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The Dicarboxylic and Basic Amino-acids of Edestin, Egg Albumin and β-Lactoglobulin

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The various investigations made in this laboratory during the few years preceding 1938 on the amino-acid composition of leaf proteins have been briefly summarized elsewhere [Chibnall, Rees & Williams, 1943]. These served to convince us that many of the methods of analysis we were then using, in particular those for the dicarboxylic acids and the bases, could not be applied to better characterized proteins with the precision demanded by the Bergmann-Niemann generalizations on protein structure [Chibnall, 1942] until their respective advantages and limitations had been more clearly defined and incidental working losses drastically reduced.

In accordance with this view we have thoroughly explored the Foreman method for the separation of the dicarboxylic acids, and an account has already been given [Bailey, Chibnall, Rees & Williams, 1943] of the various experiences which led us ultimately to devise a satisfactory procedure. This is incorporated in the general scheme of analysis for the dicarboxylic and basic amino-acids which is described in the present paper. Our aim has been to isolate these two groups of amino-acids as pure derivatives and in quantitative yield by step-wise removal without overall loss, and to overcome the solubility factors inherent in all precipitation methods it was necessary to remove also, at the appropriate stages, a large part of the monoamino-acids and proline. In formulating a procedure for doing this we have been guided to a large extent by our experience of the butanol method of Dakin [1918; 1921; 1923] and of the Cu salt method of Town [1928; 1936; 1941]. To anyone who has had experience of the earlier Fischer methods of esterification and distillation the elegance of the Dakin procedure needs no emphasis, for the monoamino-acids are obtained in the convenient form of a white crystalline powder free from both proline and hydroxyproline. In this laboratory Dakin's method has been applied to myosin [Sharp, 1939] and to cocksfoot protein [Lugg; *vide* Chibnall, 1939, p. 162], the esters subsequently being distilled under low pressure through a modern fractionating column. A certain degree of success was obtained, but the method was not proceeded with for two reasons. In the first place we found that small amounts of the dicarboxylic acids and bases were extracted by the butanol. Secondly, we found that extensive diketopiperazine formation occurred, even when the butanol extraction was carried out under reduced pressure, leading to losses amounting to several per cent of the total protein-N. The meagre data quoted by Dakin [1923] in his analysis of zein suggest that much of the N that he was unable to account for was located in the butanol-proline fraction. Our experience of the Cu salt separation has been the same as that of Town himself, in that we have found that the solubility of one particular salt or group of salts can be profoundly influenced by the presence of others. The method must therefore be used with discrimination, and unless circumstances warrant, no exaggerated idea of the purity of the products isolated must be entertained. The procedure for the estimation of the dicarboxylic acids and the bases given below embodies our cumulative experience of these two methods and it leads, incidentally, to the separation of the monoamino-acids in excellent yield but in several fractions, each of which is a mixture. These could be used, either singly or collectively, for the estimation of the indi-

vidual components by the ester method, as had been our original intention, but the new partition chromatographic method of Gordon, Martin & Synge [1941; 1943], which is applied to the hydrolysate without any preliminary fractionation, has proved so strikingly successful that it is doubtful if our products will be required for this purpose.

EXPERIMENTAL

1. *Scheme of analysis*

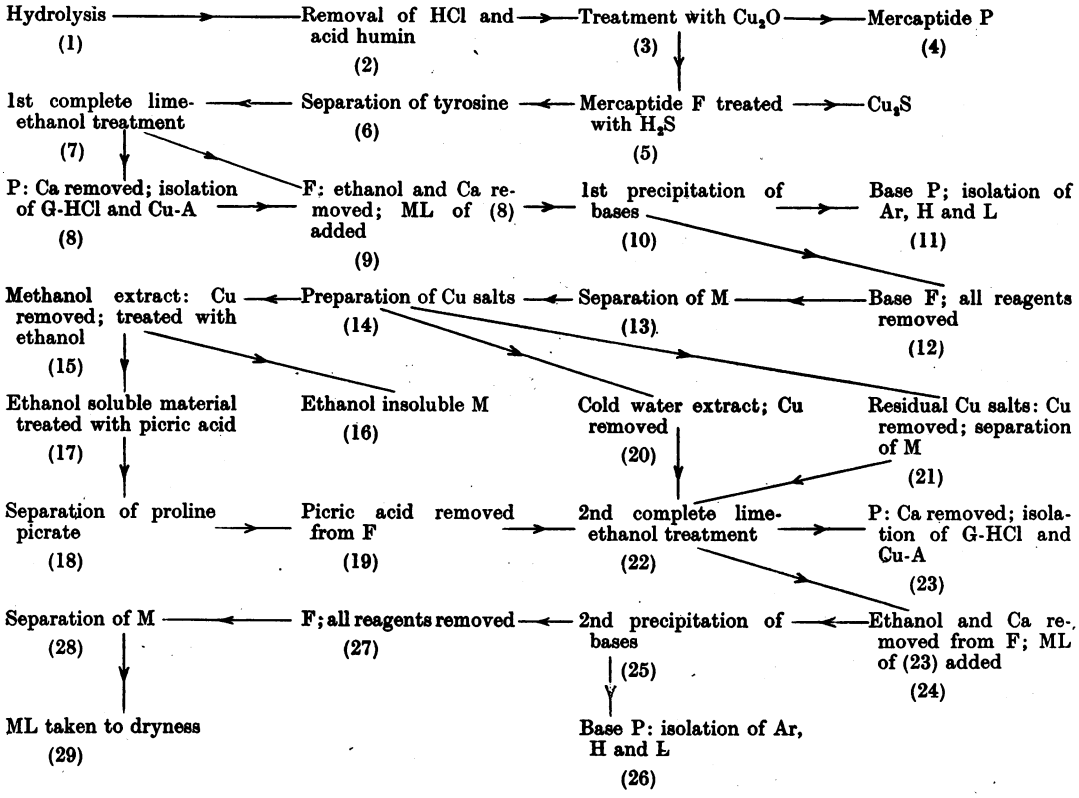
The cardinal point in the procedure is that no reagent may be introduced into the main hydrolysate unless it can be quantitatively removed at a later stage without appreciable loss of N, for in an extended scheme such as we have adopted the working losses are cumulative and can soon amount to several per cent of the total protein-N. It follows that certain reagents either cannot be used at all or only at a stage when there is reason to believe they comply with this provision.

Now it has been the experience of all protein analysts that if H_2SO_4 be used to effect hydrolysis and the mineral acid is subsequently removed with baryta, the resulting $BaSO_4$ obstinately retains N much in excess of that due to humin. We find nevertheless that after the removal of the cystine and the major part of the aspartic acid, i.e. at stage 9, Ba^{++} or SO_4^{--} can be introduced into the hydrolysate and removed in the usual way as $BaSO_4$ without appreciable loss of N, an observation which points strongly to the fact that the excess N carried down when this latter operation is conducted at an earlier stage represents these two amino-acids, very probably in the form of double salts with $BaSO_4$. Dakin [1921] mentioned the difficulty of completely removing Ba^{++} with H_2SO_4 from solutions of Ba hydroxyaspartate, but we are unaware of any corresponding citation for the two above-mentioned amino-acids. To avoid these large losses certain workers have preferred to hydrolyse the protein with HCl, remove the excess acid in the usual way

Diagram 1.

Scheme of amino-acid analysis.

Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate; Ar, arginine; H, histidine; L, lysine; M, mono-amino-acids; P, precipitate; F, filtrate; ML, mother liquor.



by evaporation *in vacuo* and then free the hydrolysate from Cl^- with Ag_2O. The precipitate of AgCl is much less bulky than the corresponding one of $BaSO_4$, and less excess N is retained, but in our experience it is still appreciable and may amount in some cases to as much as 4% of the total protein-N. Such losses are due to the insolubility of the Ag mercaptide of cysteine, even at fairly high concentrations of H^+ [Bailey, private communication], and of both Ag aspartate and glutamate, for at stage 9 Ag^+ or Cl^- can be removed as AgCl at *p*H 3 without loss of N. According to our experience CuS, Cu_2S and Ca oxalate do not retain N (except humin-N, if present); Cu^+, Cu^{++} and Ca^{++} therefore can be removed from the hydrolysate at any stage as these salts respectively. Lastly, if phosphotungstic acid be used to precipitate the bases, this acid can be removed from both precipitate and filtrate in the usual way with amyl alcohol-ether mixture without loss of N, provided that the aqueous phase be first saturated with ether and ether-water be used for all aqueous washings. If this precaution is not adopted the numerous aqueous washings soon reduce the ether content of the organic solvent mixture to such a low level that a small amount of N is extracted by the amyl alcohol on the Dakin principle.

All of the above-mentioned observations have been taken into account in devising the scheme of analysis set out in Diagram 1. The rationale is as follows. The protein is hydrolysed with HCl, the excess acid and the acid humin removed in the usual way and the clarified solution treated with Cu_2O (3) to precipitate the residual humin and the cystine as the cuprous mercaptide of cysteine. Following the separation of some crude tyrosine (6) the hydrolysate is passed through the first complete lime-ethanol treatment for the isolation of the dicarboxylic acids (7–8). The mother liquor from this is joined with the Ca-free filtrate (9) and the first precipitation of the bases is made with phosphotungstic acid (10); cystine and the major part of the aspartic acid having been previously removed the precipitate (11), according to our experience, consists almost exclusively of basic material. All reagents and the residual Cl^- are next removed from the base filtrate, which is then reduced to a small volume. On standing, a partial separation of the less soluble monoamino-acids (leucine, phenylalanine, tyrosine and methionine) occurs (13). The soluble products are then passed through the Cu salt treatment of Town, when additional amounts of the above-mentioned mixed amino-acids (21), a valine-*iso*leucine fraction (16) and a large part of the proline (18) are separated. These last few stages serve to reduce the monoamino- and imino-acid content of the hydrolysate to between one-third and one-fourth of that present at stage 7, and it is now ready for the 2nd complete lime-ethanol treatment (22). Following this a 2nd precipitation of the bases is made with phosphotungstic acid (25), and as the major part of the proline has been previously removed the precipitate (26) contains very little non-basic-N. All reagents are then removed from the base filtrate and the clarified solution reduced to a small volume, when further crops of monoamino-acids separate (28). The ultimate filtrate is taken to dryness *in vacuo* and granulated with acetone to give a fine white non-hygroscopic powder (29).

It will be seen that this extended procedure has enabled us to make a direct investigation, via stages 22 and 25, of the solubility factors operative during the first treatment for the dicarboxylic acids at stage 7, and the first treatment for the bases at stage 10 respectively. This is, of course, one of the essential features of the analysis, and as we determine the amide-N at stage 2 and the composition of the mercaptide precipitate at stage 4 we have knowledge of the gross weight and total N of the hydrolysis products present in all the various fractions. We are thus able not only to compute the overall losses and indicate from flow sheets at which stages these occur, but we can determine directly the gross weight of products given on hydrolysis of 100 g. of protein, a factor that can also be computed indirectly in terms of the total N and of that in non-peptide form [see Chibnall, 1942].

Needless to say all reagents employed must conform to certain standards of purity. $CuCO_3$ must be free from sulphate, chloride, and other metals; the commercial samples we have tested do not satisfy these requirements and we have prepared our own from A.R. $CuSO_4$. H_2SO_4 must be N-free. 12-Phosphotungstic acid must be not only N-free but also Na-free; few commercial products satisfy this latter requirement. The samples that we have used were not purified through ether by Winterstein's [1898] method as we were not specifically concerned with phosphotungstate solubilities [Van Slyke, Hiller & Dillon, 1942]. Ag_2O and Cu_2O must be washed free from salts and alkali, $Ca(OH)_2$ must be free from sulphate and iron; we have used a commercial sample but the preparation from Ca is recommended.

2. *Analysis of edestin*

An estimation of total N was made at every convenient stage in the analysis [Chibnall *et al.*, 1943] and flow sheets giving details of aliquots withdrawn were kept throughout so that factors were available to correct all values to the weight of protein (w/p) or to the total protein-N (T.P.N.) as the case may be. The numbers at the head of each section given below refer to the stage numbers in Diagram 1.

Details of analysis

1, 2. 61·741 g. of moisture- and ash-free protein, containing 11·52 g. of N or 18·65% were hydrolysed for 23 hr. with 400 ml. 20% HCl. The excess HCl was removed by evaporation *in vacuo* to a gum; water was then added and the mixture again reduced to a gum, the latter operation being repeated 6 times in all. Experience has shown that under these conditions all excess HCl is removed, and the amount present is no more than that required to bind the amino-acids and ammonia. Ammonia-N, obtained by distillation of an aliquot with alkali at *p*H 12·5 in a micro-Kjeldahl apparatus, was 10·42% T.P.N. This value is, of course, much in excess of the true amide-N, which is 9·49% [Gordon *et al.*, 1941; Chibnall, 1942]. The acid humin was too small to be separated, and it was removed later with the alkali humin in the mercaptide precipitate at stage (3). Data for the composition of the humin (Table 1) were obtained from a sample made by very cautiously treating an aliquot of the solution with lime to *p*H 7·5. Maximum precipitation was not obtained at *p*H 7 with NaOH. The separated humin was washed and dried; N and ash ($CaSO_4$) were determined.

3, 4. The main solution was treated with Cu_2O as described in a previous paper [Bailey *et al.*, 1943]. The Cu^+-free filtrate was colourless, showing that all humin had been removed. The precipitate contained S≡67·3% total protein-S and N≡2·20% T.P.N. The S is in excess of that due to cystine and must represent methionine. Colorimetric estimation before and after the precipitation showed that the precipitate contained by difference tyrosine N≡0·41% T.P.N. These two latter amino-acids and probably also very small amounts of phenylalanine and leucine must have separated, as such or as copper complexes, from the hydrolysate during the period when the mercaptide mixture was allowed to stand at 10° overnight, for the removal of Cl^- as Cu_2Cl_2 had reduced the acidity to about *p*H 3·5, in which region the solubility of these amino-acids is much depressed [Hill & Robson, 1934; Baptist & Robson, 1940].

Table 1. *Composition of the organic material in the mercaptide precipitate (edestin)*

	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
Humin	0·729	1·16	0·0759	0·61
Cystine (0·2255 g. S)	0·846	1·37	0·0985	0·85
Methionine (0·1385 g. S)	0·644	1·04	0·0605	0·53
Tyrosine	0·247	0·4	0·0191	0·21
Total	2·466	3·97	0·254	2·20

5, 6. The mercaptide filtrate and washings were collected, a few ml. HCl added and Cu^+ removed with H_2S. As the washing of inorganic precipitates such as Cu_2S must be thorough if working losses are to be kept down to the minimum, the details of our method are given fairly fully. The many precipitates of CuS obtained in later stages were treated in a similar way.

The solution is transferred to a Büchner flask fitted with a rubber bung carrying a glass tube, for the entry of H_2S, which reaches nearly to the bottom of the flask, the side limb being closed with a short length of pressure tubing and a screw clip. H_2S is then passed in under a pressure of about 18 in. water and the contents of the flask shaken until the Cu_2S coagulates to leave a clear liquid and no more gas is taken up. The mixture is then filtered on a Büchner funnel, using a hardened paper, and before allowing the precipitate to pack hard it is washed 2 or 3 times with cold water (stirring). Meanwhile the main filtrate is being concentrated *in vacuo* to a syrup to remove H_2S and excess HCl if this be present. The precipitate is removed from the filter paper, transferred to a 250 ml. centrifuge bottle fitted with a rubber bung, shaken vigorously with cold water until it is completely disintegrated and again filtered. This operation is generally repeated twice. The precipitate is next transferred to a 1 l. Pyrex beaker, 300–400 ml. of water are added and the beaker immersed in a boiling water-bath for 30–45 min. The precipitate is then filtered off and the operation repeated 5 or 6 times. Occasionally the washings become colloidal and it is necessary to add a few drops of mineral acid to induce flocculation. The collected washings are then reduced *in vacuo* to a syrup and joined with the main filtrate. The resulting solution is then tested for traces of Cu^+ by passing in H_2S, filtered if necessary from any sulphide or elementary S present, and made up to standard volume for determination of N.

The flow sheets showed that at the end of stage 5 there was no detectable loss of N. The remainder of the solution was treated with lime to *p*H 3·5 and reduced to 200 ml. On standing 4 days at 0° a small crop of crude tyrosine separated; colorimetric estimation showed that 86% of the product was tyrosine [Meyer, Field & Klemm, 1940].

7. The filtrate and washings from the tyrosine, of volume about 1200 ml., were treated with freshly prepared cream of lime until an excess remained undissolved. The mixture was then reduced *in vacuo* to a syrup to remove ammonia. Water was then added to make the volume 400 ml., followed by 3600 ml. of 95% ethanol, which were added quickly with vigorous stirring. The separated Ca salts and excess lime were filtered off without delay on a Büchner funnel, using a hardened filter paper, and the extraneous mother liquor removed by pressing the mass to a hard pad, which was then washed with a little absolute ethanol. Suction was continued until most of the ethanol passed through and the material could be reduced to a friable powder by rubbing with a spatula. Unless this precaution be adopted difficulty will be experienced, on account of the presence of ethanol, in redissolving the Ca salts in a small volume of warm water; the dry salts nevertheless are hygroscopic and the suction must not be continued too long, especially if the laboratory air is somewhat humid. The material was next transferred to a beaker, warm water added with stirring to dissolve the Ca salts, and without removing the residual excess lime the volume was adjusted to 400 ml.; 3600 ml. of 95% ethanol were then added.

The Ca salts were filtered off, washed as before and extracted with about 800 ml. warm water, the small residue of lime being washed 8 times by stirring with 150 ml. water kept near the boil for a few minutes and centrifuging. The clear liquors were collected, Ca^{++} removed as described below with oxalic acid and the mixture centrifuged. The clarified solution was reduced *in vacuo* to 200 ml. and reserved. The Ca oxalate was washed once with cold water, and then 8 times with 150 ml. hot water, the collected washings were cooled to room temperature and kept for some time to permit the recrystallization of the small amount of redissolved oxalate. The latter was removed by centrifuging,

the clear washings collected and reduced *in vacuo* to 50 ml. The small amount of oxalate that separated was centrifuged off and the clarified liquor added to the main solution mentioned above. The volume was then made up to 1 l. and 5 ml. withdrawn for analysis. The total N present (corrected) was equivalent to 20·1% T.P.N.

Without delay each of the two lime-ethanol filtrates was reduced *in vacuo* to a syrup to remove ethanol and traces of NH_3. The syrup from the first filtrate was then taken up in about 1 l. of water and oxalic acid added until no further precipitation occurred. The Ca oxalate was removed by centrifuging, washed once with hot water and reserved for treatment later. The clear centrifugate was taken to a syrup *in vacuo*, water added and again removed *in vacuo*, this treatment being applied 4 times in all to remove HCl set free from any $CaCl_2$ that had originally been present in solution. The removal of HCl at this stage is necessary, as Ca cannot be completely precipitated as oxalate in its presence. The syrup was again taken up in water and just sufficient oxalic acid added to throw out a small precipitate of Ca oxalate, which was removed by centrifuging and washed once. Both oxalate and washing were reserved. The syrup from the 2nd lime-ethanol filtrate was treated with oxalic acid, the Ca oxalate washed once as before and reserved. The clear centrifugate was reduced *in vacuo* 4 times as before to remove free HCl. Just sufficient oxalic acid was then added to throw out a fairly heavy precipitate of Ca oxalate, which was washed once and reserved. Free HCl was again removed as before, followed by precipitation of the Ca as oxalate. The number of times this operation must be repeated varies with the solution concerned; in the present case 4 treatments were necessary. All the precipitates of oxalate from both filtrates were then collected and washed thoroughly 8 times in succession with 250 ml. boiling water. The combined filtrates, including those from the initial 1st washings, were then reduced *in vacuo*, added to the two main centrifugates and the solution reduced *in vacuo* to 200 ml. Final adjustments for the removal of Ca and oxalate ions were then made, the small precipitate washed and the filtrate and washings made up to 1 l. for the determination of total N.

8. (Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate.) The solution was reduced *in vacuo* to 200 ml., the very small amount of Ca oxalate that separated was filtered off, the filtrate and washing again reduced *in vacuo* to 50 ml., filtered from a further small amount of separated Ca oxalate, and the filtrate and washings transferred to an open dish with about 30 ml. conc. HCl. Evaporation was then allowed to proceed to incipient crystallization on a water-bath. After standing at room temperature overnight the separated material was filtered off on a sintered glass crucible, washed with a little conc. HCl followed by absolute ethanol, and dried off *in vacuo* over NaOH to give G-HCl crop 1.

The filtrate was reduced *in vacuo* 6 times to remove free HCl and taken up in 600 ml. boiling water. Purified $CuCO_3$ was added in excess and the hot solution filtered immediately on a hot funnel to remove the excess carbonate. The filtrate was cooled and kept at 0° for 4 hr. Cu-A crop 1 was filtered off and washed many times with water, followed by ethanol and ether. Cu^{++} was removed from the filtrate with H_2S in the presence of HCl, and the CuS washed. The clarified filtrate and washings were collected, reduced *in vacuo* to 20 ml., transferred to an evaporating basin, a few ml. conc. HCl added and the mixture reduced to 5–6 ml. on a water-bath. On standing for 20 hr. at 0° G-HCl crop 2 separated; this was filtered, washed and dried as described above.

On further treatment in the same way Cu-A crop 2 and G-HCl crop 3 were obtained. Excess HCl was removed from the mother liquor and a 3rd lime-ethanol precipitation made from an aqueous volume of 30 ml. The filtrate was freed from Ca^{++} as described above. The precipitate was redissolved in water and Ca^{++} removed with oxalic acid until the *p*H was reduced to 3·2. The clarified solution was next reduced to about 20 ml. and kept at 0° overnight when a small crop of crude tyrosine, ≡0·30% w/P, separated. Ca^{++} was then removed from the mother liquor. The clarified solution gave Cu-A crop 3 but no glutamic acid. The lime-ethanol treatment was then applied twice in succession, from aqueous volumes of 10 and 30 ml. respectively. The clarified precipitate, on standing many weeks, gave G-HCl crop 4 containing 4% ash ($CaSO_4$), but no Cu aspartate could be separated. The final mother liquor contained 0·1173 g. N (corrected), ≡1% T.P.N. Flow sheets showed that at the end of stage 8 the overall working losses amounted to 25·8 mg. N (actual) or 30·4 mg. N (corrected), equivalent to only 0·27% T.P.N.

9, 10. The five lime-ethanol filtrates (the last two still contained Ca^{++}), together with the mother liquor mentioned above, were collected, freed from ethanol, and made up to 1 l. The total N, uncorrected, was 7·82 g. For the precipitation with 12-phosphotungstic acid (P.T.A.) two trial runs with a mixture of 50 ml. solution, 50 ml. water and 14 ml. conc. HCl showed that 20 g. P.T.A. in 20 ml. water were in slight excess. 895 ml. of the main solution were accordingly diluted to 1795 ml. with water and 240 ml. conc. HCl added. The whole was warmed to 60° and a warm solution of precipitant (216 g. P.T.A. in 400 ml. water) poured in with stirring. The two trial runs were then added and the mixture allowed to stand for 2 days at room temperature. The precipitate was filtered on a Büchner funnel using a hardened paper, suction being maintained until the material had been pressed down to a hard pad and no further liquid passed. The material, still on the paper, was next puddled to a fine cream with 100 ml. of wash solution (2·5 g. P.T.A. in 100 ml. of *N* HCl); suction was then applied and the material again pressed down to a hard pad. This pad was broken with a spatula and transferred to a cylindrical separating funnel. The Büchner funnel and hardened paper were then well washed with water, the washings being transferred to the separating funnel to give a final aqueous volume of about 1500 ml. 100 ml. conc. HCl were then added and the mixture shaken vigorously to give a fine cream. To remove the P.T.A. the following organic solvent was used: amyl alcohol (pyridine-free and well washed with approximately *N* HCl), 20 parts; 0·720 ether, 25 parts; absolute ethanol, 1 part. 200 ml. of this were added to the funnel and the mixture shaken; a further 2 lots of 100 ml., making 400 ml. in all, were required to effect a clean separation. The solvent was drawn off and washed with 400 ml. of ether-water. This aqueous washing was added to the main aqueous solution, which was then washed with 4 lots of 50 ml. and 5 of 80 ml. of the organic solvent successively. The aqueous solution was then reduced *in vacuo* to a syrup to remove excess HCl. All the organic solvent solutions were then collected (vol. about 900 ml.), 600 ml. of ether added (to depress solubility of amino-acids) and the mixture washed 10 times successively with 60–70 ml. *N* HCl saturated with ether. The aqueous washings were collected (vol. about 1·5 l.) and washed 8 times successively with 60 ml. of the organic solvent. The latter were collected

(vol. about 450 ml.), 150 ml. ether added and the mixture washed 8 times successively with 70 ml. of the *N* HCl-ether-water. The collected aqueous washings were given two final washings with 40 ml. of the organic solvent and then reduced *in vacuo* to a syrup to remove excess HCl. This syrup, together with that from the main solution, was taken up in about 300 ml. water, filtered to remove a faint mist of amyl alcohol and the clear but slightly brown filtrate made up to 1 l. The total N was 3·894 g. ≡ 33·8% T.P.N. The P.T.A. organic solvent solutions were reduced in volume to about 400 ml. and made up to 500 ml.; the total N was 6·7 mg.

11. The analysis of the base solution is described in Section 5.

12. The base filtrate was freed from P.T.A. by a procedure similar to that described above. The organic liquors retained only 6·0 mg. N. The clarified filtrate contained N ≡ 34·9% and amino-N ≡ 31·0% T.P.N. respectively. The procedure for the removal of inorganic ions was as follows.

Chloride. The excess HCl was removed by evaporation *in vacuo* to a gum, water being added and the operation repeated 6 times in all. The residue was taken up in water (300 ml.) and the bound HCl removed with moist Ag_2O, the solution being kept below *p*H 3 by the cautious addition of 0·5 *N* H_2SO_4, of which about 300 ml. were required. The AgCl was filtered off on a Büchner funnel, transferred to a beaker, brought to the boil with 700 ml. of water (stirring) and again filtered. The washing was twice repeated. The collected washings were reduced *in vacuo* to 150 ml. and allowed to stand; $CaSO_4$ separated and was filtered off. A small amount of $CaSO_4$ had also separated from the main solution on standing; this was also filtered off and joined to that mentioned above. The combined material was then washed 3 times with hot water and the washed $CaSO_4$ reserved. The AgCl was next washed 8 times in succession by bringing to the boil with about 300 ml. of *N* HCl and filtering. The washings were taken to dryness *in vacuo* to remove excess HCl, precipitated with Ag_2SO_4, the precipitate washed and the filtrate added to the main solution.

Silver. The solution and washings were treated with H_2S, the Ag_2S filtered off on a Büchner funnel and washed in the same way as the Cu_2S described above.

Calcium sulphate. The solution and washings were concentrated to the incipient crystallization of $CaSO_4$; 2 vol. of 95% ethanol were then added and the mixture allowed to stand overnight. The $CaSO_4$ was filtered off and washed 5 times successively by boiling with 100 ml. of water for 30 min. The washings were concentrated to about 60 ml. *in vacuo*, allowed to stand for a few hours, the small amount of separated $CaSO_4$ filtered off and washed 3 times with hot water.

Sulphate. The solution and washings were collected and sulphate precipitated as completely as possible with baryta, the mixture being heated on a water-bath for about 1 hr. The precipitate was filtered off on a Büchner funnel and washed 6 times successively by bringing to the boil with about 200 ml. of water. The filtrate and washings (at *p*H 7·3) were collected, reduced *in vacuo* to about 200 ml. and without removing the small amount of separated $BaSO_4$ a slight excess of Ba^{++} was carefully removed with H_2SO_4. The precipitate was filtered off and washed. A second and final adjustment for Ba^{++} and $SO_4^{=}$ was made after concentration of the filtrate and washings to a small volume.

The $BaSO_4$ and the AgCl from all these operations were collected and treated with 600 ml. *N* HCl at 80° for 8 hr. The filtrate and washings were collected, excess HCl and Cl^- removed in the usual way and the final filtrate added to the main solution. The working loss during the removal of all the above-mentioned inorganic ions was 17 mg. N actual and 23 mg. N corrected.

13, 14. In the edestin analysis stage 13 was omitted, and the solution of amino-acids was treated with $CuCO_3$ to make the Cu salts by the method of Town. These were granulated with A.R. acetone.

15, 16. (Abbreviation: M.A., monoamino-acids.) The dry salts, in a 250 ml. centrifuge bottle, were given 7 successive extractions with approximately 150 ml. carefully dried methanol (dehydrated twice over Ca) by shaking in a mechanical shaker for approximately 1 hr. each time. The insoluble residue was extracted repeatedly at room temperature with water until the extracts were colourless. These aqueous extracts were evaporated *in vacuo* and granulated with acetone as before. The dry material was then given 8 extractions as before with methanol. The residue from this last operation was taken up in water, evaporated to a syrup, and after granulation with acetone the extraction with methanol was continued, the 4th and 5th extracts being nearly colourless. The collected alcoholic extracts were reduced to a syrup, which was granulated with acetone and then re-extracted with the solvent. The alcoholic extracts were united and reduced to a syrup, which was then taken up in water and Cu^{++} removed with H_2S. After washing the CuS in the usual way the solution was reduced to 40 ml., and cooled to 0° overnight. The crystalline material that separated was filtered off, the mother liquor was reduced in volume, ethanol added to faint turbidity and the mixture warmed until the solution cleared. On standing at 0° overnight a 2nd crop of crystalline material separated. The collected products, M.A. crop 1, weighed 3·959 g. (5·318 g. corrected) and contained 11·74% N.

17, 18, 19. When present in fair concentration proline is partially precipitated by P.T.A. under the conditions of a base analysis; it was decided therefore to remove as much as possible of this imino-acid to prevent contamination of the second base precipitate at stage 25. The filtrate from the separation of M.A. crop 1 at stage 15 contained 0·165 g. amino-N and 0·204 g. non-amino-N (corrected 0·2216 and 0·2740 g. respectively). A small aliquot was first passed through the laborious $CdCl_2$ procedure of Town [1936], but the incidental losses were appreciable and the amount of non-amino-N in the final solution less than expected. Town himself found that the proline was not quantitatively precipitated by one treatment, and King [1941] records a similar finding. The remainder of the solution was therefore reduced to 30 ml. and picric acid (5·016 g. ≡ non-amino-N) in a small volume of hot water added. On standing at 0° a 1st crop of proline picrate (3·296 g.) separated and on reducing the volume a 2nd crop (1·153 g.) was obtained. The combined crops were equivalent to 1·996 g. proline (corrected). The mother liquor was acidified with HCl, the precipitated picric acid filtered off, the liquors extracted 6 times with ether to complete the removal, and reserved for stage 22.

20. Cu^{++} was removed from the solution of water-soluble salts, which was then reduced *in vacuo* to 25 ml. and kept overnight at 0°. M.A. crop 2 separated; wt. 1·9213 g. or 2·575 g. corrected; N = 10·11%, all in the amino-form. The mother liquor was reserved for stage 22.

21. The residual Cu salts were extracted repeatedly with large volumes of water in a beaker heated on the water-bath, conditions which would remove any Cu aspartate. Each extract was filtered off, and while still hot Cu^{++} was

removed with H_2S. The CuS was washed in the usual way and the collected liquors reduced *in vacuo*. Three crops of monoamino-acids were obtained; collected these gave M.A. crop 3; wt. 7·3916 g. (corrected); N = 11·26%. The mother liquor was reserved for stage 22. The final insoluble residue, which was thought at the time to be excess $CuCO_3$, almost certainly contained, according to later experience, the very insoluble Cu salt of methionine and possibly also that of phenylalanine; it also contained subordinate amounts of humin, and these products are in large part responsible for the recorded loss of N during the Cu salt treatment (stages 15–20), which amounted to 0·209 g. (corrected) ≡ 1·94% T.P.N. To guard against the possibility that the 3 crops of monoamino-acids separated at stages 16, 20 and 21 respectively contained either aspartic or glutamic acid, though none gave any significant titration for dicarboxylic acids, an aliquot of each crop was taken, dissolved in hot water, NH_3 added to *p*H 8·8 and the mixture cooled [Osborne & Liddle, 1910*a*]. The crystalline mass of monoamino-acids was filtered off and the mother liquor reduced to a small volume. The lime-ethanol treatment was then applied, but the precipitate was free from N.

22, 23. (Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate.) The collected solutions were treated with a 1/10 vol. conc. HCl and boiled gently for 2 hr. to open the pyrrolidone ring. The subsequent treatment was similar to that applied at stages 7 and 8 and calls for no special comment. The lime-ethanol treatment was applied 3 times in all, from aqueous volumes of 60, 20 and 100 ml. respectively. The clarified solution from the 1st precipitate contained 0·1935 g. N (corrected 0·2599 ≡ 2·22% T.P.N.) and 0·1538 g. amino-N; from it was isolated 0·5457 g. Cu-A and 0·4578 g. G-HCl. The 2nd treatment gave 0·1148 g. Cu-A and in 2 small crops 0·1609 g. G-HCl; the 3rd treatment 0·0848 g. G-HCl. The various lots were collected to give 0·9102 g. (corrected) of Cu-A (Table 2, crop 4) and 0·9684 g. (corrected) of G-HCl (Table 2, crop 5); the latter contained 4·96% of $CaSO_4$. The final mother liquor contained 0·0270 g. N (corrected), equivalent to only 0·27% T.P.N.

24, 25, 26. The procedure followed that of stages 10 and 11, the base precipitation being made from a total volume of 290 ml. with 25 g. P.T.A. in 20 ml. water. The base solution contained 0·1185 g. N of which 0·0778 g. was amino-N; the total N (corrected) was 0·1592 g. or 1·29% T.P.N. The analysis for the 3 bases is given in Section 5; only 0·051 g. N, all in the amino-form and equivalent to 0·45% T.P.N., was unaccounted for. The separation of proline at stage 18 had therefore been effective.

27, 28, 29. The base filtrate was freed from P.T.A. and all inorganic ions as in stages 10 and 12. The final solution was evaporated to a small volume and taken to dryness in a vacuum desiccator. The residue was a non-hygroscopic white powder; ash free it weighed 12·08 g. (corrected) and contained 13·15% N. Tests for the presence of dicarboxylic acids and bases by the methods detailed more fully in the corresponding section of the egg-albumin analysis were all negative. The fraction will presumably contain all the non-nitrogenous products, except those condensed to humin, which had been formed by deamination of certain of the amino-acids during the 20% HCl hydrolysis, evidence for which is revealed in the production of NH_3 in excess of the true amide-N.

Results of analysis

If present in the hydrolysate, tyrosine will be partially precipitated during the lime-ethanol treatment, so will phenylalanine and leucine if baryta be employed instead of lime. All three of these amino-acids, especially the former, will separate readily as the hydrochloride in the presence of 20% HCl; they may, in consequence, be serious contaminants of the glutamic acid hydrochloride isolated at stages 8 and 23. In the procedure we have adopted the only likely contaminant is tyrosine hydrochloride, and as this is fairly readily soluble in absolute ethanol we always make a point of thoroughly washing our products with this solvent. The various crops of glutamic acid hydrochloride cited in Table 2 were analysed for ash and total N [Chibnall *et al.*, 1943], and their purity was controlled by confirming that the titration value in the micro-Kjeldahl was exactly one-half of that obtained when the same weight of material in aqueous solution was titrated to *p*H 7·3 (blue tint with bromothymol blue); working to fine limits, 1% monoamino-acid impurity can be detected in this way. The necessary criteria for checking the purity of Cu aspartate have been discussed in a previous paper [Bailey *et al.*, 1943]: data for the various crops obtained in the present analysis are given in Table 2.

The detailed results of the complete analysis are given in Table 3. It is to be remembered that the

Table 2. *Analytical data for glutamic and aspartic acids from edestin*

	Glutamic acid hydrochloride			Copper aspartate			
						On anhydrous material	
Crops	Wt. (corrected) g.	N %	Rotation of glutamic acid in 9% HCl $[\alpha]_D$	Wt. (corrected) g.	H_2O %	Cu %	N %
1	10·722	7·63	+30·3	10·71	29·15	32·54	7·22
2	1·994	7·65	+23·4	2·192	29·35	32·70	7·22
3	0·590	7·67		1·354	29·02	32·43	7·21
4	1·511	7·61	+ 7·3	0·910	29·36	32·28	7·11
5	0·928	7·68					
	15·745			15·166			
Theory		7·63			29·4	32·69	7·19

Table 3. *Analysis of edestin*

Stage (diagram 1)	Fraction	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
4	Mercaptide precipitates:				
	Humin	0·729	1·16	0·0759	0·61
	Cystine	0·846	1·37	0·0985	0·85
	Methionine and tyrosine	0·891	1·44	0·0796	0·74
6	Crude tyrosine	2·549	4·13	0·1973	1·71
7, 22	Glutamic acid	12·658	20·50	1·203	10·44
7, 22	Aspartic acid	7·372	11·95	0·7765	6·74
2	Ammonia	1·44	2·36	1·205	10·42
11, 23	Arginine	10·31	16·69	3·320	28·81
11, 23	Histidine	1·464	2·39	0·3948	3·47
11, 23	Lysine	1·431	2·32	0·2802	2·39
16	Monoamino-acids, crop 1 (N = 11·74 %)	5·318	8·61	0·6243	5·43
20	Monoamino-acids, crop 2 (N = 10·11 %)	2·575	4·17	0·2603	2·26
21	Monoamino-acids, crop 3 (N = 11·26 %)	7·606	12·32	0·8282	7·21
29	Monoamino-acids, crop 4 (N = 13·15 %)	12·22	19·56	1·59	13·86
18	Proline	1·996	3·23	0·2428	2·11
	Total accounted for	69·41	112·22	11·18	97·05
	Unaccounted for	3·27(?)	5·31	0·34	2·95
	Taken for analysis (61·74 g. protein)	72·68	117·7	11·52	100·0

value for proline represents that actually isolated as picrate and is not to be regarded as maximal; Osborne & Liddle [1910*b*] found 4·1 %.

The overall loss amounted to 2·95 % T.P.N., and the individual losses at the various stages are recorded in Table 4. The major loss occurs during the Cu salt treatment covering stages 14–21, and there is reason to believe that this falls almost exclusively on the more insoluble monoamino-acids, particularly methionine, and subordinate amounts of humin. The overall loss to stage 8, which occurred

Table 4. *Losses recorded at certain stages of analysis (edestin)*

Stages	Origin	Total N (corrected) g.	Total N (corrected) % total protein-N
1– 8	Overall losses to the removal of the main bulk of glutamic and aspartic acids	0·0304	0·26
11, 12	Retained by organic wash liquors	0·0128	0·11
12	Removal of inorganic reagents	0·0230	0·20
14–21	Copper salt fractionation; loss chiefly monoamino-acids	0·20	1·76
26	Unaccounted for in base solution	0·0519	0·45
21–29	Overall loss (diff.)	0·0219	0·22
1–29	Total	0·34	3·0

in large part during the working at that stage itself, must fall in part on the dicarboxylic acids, but from stage 9 onwards the concentration of these, especially at stage 25, is so low relative to the other products present that their contribution to the remaining losses must be very small indeed. In a previous paper [Bailey *et al.*, 1943] we have discussed the solubility of the Ca salts of the dicarboxylic acids in the lime-ethanol procedure, and have given our reasons for the belief that in the present edestin analysis small solubility factors can be legitimately applied at stage 22. When account has been taken of these factors our final results for glutamic acid and aspartic acid are 20·70 and 12·0 % W/P respectively (Table 14), and in view of what has been said above we believe that these are within 1–2 % of the true values. Solubility corrections for the bases are discussed in Section 5.

Reverting to the data given in Table 3, we see that 69·41 g. of hydrolysis products have been identified, and that these represent 97·05 % of the total protein-N. If we assumed for reasons given above that the loss to stage 8 represents dicarboxylic acids with an average of 10 % N, that the loss covering stages 14–21 represents monoamino-acids with an average of 9 % N, and that the other losses represent amino-acids with an average of 12 % N, then calculation shows that the gross weight of unidentified products, containing 0·34 g. N, would be 3·27 g. This value has been incorporated in Table 3 and permits the deduction that 100 g. protein have given 117·7 g. hydrolysis products. The computed value, based on the total N and the peptide-N of the protein [Chibnall, 1942], is 117·8; agreement is good, considering the assumption made with respect to the humin and unidentified products in Table 3.

3. *Analysis of egg albumin*

The edestin procedure needed slight modification on account of the larger proportion of monoamino-acids in the egg albumin hydrolysate. In the description that follows only points of difference or of unusual interest will be noted.

Details of analysis

1, 2. The sample of coagulated protein [Chibnall *et al.*, 1943] contained 0·44 % ash, and ash- and moisture-free

15·76% N. 62·02 g. ash- and moisture-free protein containing 9·78 g. N were dissolved in 600 ml. 20% HCl by heating on a water-bath for 6 hr. The mixture was then boiled under reflux for a further 24 hr. and the excess HCl removed in the usual way. Acid humin was then removed and washed 3 times with cold and 3 times with hot water; it contained 7% ash. The ash-free product weighed 0·862 g. and contained 0·0633 g. N. To avoid the withdrawal of a large aliquot from the main hydrolysate to determine alkali humin, part of which is carried down in the mercaptide precipitate, a separate small hydrolysis under identical conditions was carried out for this purpose. Alkali humin was determined by adding CaO to *p*H 7·5. The weight, ash-free, and calculated in terms of the main hydrolysate, was 1·061 g. and the total N 0·0652 g. The filtrate was deep straw-coloured, showing that all the humin had not been removed.

3, 4, 5. As 0·0162 g. of alkali humin-N was removed in the lime-ethanol treatment at stage 8 it is assumed that the remainder of that estimated above has been carried down in the mercaptide precipitate. The latter (wt. 50·5 g.) contained organic-S in excess of that due to cystine and by difference 0·3% w/P of tyrosine. It is clear, therefore, that as in the case of edestin the conditions during the precipitation have caused the separation of the less soluble monoamino-acids or their copper complexes. To ascertain whether aspartic acid or arginine were concerned, 10 g. of the product was dissolved in 250 ml. HCl, Cu^+ removed with H_2S, the precipitate of Cu_2S and humin well washed and the clarified solution reduced *in vacuo* to remove excess HCl. Treatment with flavianic acid by the method of Vickery [1940] gave 21·4 mg. recrystallized material [Lucas & Beveridge, 1940] which was definitely not arginine monoflavianate, for it did not possess the characteristic golden lustre and on attempting a second recrystallization (see Section 5) it remained in solution. The flavianate mother liquors were collected, excess flavianic acid removed as described in Section 5, and the lime-ethanol treatment for dicarboxylic acids applied from an aqueous volume of 18 ml. The Ca precipitate, when treated as in stage 8, gave a solution containing 42·2 mg. N and from this various crops of impure Cu cysteate were separated, but a careful search failed to give evidence for the presence of Cu aspartate. The final data for the composition of the mercaptide precipitate are given in Table 5. The mercaptide filtrate, still somewhat coloured, was freed from Cu^+ with H_2S without the addition of HCl, consequently the concentration of Cl^- at the 1st lime-ethanol stage was very low.

Table 5. *Composition of the organic material in the mercaptide precipitate (egg albumin)*

	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
Alkali humin	0·74	1·20	0·0490	0·50
Cystine	1·10	1·78	0·1282	1·31
Methionine	1·60	2·59	0·1582	1·62
Monoamino-acids (diff.)	0·59	0·95	0·0589	0·60
	4·03	6·52	0·3943	4·03

7, 8, 9. Two successive treatments with lime-ethanol were made from aqueous volumes of 500 ml. There was insufficient Cl^- present at the second operation to flocculate the precipitate, and it was necessary to add 8 g. crystalline $CaCl_2$. On redissolving the precipitate the residual lime retained 0·0162 g. alkali humin-N. During the treatment with HCl to effect the separation of G-HCl much darkening of the solution occurred, due presumably to the decomposition of the glucosamine moiety of the polysaccharide present in egg albumin [cf. Neuberger, 1938]. As a result 0·157 g. acid humin, containing 0·0122 g. N, was removed prior to the 3rd treatment with lime-ethanol, which was made from an aqueous volume of 500 ml. The 4th treatment was also made from a similar volume, and in the final clearing of the material recovered from the precipitate it was necessary to remove $SO_4^=$ (from the protein ash) as $CaSO_4$ with ethanol. From all these operations 4 crops of Cu-A, containing aspartic acid equivalent to 7·74% w/P, and 4 crops of G-HCl, containing glutamic acid equivalent to 13·89% w/P, were obtained. The final filtrate contained (corrected) 0·0932 g. N of which 0·0664 g. (≡0·68% T.P.N.) was amino-N. The overall loss to the end of stage 8 was 14·7 mg. N (17·9 mg. N corrected) and the flow sheets showed that this occurred in large part during the separation of the glutamic and aspartic acids.

9, 10, 11. The precipitation of the bases with P.T.A. was made from a total volume of 2 l. As the hydrolysate contained such a high proportion of monoamino-acids the base solution, freed from P.T.A., was reprecipitated from a total volume of 1450 ml., the mixture being allowed to stand at 0° overnight. It is doubtful if any benefit resulted from taking this precaution, as the residual solubility of the arginine phosphotungstate was considerable: the final base solution nevertheless was free from extraneous-N (see Section 5). The whole operation, including the removal of P.T.A. from the two filtrates, resulted in a loss of only 8·7 mg. N (11·1 mg. N corrected).

The loss on removing all inorganic reagents and excess Cl^- was 7·4 mg. N (10·1 mg. corrected). On evaporation of the clarified solution 3 crops of monoamino-acids were removed.

14–21. The filtrate from stage 13 was brownish, and clearly contained humin, part of which probably may have resulted from the operations of stage 8. On treating the solution with excess $CuCO_3$ to make the Cu salts a small reduction to Cu_2O occurred, presumably due to glucosamine or its derivatives. The extraction of the dry salts with ethanol and cold water proceeded smoothly and but little material was subsequently removed from the residue by treatment with boiling water, probably because the amino-acids concerned had been separated at stage 13. On removal of Cu^{++} from the various fractions the CuS retained 50 mg. humin-N. The separation of proline, at the picric acid stage, was more difficult than in the case of edestin on account of the presence of hydroxyproline, and fractional crystallization was necessary. The yields of the two picrates (m.p. 153° and 189°) were equivalent to 1·632 g. of proline or 2·63% w/P and 0·262 g. hydroxyproline or 0·42% w/P respectively. The overall loss in the complete Cu treatment was 0·144 g.; as in the case of edestin we believe the major part of this to be due to the insoluble Cu salt of methionine.

22, 23. The collected solutions were treated with a 1/10 vol. conc. HCl and gently boiled for 2 hr. to convert any pyrrolidone carboxylic acid present to glutamic acid. Excess HCl was then removed and the lime-ethanol treatment applied 3 times in succession from 200 ml. of aqueous solution. The clarified solution from the ultimate precipitate contained 0·2057 g. N (0·385 g. corrected) of which only 0·147 g. was amino-N, indicating the presence of much

arginine due to recrystallization of the base precipitate at stage 10. G-HCl crop 5, and a fraction of Cu-A were separated: the latter needed recrystallization. The lime-ethanol treatment was then applied once more from 200 ml.; the clarified solution from the precipitate contained 0·0807 g. N and 0·0597 g. amino-N. G-HCl crop 6, and a fraction of Cu-A were separated; the latter was added to that mentioned above to give Cu-A crop 5. The final mother liquor contained 58 mg. of amino-N (corrected) equivalent to 0·59% T.P.N. Much of this was probably due to serine, as egg albumin contains many per cent of this hydroxy-amino-acid [Martin & Synge, 1941]. The loss during these operations was 18·7 mg. (corrected), part of which must fall on the dicarboxylic acids.

24, 25, 26. The base precipitation was made from a total volume of 290 ml. The precipitate was filtered off, washed on the filter paper and then redissolved (incompletely) by warming with 180 ml. of P.T.A. wash solution for 10 min. at 80°. The mixture was allowed to stand at room temperature for 2 hr. and then filtered off. The filtrate was shown later to contain much arginine, and as in stage 10, it is doubtful if this recrystallization was of any advantage.

27, 28, 29. The clarified solution on evaporation to a small volume gave M.A. crop 5, containing 12·31% N and on taking the mother liquor to dryness in a vacuum desiccator there was obtained a final fraction containing 3·7% ash. Ash-free, this residual product weighed 4·34 g. (9·297 g. corrected).

As the base precipitate at stage 25 had been recrystallized we suspected that a small amount of arginine phosphotungstate had remained in solution. Accordingly 2·604 g. of the final residue was dissolved in 75 ml. water and arginine flavianate precipitated by the method of Vickery [1940], using 2·4 g. flavianic acid. The yield of arginine flavianate (tested for purity as described in Section 5) was equivalent to 0·1358 g. arginine (corrected). The excess flavianic acid was removed from the filtrate by strongly acidifying with HCl and extracting with amyl alcohol-ether mixture. The aqueous solution freed from excess HCl was then submitted to the lime-ethanol treatment from a volume of 50 ml. The clarified solution from the precipitate contained 38·7 mg. N, equivalent to 0·88% T.P.N. and it was considered that the major part of this would be due to serine. Abderhalden & Kautzsch [1912] observed that aspartic and glutamic acids were precipitated by Hg acetate, and we have found that this is so even in the presence of 10 times the equivalent of serine. The above-mentioned solution was treated accordingly and the precipitate freed from Hg^{++} with H_2S. The clarified solution (8·9 mg. N ≡ 0·3% T.P.N.) gave a small amount of good crystallin Cu aspartate, in amount (corrected) equivalent to only 17 mg. of aspartic acid or 0·03% W/P and in excellent agreement with the calculated solubility correction [Bailey *et al.*, 1943] for stage 23. No glutamic acid hydrochloride could be isolated.

The mother liquors and the various filtrates were then collected, all reagents removed, and the clarified solution submitted to electrodialysis by Mr A. H. Gordon. Only 3 mg. N (10·6 mg. N corrected ≡ 0·11% T.P.N.) passed into the cathode compartment, showing that no appreciable basic-N was present. The object of this last experiment was to demonstrate the absence of ornithine, for the properties of this base are such that it would almost certainly have escaped precipitation with phosphotungstic acid at stages 10 and 25. In Table 7 monoamino-acids crop 7 represents the residual 9·297 g. less the amount of arginine and aspartic acid subsequently isolated.

Results of analysis

The analytical data for the various crops of glutamic acid hydrochloride and copper aspartate are given in Table 6. Calculation shows that the glutamic acid hydrochloride isolated at stage 8 (i.e. crops 1–4) represents 8·269 g. of *l*(+)-glutamic acid and 0·384 g. of *dl*-glutamic acid; the corresponding weights for stage 23 are 0·721 and 0·6 g. respectively, showing that the solubilities of the two Ca salts in the modified Foreman procedure are similar [Bailey *et al.*, 1943] and refuting the contrary claim of Kögl, Erzleben & Akkermann [1939]. 9·8% of the total glutamic acid isolated at stages 8 and 23 can be regarded as fully racemized.

The detailed results of the analysis are given in Table 7. The main working losses have been indicated in the text and if these are evaluated as in the case of edestin it is possible to suggest the data given in the table for the products unaccounted for. The weight of hydrolysis products given by 100 g. protein is 117·8 g., a value that cannot be accurate to ± 0·5 g. on account of the difficulty of assessing the alkali humin fractions, into which, with the acid humin, most of the mannose and glucosamine of the polysaccharide moiety must have passed through condensation with tryptophan and subordinate amounts of cystine and tyrosine [Lugg; see Chibnall, 1939, p. 162]. The computed value is 117·2 g. [Chibnall, 1942]. When account is taken of the small solubility corrections applicable at stage 22

Table 6. *Analytical data for glutamic and aspartic acids from egg albumin*

	Glutamic acid hydrochloride			Copper aspartate			
						On anhydrous material	
Crops	Wt. (corrected) g.	N %	Rotation of glutamic acid in 9% HCl $[\alpha]_D$	Wt. (corrected) g.	H_2O %	Cu %	N %
1	7·425	7·65	+31·7	6·319	29·14	32·5	7·15
2	1·762	7·65	+31·3	2·272	29·32	32·5	7·20
3	0·735	7·64	+29·7	0·845	29·3	32·5	7·19
4	0·770	7·61	+13·6	0·479	28·6	32·6	7·15
5	0·884	7·67	+26·1	0·459	29·2	32·4	7·18
6	0·727	7·66	+ 5·5	—	—	—	—

Table 7. *Analysis of egg albumin*

Stage (diagram 1)	Fraction	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
2, 8	Acid humin	1·019	1·64	0·0755	0·77
3, 8, 20, 21	Alkali humin	2·110	3·40	0·1344	1·37
4	Mercaptide precipitates:				
	Cystine	1·100	1·77	0·1282	1·31
	Other amino-acids	2·190	3·54	0·2171	2·22
6	Crude tyrosine	1·511	2·44	0·1184	1·21
2, 8, 26	Ammonia	1·071	1·73	0·8909	9·11
7, 22	Glutamic acid	9·913	16·00	0·9428	9·64
7, 22	Aspartic acid	5·019	8·10	0·5294	5·41
11, 23, 29	Arginine	3·483	5·62	1·1211	11·46
11, 23	Histidine	0·901	1·45	0·2443	2·50
11, 23	Lysine	3·136	5·06	0·6010	6·15
13	Monoamino-acids, crop 1 (N = 10·71 %)	10·120	16·32	1·0830	11·08
13	Monoamino-acids, crop 2 (N = 11·04 %)	2·557	4·12	0·2822	2·89
13	Monoamino-acids, crop 3 (N = 11·35 %)	5·443	8·78	0·6179	6·32
16	Monoamino-acids, crop 4 (N = 10·88 %)	3·466	5·59	0·3772	3·86
16	Monoamino-acids, crop 5 (N = 10·85 %)	2·589	4·18	0·2826	2·89
28	Monoamino-acids, crop 6 (N = 12·31 %)	3·624	5·84	0·4650	4·76
28	Monoamino-acids, crop 7 (N = 12·25 %)	9·142	14·74	1·1381	11·64
18	Proline	1·632	2·63	0·1920	1·96
19	Hydroxyproline	0·262	0·42	0·0383	0·40
1–29	Total accounted for	70·29	113·35	9·50	96·95
	Unaccounted for	2·55(?)	4·13	0·28	2·86
	Taken for analysis (62·02 g. protein)	72·84	117·5	9·78	100·0

the final results for glutamic acid and aspartic acid are 16·1 and 8·13 % w/P respectively (Table 14). As the gross losses at stages 8 and 23 totalled only 36·6 mg. N we believe that these results are within 1–2 % of the true values.

Throughout the analysis we have kept in mind Fearon's [1939] recent claim that egg albumin contains citrulline. The unhydrolysed protein gave the bright purple-red colour with diacetyl monoxime, as he states. After acid hydrolysis the colour was redder, yet it did not match the characteristic red given by citrulline itself; the colour moreover faded rapidly, whereas that given by citrulline is stable for many days. This last difference became more pronounced as the hydrolysate was fractionated, and the various crops of monoamino-acids described in the text and in Table 7 failed to give the test at all. Citrulline is not very stable on acid hydrolysis, as shown by the fact that a sample of 0·2 g., when boiled with 10 ml. of 20 % HCl for 22 hr., yielded 14·2 % of the total N as amide-N; even so we should have expected to find evidence for it in the amino-acid mixtures recovered from the Cu salts at stages 16, 20 or 21 if any appreciable amount had been present in the hydrolysate. Our evidence therefore does not support Fearon's claim.

4. *Analysis of β-lactoglobulin*

In light of the experience gained with the other two proteins the procedure used in this case was considerably simplified.

Details of analysis

1, 2. 30·61 g. moisture- and ash-free coagulated protein, containing 4·76 g. N or 15·55 %, were heated on the water-bath with 250 ml. 20 % HCl until solution was effected and then boiled under reflux, for an inclusive period of 24 hr.

4. The mercaptide precipitated was analysed for N but not for S or for humin.

7, 8, 9. Three lime-ethanol treatments were made in succession from an aqueous volume of 250 ml. before the separation of the dicarboxylic acid derivatives was attempted. The clarified precipitate contained 1·042 g. N (corrected) ≡ 21·9 % T.P.N. Repeat precipitations were made in due course from 200 and 100 ml. respectively.

9, 10, 11. The base precipitation was made from a total volume of 1600 ml. The total N in the precipitate was 0·9282 g. (corrected) (see Section 5).

13. Four crops of monoamino-acids were obtained, weighing 4·062, 1·360, 2·951 and 1·557 g. and containing 10·07, 10·67, 11·02 and 11·81 % N respectively.

14–21. The Cu salt procedure was omitted, as a determination of amino-N on the filtrate from 13 indicated that the proline and hydroxyproline content must be fairly small.

22, 23, 24. The lime-ethanol treatments were made in succession from aqueous volumes of 100, 200 and 200 ml. respectively before the separation of the dicarboxylic acid derivatives was attempted. The clarified precipitate contained 0·0708 g. N ≡ 1·49 % T.P.N. A final treatment from 50 ml. was made in due course. The ultimate mother liquor contained 13·3 mg. N (corrected), equivalent to only 0·28 % T.P.N.

25, 26. The 2nd base precipitation was made from a total volume of 265 ml. The total N in the precipitate was 54·2 mg. all of which was accounted for as base (see Section 5).

28, 29. Two crops of amino-acids and a final residue were obtained; these weighed 1·745, 3·59 and (ash not deducted) 5·94 g. and contained 11·37, 12·38 and 10·5 % N respectively. The latter product was treated with flavianic acid, but no arginine salt could be separated.

Results of analysis

The data given in Table 8 summarize the fractionation of the N; as the mercaptide precipitate was not examined in detail it is not possible to summarize the hydrolysis products as in the case of the other two proteins. The overall loss was 59·9 mg. N (corrected), while the losses on working stages 8 and 23 were 5·0 and 1·6 mg. N respectively, jointly equivalent to 0·14 % T.P.N., part of which must fall on the dicarboxylic acids. The final values for arginine, histidine, lysine, glutamic acid and aspartic acid are 2·89, 1·54, 9·75, 21·32 and 9·83 % W/P respectively. If the small solubility corrections for stage 22 be applied [Bailey *et al.*, 1943] the two latter values are raised to 21·51 and 9·88 % respectively (Table 14) and we believe these are accurate to 1–2 %. The solubility corrections for the bases are discussed in Section 5.

Table 8. *Analysis of β-lactoglobulin*

Stage (diagram 1)	Fraction		N g.	N as % total protein-N
2	Ammonia		0·3593	7·55
3	Mercaptide precipitate		0·2090	4·39
8	Glutamic acid	0·5982 g.	0·6199	13·03
23	Glutamic acid	0·0217 g.		
8	Aspartic acid	0·2912 g.	0·3159	6·65
23	Aspartic acid	0·0247 g.		
8	Crude tyrosine		0·0220	0·46
11	Arginine	0·2623 g.	0·2799	5·88
26	Arginine	0·0176 g.		
11	Histidine	0·1076 g.	0·1247	2·62
26	Histidine	0·0171 g.		
11	Lysine	0·5537 g.	0·5719	12·01
26	Lysine	0·0182 g.		
13	Monoamino-acids in 4 crops		1·0637	22·35
28, 29	Monoamino-acids in 3 crops		1·1332	23·81
	Total accounted for		4·6995	98·75
1, 29	Unaccounted for		0·0599	1·25
	Taken for analysis		4·76	100·0

5. *Analysis of the base fractions*

The present study is to be regarded as a continuation of that of Tristram [1939] on the reliability of various methods for estimating the basic amino-acids in protein hydrolysates. Tristram favoured the use, with certain minor modifications, of Block's [1938] small-scale adaptation of the modified Kössel procedure devised by Vickery and his co-workers [Vickery & Leavenworth, 1928; Vickery & Shore, 1932; and Vickery & White, 1933] and he tested this, *inter alia*, against appropriate mixtures of the bases representing 2–3 g. of protein. The losses at the various stages in the analysis were determined. Having allowed for the solubility of arginine silver [1·1 mg. N/100 ml., Gulewitsch, 1899], the overall loss in the estimation of arginine was 2·6 mg. N, due in part to precipitation with the histidine silver at *p*H 7·2 and in part to incomplete precipitation as arginine silver at *p*H 12–13. The overall loss in the estimation of histidine was small (0·3 mg. N/100 ml.) while that for lysine (1·6 mg. N/100 ml.) was but little more than that due to the solubility of lysine phosphotungstate. Tristram considered that corrections to allow for these overall losses could be legitimately applied when analysing a mixture of the three bases, but that in the case of protein hydrolysates the validity of such corrections must remain a matter of conjecture until new methods of analysis were found which would permit a direct determination of the solubility factors concerned. Examination of the base solutions obtained during the fractionation of the hydrolysates described in Sections 2–4 has enabled us to investigate these solubility factors in detail.

Base fraction of β-lactoglobulin

The two base fractions described in Section 4 were analysed by the Tristram-Block procedure mentioned above, the only modifications being the employment of Block's [1940] nitranilic acid method for the estimation of histidine and the thorough washing of all inorganic precipitates as described in Section 2. The purity of the arginine flavianate, which might be contaminated with the histidine salt, was controlled by recrystallization under standard conditions detailed later in our discussion of Vickery's method for arginine. Arginine nitranilate is fairly soluble under Block's conditions, but the lysine salt is as insoluble as that of histidine itself. As nitranilic acid is readily decomposed in hot water [Town, 1936] the purity of the isolated histidine salt was controlled in the following way. 0·1 g. material and 25 ml. water were placed in a 100 ml. beaker, which was then partly immersed for exactly 4 min. in a water-bath at 65°. Solution of the salt is readily effected on stirring, and if there be an insoluble residue the presence of Ba nitranilate may be suspected. The beaker was then removed from the bath, 25 ml. of methanol were run in with stirring, and crystallization allowed to proceed for 2 days at 2°. The filtered, washed and dried product should weigh 0·0980 g. Under the conditions of a base analysis it is improbable that lysine would pass into the histidine fraction, but the procedure serves to detect the presence of the Ba salt, a possible contaminant.

Analysis of 1st base solution. The aliquot used contained 0·3664 g. N; as this amount of basic-N was approximately 3 times that present in the hydrolysate of 2–3 g. protein the separation into the 3 base fractions was made from 3 times the respective volumes employed by Tristram. Excess HCl was removed by evaporation *in vacuo*, and the solution freed from Cl^- with Ag_2O and H_2SO_4 at *p*H 3·0

in the usual way. The arginine, histidine and lysine fractions contained 0·1034, 0·0394 and 0·2207 g. of N respectively. The N recovered in the form of base derivatives in this first analysis is given in Table 9, and allowing solubility corrections (300 ml.) for lysine phosphotungstate and arginine-silver the N unaccounted for was 9·5 mg. The mother liquors of the 3 derivatives were collected, reduced *in vacuo* to a few ml., strongly acidified with HCl and the 3 acidic reagents extracted by the amyl alcohol-ether procedure for the removal of excess phosphotungstic acid, an operation that can be conducted without loss of basic-N. The clarified solution, freed from HCl and Cl^- and containing 7·0 mg. N, was then passed once again through the Tristram-Block procedure, using a third of Tristram's volumes. The weight which may not be true as the amino-acid composition and concentration in the respective solutions was not the same, the amounts of the bases isolated from the 2nd base solution (column 1) represent the solubilities in 1335 ml. From these the solubility corrections for the 2nd and 1st precipitations (columns 2 and 3) have been calculated. The original Van Slyke corrections for the latter are given in column 4. Comparison is not strictly valid as we did not use P.T.A. purified by Winterstein's method, but our low value for histidine is what would be expected from the more recent work of Van Slyke *et al.* [1942]. The two arginine values show fair agreement, while the great difference between those for lysine suggests that the amino-acid mixture has influenced the solubility in our case. The final values for

Table 9. *Analysis of the base fractions of β-lactoglobulin*

	1st base solution		2nd base solution		
	N g.	N (corrected) as % total protein-N	N g.	N (corrected) as % total protein-N	Total base N as % total protein-N
Aliquot taken for analysis	0·3664	19·50	0·0496	1·10	20·60
Arginine isolated as flavianate, 1st analysis	0·0975	—	0·0135	—	(5·43)
Correction for solubility of arginine silver, 1st analysis	0·0033	—	0·0011	—	—
Correction for overall losses, 1st analysis	0·0026	—	0·0026	—	—
Total arginine	0·1034	5·51	0·0172	0·37	5·88
Histidine isolated as nitranilate, 1st analysis	0·0375	—	0·0162	—	—
Histidine isolated as nitranilate, 2nd analysis	0·0048	—	—	—	—
Total histidine	0·0423	2·26	0·0162	0·36	2·62
Lysine isolated as picrate, 1st analysis	0·2170	(11·53)	0·0159	—	(11·88)
Correction for solubility of lysine phosphotungstate	0·0015	—	0·0008	—	—
Total lysine	0·2185	11·63	0·0166	0·37	12·0
Total base accounted for	0·3642	19·38	0·50	1·11	20·49

of histidine nitranilate obtained in the 2nd analysis was 40·8 or 43·8 mg. corrected to the 1st base solution, equivalent to 4·8 mg. histidine-N. This amount of histidine must have passed into the arginine fraction in the 1st analysis. No flavianate was obtained from the arginine fraction of the 2nd analysis. The complete results for the 1st base solution are set out in Table 9. As it is clear that no appreciable amount of extraneous non-basic material is present we have included Tristram's correction of 2·6 mg. for overall losses of arginine-N, though we worked to 3 times his volumes; such an amount is, in fact, insufficient to lead to any recovery of arginine flavianate in the 2nd analysis.

The 2nd base solution was passed once through the Tristram-Block procedure, working to Tristram's volumes. Again allowing a correction of 2·6 mg. for overall losses of arginine-N the recovery of basic-N is complete, as shown in Table 9. It may be mentioned that in all the above-mentioned analyses the arginine flavianate and histidine nitranilate were shown to be pure by the solubility test mentioned earlier, and the lysine picrate, without recrystallization, exploded sharply at 266–267°. The overall correction for working losses applied to arginine accounts for 0·2% T.P.N. and this must be considered the one uncertainty in the full analysis.

It is of interest to consider the solubility of the phosphotungstates on precipitation of the bases. Relevant data are collected in Table 10. The volume employed in the 1st precipitation was 1600 ml. and in the 2nd 265 ml. On the assumption that the phosphotungstate solubilities in the two cases were proportional to the volumes employed, the bases given in Table 14 include the solubility corrections (Table 10, column 2) for the 2nd precipitation. We believe that these will be found to be within 2% of the true values.

Table 10. *Base phosphotungstate solubility corrections for β-lactoglobulin*

(Values quoted are in percentages of total protein-N.)

	Isolated from 2nd base solution	Solubility corrections		Van Slyke solubility corrections
		2nd base precipitation	1st base precipitation	1st base precipitation
	(1)	(2)	(3)	(4)
Arginine	0·37	0·07	0·44	0·51
Histidine	0·36	0·07	0·43	0·64
Lysine	0·37	0·07	0·44	0·08

Direct Tristram-Block analysis. 3·803 g. moisture- and ash-free protein, containing 0·5895 g. N, were hydrolysed with H_2SO_4 for 24 hr. The analysis followed the lines of those mentioned above, including the thorough washing of all inorganic precipitates. The arginine flavianate and histidine nitranilate were of satisfactory purity; the lysine picrate was recrystallized from 15 ml. water and corrected for solubility (3·4 mg. N/ml. at 0°); it exploded at 266–267°.

The collective results given in Table 11 bring out some interesting comparisons with Table 9. In the Tristram-

Table 11. *Tristram-Block analysis of β-lactoglobulin*

	Total N g.	N as % total protein-N
Arginine fraction	0·0394	
Arginine isolated as flavianate	0·0316	(5·36)
Correction for arginine silver (150 ml.)	0·0016	
Correction for overall losses	0·0026	
Total arginine	0·0358	6·07
Histidine fraction	0·1292	
Histidine isolated as nitranilate	0·0133	2·26
Lysine fraction	0·1186	
Lysine isolated as picrate	0·0681	(11·55)
Correction for overall losses	0·0016	
Total lysine	0·0697	11·82

Block analysis the arginine flavianate isolated was equivalent to 5·36% of the total protein-N, while our comprehensive procedure gave 5·43%; yet the corrected values were 6·07 and 5·95% respectively, suggesting that in the direct analysis the amount of N allowed for overall losses was a little too high. The histidine value in the Tristram-Block analysis is low and as according to our experience the total N in the histidine fraction varies enormously from protein to protein it is difficult to suggest any general overall corrections. With a mixture of bases Tristram's losses by his procedure were negligible (0·3 mg. N) and we confirm Block's finding that under the conditions that he applies nitranilic acid the precipitation of histidine in the absence of extraneous material is quantitative. Whether the low yield in the present case is to be ascribed to the increased solubility of histidine silver in the presence of the protein hydrolysis products or to that of histidine nitranilate in the presence of nearly 10 equivalents of extraneous N cannot be stated with certainty; both factors are probably operative, each to a degree that will vary from protein to protein. In the Tristram-Block analysis the lysine value based on the isolated picrate was 11·55%, in excellent agreement with the 11·53% obtained from the first phosphotungstate precipitate in the comprehensive procedure. The corrected value is a little low, as one would expect from our demonstration that the solubility of the phosphotungstate is enhanced by the presence of the amino-acids of the hydrolysate: even so the presence of much extraneous material in the lysine fraction has probably prevented the quantitative separation of the lysine as picrate in one operation [Vickery & Leavenworth, 1928].

Base fractions of edestin and egg albumin

The procedure used was the same as that described for lactoglobulin, and the results are given in Table 12. In the case of edestin the volume ratio in the two base precipitations was 9 to 1, so that the calculated corrections for the phosphotungstate solubilities in the 2nd were 0·04, 0·03 and 0·05% T.P.N. respectively. The final values for the bases are given in Table 14.

Conditions were different in the case of egg albumin, for both phosphotungstate precipitates were recrystallized. From the results given in Table 12 it would appear that on recrystallization of the 1st precipitate the arginine phosphotungstate separated as a gelatinous rather than a crystalline precipitate [Van Slyke *et al.*, 1942] and its solubility has been much enhanced. As the 2nd base precipitate was also recrystallized we inspected a similar occurrence and accordingly estimated the amount of arginine in the amino-acid fraction at stage 29 by the Vickery flavianic acid method discussed later. This fraction contained 12·1% of the T.P.N. and from it was isolated arginine equivalent to 0·41% of the T.P.N. After removal of the excess flavianic acid, and also of the residual dicarboxylic acids, from the arginine flavianate filtrate, the clarified solution gave on electrodialysis 0·11% T.P.N. in the cathode compartment. This value sets a definite limit to the residual solubilities of the 3 bases, as it will include any monoamino-acid-N which had migrated by simple diffusion. Our final results for egg albumin are therefore those given in Table 12, and are believed to be not more than 2% low.

Direct arginine analysis by the method of Vickery

In the comprehensive analyses mentioned above our original plan had been to effect a preliminary removal of arginine as flavianate by Vickery's [1940] new method so that the histidine and lysine fractions in the subsequent Tristram-Block procedure would be free from this base. The expectation was not realized, for in the absence of all the non-basic products of hydrolysis the recrystallized product was found to be heavily contaminated with the flavianates of the other two bases, and repeated recrystallization was necessary to obtain pure arginine flavianate. As this latter substance has an appreciable solubility under the conditions employed small amounts of it passed into the various mother liquors and washings, so that the incorporation of this additional step in the analysis of the base solutions defeated its own end. These findings prompted us to investigate the purity of the arginine flavianate obtained when the simple Vickery procedure for this base is applied directly to solutions containing known amounts of the three bases, and to protein hydrolysates.

Table 12. *Analysis of the base fractions of edestin and egg albumin*

	Edestin				Egg albumin			
	N as % of total protein-N			Wt. (% of protein)	N as % of total protein-N			Wt. (% of protein)
	1st base solution	2nd base solution	Total		1st base solution	2nd base solution	Total	
Arginine	28·47	0·33	28·80	16·69	8·20	2·85	11·46*	5·63
Histidine	3·20	0·27	3·47	2·39	2·27	0·23	2·50	1·45
Lysine	2·01	0·38	2·39	2·32	5·98	0·17	6·15	5·06

* Included 0·41% isolated as flavianate at a later stage than the 2nd base precipitation.

Precipitation of arginine as flavianate. 30 ml. of a solution of arginine dihydrochloride containing 0·2084 g. arginine were treated with 1·84 g. (5 mol.) flavianic acid according to Vickery's directions. After 4 days at 2° the precipitate was filtered off, washed with 30 ml. of water saturated at room temperature with arginine monoflavianate and recrystallized from 25 ml. via NH_3 and H_2SO_4. The precipitate weighed 0·5805 g. (theory 0·5845 g.) and on further recrystallization 5 times successively under the same conditions, the losses in weight were 3·0, 3·7, 2·2, 3·2 and 2·4 mg. respectively, or an overall mean of 3·0 mg. If this correction be applied to the weight of flavianate obtained after 1 recrystallization, giving a total of 0·5840 g. in all, the recovery is almost theoretical and confirms Vickery's claim that the diflavianate, in which form most of the arginine separates in the initial stage, is insoluble in the presence of an excess of flavianic acid. A similar experiment, using a volume of 50 ml. for the preliminary separation and for all subsequent recrystallizations, gave a mean overall loss of 4·0 mg.

Precipitation of arginine as flavianate from a mixture of bases. (1) The solution (30 ml.) contained 0·2084 g. arginine, 0·171 g. lysine and 0·0505 g. histidine, the relative proportions being approximately the same as in egg albumin. It was treated with 1·84 g. flavianic acid and the recrystallized product (from 25 ml.), which appeared to consist of plates with the golden yellow lustre characteristic of arginine monoflavianate, weighed 0·6465 g. The losses on successive recrystallization were 46·9, 15·8, 6·8, 7·9, 3·1, 2·2 and 3·1 mg. suggesting that 5 recrystallizations in all were necessary to give pure arginine monoflavianate. Applying the overall correction ($8 \times 3{\cdot}0$ mg.) the corrected weight was 0·5848 g. giving a theoretical estimation of the arginine present. (2) The solution (30 ml.) contained the same weight of arginine as before, and the other two bases in the relative proportions that occur in edestin. After 1 recrystallization the weight of the product was 0·595 g. and the losses in 2 subsequent recrystallizations were 19·2 and 3·0 mg. respectively. Applying the overall correction of 9·0 mg. the yield of pure monoflavianate was 0·5818 g. In both these experiments the arginine content, calculated from the product obtained on 1 recrystallization, was in excess of that known to be present, whereas if the recrystallization be continued until the operational loss is no more than 3·0 mg. and the appropriate correction be applied, the recovery is satisfactory. These results are not strictly in keeping with Vickery's deductions, and it became of interest to apply a similar treatment to products obtained directly from protein hydrolysates. In the following experiments Vickery's directions were adhered to as closely as possible and the flavianate was separated by using 5 mol. flavianic acid/mol. arginine present.

Arginine content of edestin. Details of four experiments, using different concentrations of protein, are given in Table 13. In the 1st the arginine content of the protein, calculated from the weight of recrystallized flavianate, was 16·75%, a value in excellent agreement with that of Vickery [1940] and also with that obtained in our comprehensive analysis (Table 3). The product, nevertheless, was not pure arginine monoflavianate, and in each of the four replicate determinations made it was necessary to recrystallize 5 times in all before the operational loss in weight was reduced to 4 mg. Allowing for overall losses ($5 \times 4 = 20$ mg.) the corrected arginine content of the protein was $16{\cdot}23 \pm 0{\cdot}03$%. The other three experiments, in which different concentrations of protein were used, gave corrected values in close agreement with this figure.

This result was somewhat disconcerting, for our own experiments with a solution of arginine, confirming those of Vickery, show that the product obtained after only 1 recrystallization does not contain any arginine diflavianate. Appropriate Tristram-Block analysis of the collected edestin mother liquors from the 2nd to the 5th recrystallization moreover failed to disclose the presence of any appreciable amount of histidine. The extraneous material was clearly some other amino-acid flavianate, and indirect evidence in support of this is furnished by the fact that in these edestin experiments, and also in those of other proteins quoted later, the 1st recrystallization product was slightly hygroscopic (as Vickery records). According to our experience pure arginine monoflavianate, after drying in the oven at 102° and cooling to room temperature in a desiccator, gains no more than 1 mg./0·5 g. when exposed for 3 days to the atmosphere of the laboratory. In a recent paper, Vickery, Smith, Hubbell & Nolan [1941] cite experiments with water-melon globulin in which low results (17·44% as against 17·90%) were obtained when the original hydrolysate-flavianic acid mixture, after standing at 6–7° for 4 days, was allowed to stand at room temperature for a short time before filtration. They attributed this to resolution of arginine diflavianate, but from our experience we would suggest that it was the contaminants mentioned above that had been redissolved and that the lower value of 17·44% is the more reliable. In the experiments quoted in Table 13 the hydrolysate-flavianic acid mixture was allowed to stand for 4 days at +2° before filtration; a slightly higher temperature (6–7°) did not significantly affect the results.

Arginine content of egg albumin. The value based on the once recrystallized product is in close agreement with that obtained by Vickery [1940] if allowance is made for the N contents of the proteins concerned. The corrected value, based on the 4 times recrystallized product, is in agreement with that obtained during our comprehensive analysis (see Table 12). For this protein the differences concerned are small.

Arginine contents of horse and cattle haemoglobin. Clarification with charcoal of these two protein hydrolysates did not remove all the haem, which separated at once on adding flavianic acid to the aliquot (25 ml.) used for analysis. The precipitate was centrifuged off immediately and well washed. The collected centrifugate and washings (45 ml.), on standing, gave a voluminous precipitate of yellow crystalline material containing large amounts of histidine and lysine flavianates. After filtering off, the product was liberally washed with the usual wash solution, but in spite of this the recrystallized material was still heavily contaminated and 4 additional recrystallizations were needed to give pure arginine monoflavianate. The corrected value (Table 13) for the arginine content of horse carboxyhaemoglobin is a little higher than the $3{\cdot}59 \pm 0{\cdot}07$% recorded by Vickery [1940], while that for cattle haemoglobin is much higher than the 3·1% (on the globin) recorded by Bergmann & Niemann [1937].

Arginine content of amandin. Accepting 18·75% as the total N content of this protein [Chibnall *et al.*, 1943] our corrected value for arginine is $12{\cdot}54 \pm 0{\cdot}05$%. The value for arginine-N is 21·63% T.P.N.; Vickery [1940] records 22·1% T.P.N.

All these findings lead us to the conclusion that the Vickery procedure, if modified as we suggest, will give a high yield of arginine monoflavianate of unquestionable

Table 13. *Results of arginine analyses by Vickery's method*

Protein used	Edestin				Egg albumin	Horse carboxy-haemoglobin	Cattle haemoglobin
Wt. of protein in aliquot of hydrolysate (g.)	2·935	0·8288	0·97	0·97	2·665	3·565	3·76
Vol. used for precipitation (ml.)	50	50	25	50	25	45	45
Conc. of protein (g./100 ml.)	5·86	1·66	3·88	1·94	10·66	7·92	8·35
No. of determinations	4	2	4	2	4	2	3
Vol. used for recrystallization of flavianate (ml.)	50	50	25	50	25	50	50
Wt. of recrystallized flavianate (g.)*	1·379	0·381	0·5	0·445	0·4264	0·4593 0·4895	0·4516
Arginine content of protein (%)	16·75	16·4	16·5	16·34	5·71	—	—
No. of additional recrystallizations of flavianate	4	2	3	2	3	4	3
Final wt. of flavianate (g.)†	1·316	0·366	0·431	0·428	0·4105	0·3571	0·3659
Overall correction (g.)	0·02	0·012	0·012	0·012	0·012	0·02	0·016
Corrected arginine content of protein (%)	16·23	16·31	16·31	16·20	5·65	3·75	3·62

* In each analysis (except those of the haemoglobins) the replicates did not differ by more than 10 mg.
† In each analysis the replicates did not differ by more than 1 mg.

purity, yet we are not convinced that in all cases, especially when the hydrolysate contains a large amount of arginine, the separation is strictly quantitative. In other words, the method may not indicate with absolute certainty the number of arginyl residues in the protein molecule.

DISCUSSION

It will be seen from Tables 3, 4, 7 and 8, and from the text, that the working losses in the procedure we have adopted for the separation and estimation of the dicarboxylic acids and bases in protein hydrolysates have been reduced to what are probably the lowest possible limits. In the analysis of β-lactoglobulin, which required about 900 working hr. to take to completion, the gross overall loss of N amounted to only 1·25% T.P.N., a remarkable achievement considering the number of operations concerned, for it is less than that which most previous workers have incurred during the preliminary removal of the mineral acid used for hydrolysis. With edestin and egg albumin the corresponding stepwise losses were no greater, but the total overall loss was enhanced by the application of the Cu salt procedure for the removal of certain of the monoamino-acids and proline. Later experience suggests that this loss was greater than it need have been, for we used an unnecessarily large amount of $CuCO_3$ to make the Cu salts with the result that the relatively small amount of N retained by the bulky residue was not readily characterized. At the time these operations were performed the almost complete insolubility of the Cu salt of methionine in boiling water was not realized. In any future analysis we would suggest that the Cu salts at stage 14 be made by the cautious addition of $CuCO_3$ to the boiling solution under conditions that would permit of rapid filtration of the excess before the separation of this very insoluble salt of methionine, which could thus be obtained free from extraneous material. If the protein contains a polysaccharide moiety, as in egg albumin, or polyuronide impurities as in our forage crop preparations, reduction of Cu^{++} to give Cu_2O will occur almost immediately, but it should be possible to remove this with the slight excess of $CuCO_3$ by filtration.

The complete procedure is admittedly very laborious, yet we feel that our final data (Table 14) for edestin, egg albumin and β-lactoglobulin will be of value to those interested in protein analysis, for the errors involved are known within small limits and the results can thus be used to assess the accuracy of new or existing methods of estimation which are less onerous to perform. We have, indeed, already attempted this in our investigations on the dicarboxylic acids [Bailey *et al.*, 1943] and the bases (Section 5).

Table 14. *Dicarboxylic and basic amino-acid analysis of edestin, egg albumin and β-lactoglobulin*

(Small residual solubility corrections have been applied.)

	Edestin		Egg albumin		β-Lactoglobulin	
	N as % total protein-N	Wt. as % of protein	N as % total protein-N	Wt. as % of protein	N as % total protein-N	Wt. as % of protein
Glutamic acid	10·54	20·70	9·70	16·10	13·14	21·51
Aspartic acid	6·77	12·0	5·43	8·13	6·68	9·88
Arginine	28·85	16·71	11·44	5·63	5·95	2·89
Histidine	3·50	2·41	2·50	1·45	2·69	1·54
Lysine	2·44	2·37	6·15	5·06	12·07	9·75

SUMMARY

1. A method for the estimation of the dicarboxylic and basic amino-acids in protein hydrolysates is described, in which these amino-acids are isolated as pure derivatives and in quantitative yield by step-wise removal without overall loss.

2. The cardinal point in the procedure is that no reagent may be introduced into the main hydrolysate unless it can be quantitatively removed at a later stage without appreciable loss of N. Cu^+ and Cu^{++} may be introduced into the hydrolysate and subsequently removed as Cu_2S and CuS respectively at any stage in the analysis; $SO_4^=$ must not be removed as $BaSO_4$, or Cl^- as AgCl until the hydrolysate has been cleared of cystine and the main part of the dicarboxylic acids.

3. In the analytical procedure adopted the cystine is first removed as the cuprous mercaptide of cysteine. The major part of the dicarboxylic acids is then isolated by a modification of Foreman's lime-ethanol method. Following this the bases are removed by precipitation with phosphotungstic acid and then estimated by the gravimetric procedure of Tristram-Block. The solubility factors inherent in the Foreman treatment for the dicarboxylic acid and in the phosphotungstate precipitation of the bases are overcome by repeating these operations at a later stage when the major part of the monoamino-acids and proline has been removed by various means from the collected mother liquors.

4. The method has been applied in turn, with modification suggested by experience, to hydrolysates of edestin, egg albumin and β-lactoglobulin; the overall losses of N were 2·95, 2·86 and 1·25% of the total protein-N respectively. The final values for glutamic acid, aspartic acid, arginine, histidine and lysine are given in Table 14.

5. The accuracy of the Tristram-Block procedure for the direct estimation of the bases and of Vickery's flavianic acid method for the direct estimation of arginine are criticized in light of experience.

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