medium and has been shown to be due to an *N–O* acyl shift in serine and threonine residues.

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Studies on the Amide and *C*-Terminal Residues in Proteins

4. SEPARATION AND QUANTITATIVE DETERMINATION OF β-AMINO ALCOHOLS

BY M. W. REES*
Department of Biochemistry, University of Cambridge

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The procedure for characterization of the *C*-terminal residues in proteins which was described in the first paper of this series (Chibnall & Rees, 1958) called for a method for the separation and estimation of the β-amino alcohols present in the hydrolysate of the esterified and reduced protein. At the time the investigation was started no such method was available, although subsequently the isolation and chromatographic separation of dinitrophenylamino alcohols has been described by a number of workers (Luck & Wilcox, 1953; Jutisz, Privat de Garilhe, Suquet & Fromageot, 1954; Jatzkewitz & Tam, 1954; Grassmann, Hörmann & Endres, 1954; Crawhall & Elliott, 1955).

A partition column of silica gel on gradient elution with ethanol–chloroform has been found to effect a good separation of such of the β-amino alcohols as were encountered when dealing with insulin, lysozyme and β-lactoglobulin. The identities of the products giving rise to the effluent peaks were checked by paper chromatography, and these products could be quantitatively estimated as formaldehyde after periodate oxidation. Because of the limitations to which the reductive procedure is prone (Chibnall & Rees, 1958), no attempt has yet been made to test the behaviour of such other β-amino alcohols as might occur, but there is no doubt that the method of separation is capable of further development. Early experiments on the use of Dowex-50 were not continued because of low recoveries; paper chromatography was also tried but had to be abandoned because of persistent streaking due to traces of inorganic salts and low recoveries.

* Member of the Scientific Staff of the Agricultural Research Council.

MATERIALS AND METHODS

β-Amino alcohols. Alaninol, aspartidiol and glutamidiol were prepared by reduction of the corresponding amino acid ester hydrochloride. They were purified by distillation *in vacuo*. The other β-amino alcohols used were obtained from L. Light and Co., Wraysbury. As most of the products are oils, aqueous solutions were prepared and standardized for total N and formaldehyde given on periodate oxidation. Suitable portions containing 1–3 μmoles of each β-amino alcohol were then taken for chromatography.

Buffer solution (pH 4·85). $Na_2HPO_4,12H_2O$ (A.R., 184·5 g.) and citric acid monohydrate (A.R., 51 g.) were dissolved in water and made up to 1 l. Chloroform (1–2 ml.) was added as preservative.

Solvent A. Lower phase of the mixture: buffer solution–ethanol–chloroform (1:1:1).

Solvent B. Upper phase of the mixture: buffer solution–ethanol–chloroform (1:2:1). The mixtures were shaken in separating funnels and left overnight at 27·5±1° to equilibrate; the solvent phases were filtered before use.

Column chromatography

Preparation of column. Silica gel (5 g., Tristram, 1946) was ground with 2·5 ml. of the upper (aqueous) phase from solvent *A* and a slurry was then made with 40 ml. of the

lower layer. The slurry was pipetted into a siliconed chromatography tube, 25 cm. long and 1·3 cm. int. diam., in five portions, each section being packed under pressure, and the walls of the tube were washed with solvent after each addition. The pressure was maintained for a few seconds after the liquid level had cleared the surface of the column, but care was taken not to let the surface crack. The column should be 9·3–9·5 cm. high and it should have a firm level surface. 8-Hydroxyquinoline (25 mg. dissolved in 1 ml. of solvent *A*) was then added to the column and washed through with fresh solvent. This removed metal ions and was also a visible test of the adequacy of packing. The column was then clamped over a drop-counting fraction collector.

Fractionation on the column. A solution containing the β-amino alcohol hydrochlorides was run into an evaporating basin and taken to dryness in a vacuum desiccator. The residue was then dissolved in 1·5 ml. of solvent *A* and transferred to the column. After the solvent had drained into the column the evaporating dish and sides of the chromatography tube were washed five times with approx. 1 ml. lots of the solvent. A volume (6 ml.) of the solvent was then pipetted on to the column and the chromatography tube was attached by a rubber stopper to a glass-capillary tube that led to a 430 ml. mixing flask containing a glass-covered stirring bar mounted over a magnetic stirrer. The inlet to the mixing flask was a 500 ml. separating funnel having a capillary stem. Solvent *A* (400 ml.) was added to the mixing flask and about 20 ml. to the separating funnel. Both inlet and outlet tubes should be well under the surface of the solvent. Pressure was then gradually applied from a cylinder of nitrogen fitted with a reducing valve and outlet-pressure gauge, until the rate of flow was about 8 ml./hr. The top of the separating funnel was then closed and the cylinder valve shut without further adjustment of the reducing valve. The remaining solvent in the separating funnel was then removed and the funnel was filled with solvent *B* to which had been added 15 ml. of ethanol/100 ml. of solvent. The gas cylinder was again connected and the valve opened. After waiting for the pressure to build up, the separating-funnel tap was opened and fractions of 2·6 ml. were collected in 13 cm. × 1·6 cm. test tubes. All the above operations were carried out in a room controlled at 27·5 ± 1°.

The fractions, in racks of 25 tubes, were evaporated almost to dryness in a water bath after the addition of 1 drop of 5% (w/v) phosphoric acid in ethanol and a small glass bead. The temperature of the bath was raised slowly so as to eliminate the danger of loss by ebullition. The tubes were finally dried for 30 min. *in vacuo* over P_2O_5 and KOH.

Comments. When solvents *A* and *B* are mixed the buffer solution tends to precipitate. The addition of ethanol minimizes the effect and the small amount of aqueous phase that separates in the mixing flask during the first few hours of the chromatographic run is gradually re-adsorbed and does not interfere with the fractionation.

The air locks formed between the solvent and stoppers in the mixing flask and chromatographic tube are necessary to prevent contact of the solvent with the rubber. A mixing chamber of the type described by Moore & Stein (1954) could not be used in the present work because the plasticizer which is a component of the synthetic (polyvinyl chloride) tubing was extracted in the solvents and caused interference.

Analysis of fractions. β-Amino alcohols react with ninhydrin to give the familiar blue colour, but the colour yield depends on the amino alcohol concerned and is always lower, sometimes much lower, than that given by the corresponding amino acid; ethanolamine, for instance, gives 70% and alaninol 16% of the colour yield obtained from leucine. β-Amino alcohols also react with periodate to give formaldehyde, and all those that we are concerned with give 1 mol. equivalent except ethanolamine and serinol, which give 2. A simple colorimetric method of estimating formaldehyde, based on the specific reagent (chromotropic acid) of Eegriwe (1937), has been developed, and has been used in the present research as an indirect method for the quantitative estimation of β-amino alcohols.

Estimation of β-amino alcohols as formaldehyde after periodate oxidation

Reagents. Saturated solutions of K_2HPO_4 and $Na_2S_2O_5$. Periodate reagent: $NaIO_4$ (1·07 g.) was dissolved in water (44·7 ml.) and 5·3 ml. of 2N-NaOH added. Chromotropic acid: the acid (0·9 g.; Judex special reagent) and $Na_2S_2O_5$ solution (0·5 ml.) were adjusted to 25 ml.; the solution was stored in a dark bottle. H_2SO_4: 98–100%, N-free. All reagents are stable over several weeks.

Procedure. To each tube in the rack of 25 was added 0·1 ml. of the K_2HPO_4 solution followed by 0·8 ml. of water and, after mixing, 1 drop (approx. 0·02 ml.) of periodate solution. The oxidation was allowed to proceed for 4 min., when 1 drop of $Na_2S_2O_5$ solution was added to destroy the excess of periodate. Chromotropic acid solution (0·1 ml.) was then pipetted into each tube and, after cooling in an ice bath, 4 ml. of H_2SO_4 was slowly added from a burette. The tubes were shaken and the colour was developed by heating in a boiling-water bath for 10 min. The tubes were again cooled, diluted with 5 ml. of water and the optical density determined at 570 mμ or in a photoelectric colorimeter fitted with a suitable filter.

Values for each peak were obtained from a standard curve (Fig. 1) plotted from readings obtained with a series

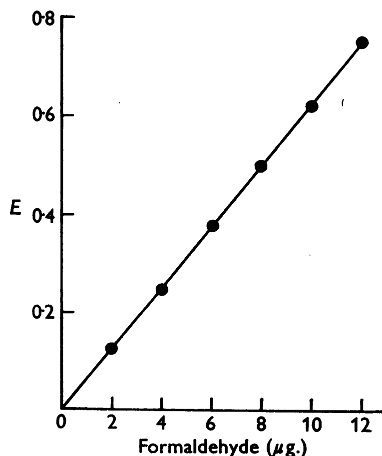

Fig. 1. Typical standard curve from formaldehyde given on periodate oxidation of serine. Sulphuric acid free from nitrogen was used.

of solutions of serine of known concentration or a standardized formaldehyde solution. The standard curve should be checked at frequent intervals and blank readings for the reagents should be of the order of 0·02 on the optical-density scale.

Comments. The colour developed from formaldehyde is stable for 24 hr. provided that the tubes are screened from direct sunlight. Care must be taken to check the standard curve whenever a fresh lot of H_2SO_4 is used, as on two occasions gross interference has been observed. In each case the colour yield was approximately half the maximum given by other lots of H_2SO_4, though it was still proportional to the amount of formaldehyde present. Russell (1944) has reported a similar anomaly with the colorimetric determination of acetaldehyde by *p*-hydroxydiphenol in the presence of H_2SO_4, which she traced to the presence of traces of nitrates or nitrites in the particular sample of H_2SO_4 used.

Identification of β-amino alcohols

Paper chromatography. To check the identity of the amino alcohol peaks the fractions concerned are collected and the solvent is removed. Water (1 ml.) is added to each fraction and 0·5 ml. taken for the estimation of formaldehyde given on periodate oxidation. The remainders of the fractions from each peak are then bulked and, after concentration to 1–2 ml., passed through a column (1 cm. × 5 cm.) of Dowex-2 × 10 in the free-base form. The eluate is then acidified with HCl, evaporated to dryness and the β-amino alcohol hydrochloride subjected to paper chromatography with either propanol–water (80:20, v/v) or the solvents described by Fromageot, Jutisz, Meyer & Penasse (1950), in the manner described below.

When dealing with products derived from esterified and reduced proteins or obtained from the buffered columns the presence of inorganic salts can interfere with the paper chromatography. In such cases the following procedure was found useful. In phenol (70 g.)–water (30 ml.) to which had been added 5% (v/v) aq. NH_3 soln. (sp.gr. 0·880), the amino alcohols travelled with the solvent front and the clearly visible salt spot was some distance behind. A sample of material from the amino alcohol peak, and several suitable marker spots, were placed on a line 1·5 cm. from the edge of a sheet of Whatman no. 1 filter paper and run as an ascending chromatogram for 6 cm. with the phenol solvent just mentioned. After removal of the phenol by drying at 45° and washing the paper with dry ether, the section containing the salt was cut off and the remainder of the paper chromatographed in the usual way with propanol–water or the other solvent mixtures mentioned above. The colour development with ninhydrin is slower than with α-amino acids and in view of the differences in intensity of colour given by different β-amino alcohols, to which attention has already been drawn, a visual comparison of spots can be very misleading.

Oxidation to α-amino acids. In most cases this can be effected smoothly with Ag_2O and the procedure can be useful. The amino alcohol fraction containing from 1 to 10 μmoles (10 ml.) was run into a glass tube and Ag_2O (400 mg.) added. The tube was then sealed and heated overnight at 100°, the contents being shaken occasionally during the first 2 hr. After cooling, the tube was opened, HCl (0·4 ml.) added and the contents of the tube heated in a boiling-water bath for 5 min. to precipitate AgCl. After centrifuging and washing twice with water the combined liquors were evaporated to dryness *in vacuo* at 40° and the residue was taken up in water. The amino acids present were then separated from any unoxidized material and salts by adsorption on a column (1 cm. × 5 cm.) of Dowex-2 × 10 in the free-base form and washing the column with water until it was salt-free. The amino acids were then eluted from the column with N-HCl (20 ml.) and, after removal of excess of mineral acid by evaporation, they were identified by paper chromatography. Ethanolamine and leucinol gave the corresponding amino acids in 75% yield, but, as one might expect, the recoveries of tyrosine from tyrosinol were low and erratic.

RESULTS

A typical effluent concentration curve from a mixture of the β-amino alcohols listed is given in Fig. 2 (*a*), and the recoveries are summarized in Table 1. The effluent volume for a given amino alcohol was found to be reproducible to ± 5% and the position of the peaks relative to one another varied by no more than ± 2%. Rates of flow between 4 and 12 ml./hr. appeared to have no effect on the shape of the peaks but increasing the temperature over the range 18–30° gave progressively sharper peaks.

Fig. 2 (*b*) shows the concentration curves obtained from the β-amino alcohol hydrochloride fractions of hydrolysates of 16 mg. of reduced insulin and reduced insulin ester that had been separated from the amino acids by the method described in the first paper of this series (Chibnall & Rees, 1958). Before chromatography most of the lithium was removed from the β-amino alcohol hydrochloride fraction by passing it through a column (20 cm. × 1·5 cm.) of Dowex-2 × 10 in the free-base form. The amino alcohol hydrochloride fraction, in a volume of 1–2 ml., was added to the column and eluted with CO_2-free water. The eluate was collected in fractions of 1·3 ml. and the lithium was located either by a flame test or by spot-testing the fractions on Universal Indicator paper. The lithium came through as a narrow band in fractions 9 and 10, and the β-amino alcohols as

Table 1. *Recoveries of β-amino alcohols from mixtures chromatographed on buffered silica gel*

β-Amino alcohol	β-Amino alcohol (μg.)		Recovery (%)
	Taken	Found	
Phenylalaninol	147	144	98
Leucinol / Isoleucinol	141	136	96
Valinol	90·4	88·6	98
Tyrosinol	171	165	96
Propanolamine	153	150	98
Aspartidiol	142	138	97
Ethanolamine	62·7	61·1	99
Mixture	907·1	882·7	97·4

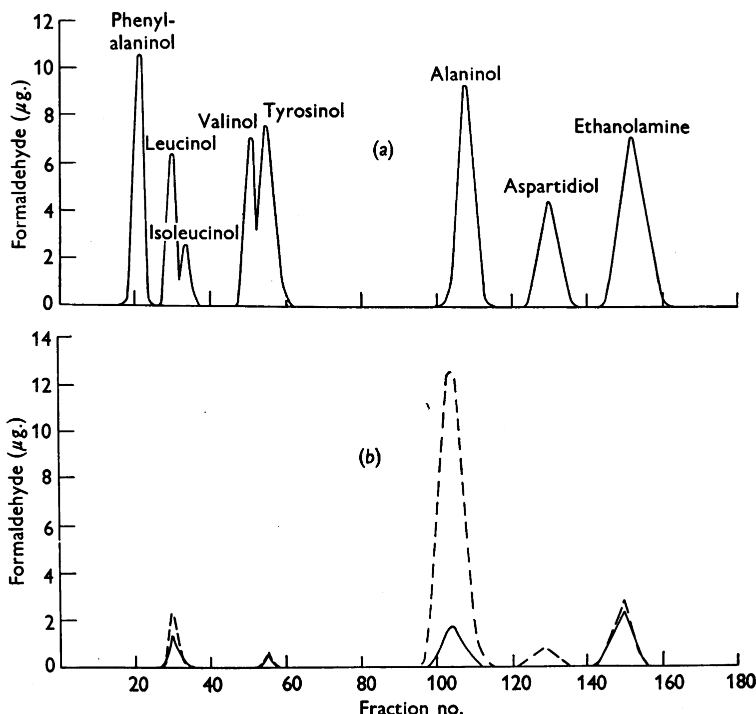

Fig. 2. (*a*) Chromatographic separation of β-amino alcohols from a synthetic mixture on a column of buffered silica gel (1·3 cm. × 9·5 cm.), gradient elution with ethanol–chloroform being used. (*b*) Separation of β-amino alcohols from hydrolysates of insulin ester hydrochloride reduced with $LiBH_4$ (broken line) and insulin reduced with $LiBH_4$.

one diffuse peak extending from fractions 9 to 46. Fractions 11 to 46 were bulked and, after the addition of 2–3 drops of conc. HCl, taken to dryness for chromatography. The addition of acid to solutions of β-amino alcohols before concentration is necessary to prevent losses due to volatility. Periodate formaldehyde estimations were carried out on portions from fractions 5 to 10 and the final results were corrected for the amount of β-amino alcohols in fractions 9 and 10, usually of the order of 2–3 %.

DISCUSSION

The conditions used in the column-chromatographic procedure outlined above were chosen to give an adequate separation, in the minimum number of fractions, of the β-amino alcohols obtained from certain proteins that were being investigated primarily for other purposes. Under such conditions, and with a more complex mixture of β-amino alcohols than that portrayed in Fig. 1, some overlapping occurred. Glutamidiol, for instance, was located between aspartidiol and ethanolamine and was not completely separated from them, and serinol occupied a similar position. By making use of a larger column, by increasing the capacity of the mixing flask, by altering the pH of the buffer solution or by a combination of these changes it should be possible to obtain a separation of the components of a mixture different from that found in the present work.

From preliminary experiments with a buffered column of Celite it is clear that adsorption as well as partition was taking part in the separation with silica gel. When using a column (1·3 cm. × 17 cm.) of buffered Celite (8 g.) packed by the method of Chibnall & Spahr (1958) a separation similar to that shown in Fig. 2 was obtained. Nevertheless, under the conditions used, no separation was obtained between leucinol and isoleucinol, and tyrosinol and valinol overlapped to give an asymmetrical peak.

SUMMARY

1. The quantitative chromatographic separation of β-amino alcohols on partition columns of buffered silica gel, with ethanol–chloroform mixtures as eluting agents, is described. The recoveries vary between 96 and 100 %.

2. A method for the quantitative estimation of β-amino alcohols, based on the formaldehyde given on periodate oxidation, has been developed.

3. The procedure has been used to separate and identify the β-amino alcohols present in the hydrolysates of esterified and reduced proteins.

4. The use of silver oxide to oxidize β-amino alcohols to the corresponding α-amino acid is described.

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Studies on the Amide and *C*-Terminal Residues in Proteins

5. ESTIMATION OF ASPARAGINE AND GLUTAMINE RESIDUES

BY A C. CHIBNALL, C. HASELBACH, J. L. MANGAN AND M. W. REES*
Department of Biochemistry, University of Cambridge

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In the introduction to the first paper of this series (Chibnall & Rees, 1958) an outline was given of the modifications that an esterified protein could undergo on reduction with lithium borohydride, and how the various changes produced would become manifest after hydrolysis. In the present paper we deal with one particular aspect of this phenomenon, which offered the possibility of estimating the total number of asparagine and of glutamine residues in the protein molecule concerned. Subject to certain qualifications to be discussed later, asparaginyl and glutaminyl residues which occur in the peptide chains will remain intact until hydrolysis, when they will be converted into aspartic and glutamic acids respectively, whereas those occupying *C*-terminal positions (I) will undergo reduction at the esterified α-carboxyl group to give, eventually, γ-hydroxy-β-aminobutyric acid (γ-β-HAB) and δ-hydroxy-γ-aminovaleric acid (δ-γ-HAV) respectively (II). Aspartyl and glutamyl residues in the peptide chains (III) will undergo reduction at esterified ω-carboxyl groups to give γ-hydroxy-α-aminobutyric acid (γ-α-HAB) and δ-hydroxy-α-aminovaleric acid (δ-α-HAV) respectively (IV), whereas if they occupy *C*-terminal positions (V) they will give the corresponding diols (VI). Products (II) and (VI), having hydroxyl and amino groups attached to adjacent carbon atoms, are susceptible to periodate oxidation, which makes it possible to devise a procedure for determining the various residue species in the protein concerned.

Aspartic and glutamic acids (estimated with ninhydrin), as well as δ-γ-HAV and γ-β-HAB (estimated as periodate-formaldehyde), are separated on a column of Dowex-50 by elution with hydrochloric acid. There is a partial overlap between δ-α-HAV and γ-β-HAB so that the former cannot be estimated by ninhydrin. δ-α-HAV and γ-α-HAB have been computed by difference. The two diols (VI) are estimated after separation on a column of silica gel (Rees, 1958).

Before presenting evidence that such a scheme is of practical value, however, it is necessary to mention five points which may adversely affect the issue. In the first place it has already been shown that full esterification of a protein cannot be achieved without a small concomitant loss of about 6% of the amide N (Chibnall, Mangan & Rees, 1958*b*), which sets a limit to the accuracy with

* Member of the Scientific Staff of the Agricultural Research Council.