample, in 0.2 *M* sodium citrate buffer at pH 2.2, was washed in with 1 ml of 0.01 *M* hydrochloric acid. The appearance of two peaks was attributed to differences in sample to buffer ratio and in the volume of sample applied to the column, since a single peak was obtained under certain conditions However, the reasons for this were not clear, and similar glutamyl peptides were eluted as single peaks.

Resolution problems due to the presence of extra peaks can occur, following some extraction procedures carried out to isolate the samples to be analysed. For

example, Joy *et al.*[79] used organic buffers, such as tricine, during a study of amino acids from pea leaf chloroplasts. This resulted in the elution of aspartic acid as two peaks, and in the distortion of other peaks in this region. No explanation could be given for this dramatic effect, which occurred with both lithium and sodium citrate buffers but not with all the organic buffers studied. The pH of the sample when loaded, ideally pH 2.1, was considered important and other examples of the distortion of aspartic acid associated with sample pH have been reported[37,80].

4.2. Incorrect identification of amino acids

Ambler[63] considered that major errors in published amino acid compositions are sometimes caused by incorrect identification of peaks. An example was given where a peak identified as cystine was really valine, and the reported value for valine which was, unusually, lower than cystine, was derived from a peak which was a buffer-change artefact. Cystine was either absent or had been eluted with the alanine peak. Mackie[81] also found that Ibrahim and Ingalls[82] had incorrectly identified 2-aminoethylphosphonic acid (AEP) and another peak attributed to phosphoserine Mackie[81] showed that AEP was eluted early in the chromatogram rather than at the end as reported by Ibrahim and Ingalls[82]. It is also clear that the correct elution time for AEP had already been well documented. The need for care in identifying peaks has also been stressed by Beckerton *et al.*[83], who detected a new peak in deproteinized sheep plasma that eluted at the same time as lysine. Careful inspection of the chromatogram revealed that two compounds were being eluted together, lysine and the unexpected amino acid N^{ε}-monomethyllysine. The chromatographic conditions were therefore changed to resolve these two peaks and ornithine from one another.

5 CONCLUSION

Some of the problems of amino acid analysis can be readily avoided if the analyst is aware of them. Thus care should be taken in the use of antibiotics, and in the storage of samples where physiological fluids are involved. It is also necessary to be aware of the non-protein components of the samples, particularly with protein hydrolysates, and if necessary to extract the interfering compounds.

There are however certain problems that are less readily avoided and these include the resolution and correct identification of amino acids. As modern instrumental procedures become faster, and the amount of material required for analysis becomes smaller, such errors are generally becoming more difficult to recognize. Baltes[84] has highlighed these problems with examples of costly mistakes, in areas other than amino acid analysis, which he suggested could be even more catastrophic when instruments were linked to computers for data processing. This situation may not be much improved by the more recent application of HPLC techniques to amino acid analysis, particularly those using pre-column derivatization. Somack[85] suggested that many laboratories using phenylthiohydantoin derivatives were unable to reproduce published procedures owing to confusion in the position of the peaks, particularly of aspartic acid, glutamic acid, histidine and arginine. Others[86,87] have also reported poorer resolution and recoveries with high-performance liquid chromatography (HPLC) in comparative studies with ion-exchange chromatography. Even some instrument manufacturers[88] conclude that HPLC is currently attractive only for applications not requiring extreme accuracy.

Some other problems could be solved by the appropriate commercial companies, if improvements could be achieved in the quality of reagents and calibration standards used in amino acid analysis. This aspect requires vigilance by the analyst and constant feed-back to his suppliers if contamination is detected.

Unfortunately, major problems arising from deproteinization and hydrolysis remain, and are unlikely to be solved in the near future. The most effective procedure for deproteinization appears to be equilibrium dialysis[31] though further investigation is required. The problems of hydrolysis, particularly those of the sulphur amino acids and tryptophan, may never be adequately resolved although greater efforts at standardizing existing methods would lead to useful improvements[6].

6 SUMMARY

Some of the general problems commonly encountered with the analysis of amino acids have been described. These include problems associated with the preparation and storage of samples prior to analysis and those associated with the analytical stage itself. The effects on the accuracy of the determination of amino acids have been discussed, together with possible solutions to some of the problems.

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