depends, in the first place, on the availability of the substrates. The occurrence of 2-(2-aminophenyl)-ethylamine in plants has apparently not been recorded, and Baker, Happold & Walker (1946) have shown that 2-(2-aminophenyl)acetaldehyde is not an intermediate in the formation of indole from tryptophan by tryptophanase. Nevertheless, it is clear from the present results and from those of Mann & Smithies (1955) that the products of the reactions catalysed by plant amine oxidase frequently undergo spontaneous cyclization. Some of these cyclizations may be of importance *in vivo*. The possibility that the products formed by the action of plant amine oxidase on 1:4-diaminobutane and 1:5-diaminopentane may condense with plant metabolites to form alkaloids has already been referred to by Mann & Smithies (1955). It is possible that such condensations may also occur when phenylalkylamines are the substrates of the enzyme. In this connexion Blaschko (1952) has drawn attention to a particularly interesting model alkaloid synthesis by Späth & Berger (1930) in which 2-(3:4-dimethoxyphenyl)ethylamine condenses with the corresponding 2-(3:4-dimethoxyphenyl)acetaldehyde to give a compound which re-arranges itself to form tetrahydropapaverine. The amine is a substrate of animal amine oxidase (Bhagvat, Blaschko & Richter, 1939) and the aldehyde is presumably formed as a result of the enzyme-catalysed oxidation. Blaschko (1952) points out that the amine and aldehyde are, therefore, present simultaneously and that the presence of the amine oxidase creates the conditions under which alkaloid synthesis can take place.

Lastly it should be pointed out that the reaction described in the present work may be catalysed not only by plant amine oxidase but also by the plant monoamine oxidase described by Werle & Roewer (1950).

SUMMARY

1. The oxidation of 2-(2-aminophenyl)ethylamine is catalysed by plant amine oxidase. The oxidation product accumulates in the reaction mixture as indole.

2. The oxidation of indole by hydrogen peroxide is catalysed by peroxidase.

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Biosynthesis of Proteins

3. PRECURSORS IN THE SYNTHESIS OF CASEIN AND β-LACTOGLOBULIN*

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During recent years there has been much discussion of the role of peptides in protein synthesis. It has been suggested that one body protein can be converted into another without complete degradation to amino acids and that, for example, blood-plasma proteins may be used by the tissues without undergoing complete hydrolysis (Yuile, Lamson, Miller & Whipple, 1951). Evidence has been obtained also by Francis & Winnick (1953) that proteins present in embryo extracts can be used by tissue cultures without undergoing degradation and resynthesis. The idea of the conversion of one protein into another through peptide intermediates has also gained indirect support from the

* Part 2: Askonas, Campbell & Work (1954*a*).

demonstration that intracellular cathepsins can catalyse transpeptidation reactions; such reactions would allow the conversion of one protein into another without degradation to amino acids (Fruton, 1950; Hanes, Hird & Isherwood, 1952).

In the present investigations, advantage was taken of the remarkable efficiency of the goat mammary gland as a protein factory and experiments were designed to answer the question, can plasma protein be converted into milk protein without complete degradation to amino acids?

During full lactation a goat may produce between 100 g. and 150 g. of milk protein daily. Nitrogen for the synthesis of this protein could be derived either from blood-plasma protein or from free amino acids of the blood (or from both). Campbell & Work (1952) and Barry (1952) showed that in the rabbit and the goat the free circulating amino acids were the most important precursors of milk proteins, but the possibility remained that some peptides for milk-protein synthesis might be supplied by partial degradation of plasma protein. Later, it was shown that although casein and β-lactoglobulin were synthesized mainly from amino acids, the milk immune globulin was not synthesized in the mammary gland (Askonas, Campbell, Humphrey & Work, 1954; Askonas, Campbell & Work, 1954*a*).

In the present investigation two experiments were carried out. In the first, a lactating goat was given a single injection of a mixture of radioactive glycine, valine and lysine, milk was collected 4 hr. later and the casein partially hydrolysed. From the partial hydrolysate nine radioactive peptides were isolated. In the second experiment, the same animal was given a single injection of a mixture of radioactive valine and lysine and milked after 3 hr. Crystalline β-lactoglobulin was isolated from this milk, partially hydrolysed, and eighteen radioactive peptides were isolated. The distribution of radioactivity in these peptides was such as to permit a decision between alternative theories of milk-protein synthesis.

Preliminary accounts of this work have already appeared (Askonas, Campbell & Work, 1954*b*; Askonas, Campbell, Godin & Work, 1954).

MATERIALS AND METHODS

Animal. A British Saanen goat with a daily milk yield of about 5 lb. was used.

Radioactive amino acids. These were the same as used previously. All injections were intravenous (cf. Askonas *et al.* 1954*a*).

Separation of milk proteins. Casein was precipitated twice at pH 4·5 and washed as described previously (Askonas *et al.* 1954*a*). β-Lactoglobulin was crystallized by the method of Askonas (1954).

Peptides from casein

Partial hydrolysis of casein. Casein (150 mg. N) was suspended in 12 N-HCl (15 ml.) and heated in a sealed tube for 12 hr. at 56°. The bulk of the HCl was removed in a rotary evaporator (Craig, 1950). The residue was dissolved in water (30 ml.) and amino N determined on a small sample using the nitrous acid method of Van Slyke (1913). The proportion of amino N varied slightly in different experiments but was about 45% of total N.

To remove residual HCl, the hydrolysate was shaken with successive small portions of De-Acidite E (The Permutit Co., Gunnersbury Avenue, London, W. 4) (before use the resin was activated by washing with excess N-Na_2CO_3 followed by distilled water until the washings were neutral). When the pH of the hydrolysate had risen to about 3·5 the resin was filtered off and washed twice with 15 ml. water, the washings being added to the main hydrolysate. This fraction (C_1) contained 83% (120 mg.) of the original nitrogen. The resin was washed with N-HCl (20 ml.) followed by water (15 ml.). The combined washings contained 10·6 mg. N and were discarded.

Fractionation of casein hydrolysate (C_1) on charcoal. The charcoal was prepared from Sutcliffe Speakman grade 130 (Sutcliffe Speakman and Co., Leigh, Lancs), by the method of Schramm & Primosigh (1943). Washed charcoal (3·5 g.) was suspended in 5% (v/v) acetic acid and poured into a column (diam. 1·3 cm.). Fraction C_1 dissolved in 5% (v/v) acetic acid was allowed to percolate through the charcoal and washed through with a further 130 ml. of 5% acetic acid. The combined effluents (C_2) contained 69 mg. N. By washing the charcoal with a mixture of phenol, acetic acid and water (5:20:75, v/v) another fraction (C_3, 32 mg. N) was obtained. Fractions C_2 and C_3 were kept separate.

Fractionation of casein hydrolysate (C_2) on Dowex-50 resin. Dowex-50 × 12 resin (200–400 mesh/in., 750 g. wet wt., Microchemical Specialities Co., Berkeley, California) was prepared as described by Hirs, Moore & Stein (1952) and the ammonium salt of the resin equilibrated with 0·1 M ammonium formate buffer (pH 3·0) (all molarities are referred to the cation; the buffer was made from 112 ml. redistilled 98% (w/v) A.R. formic acid, 200 ml. 2 N-NH_3 made from pure NH_3, 1320 ml. ethanol and water to 4 l.). It is essential to degas all buffers at the water pump, since otherwise air bubbles form on the resin and break up the column. The column (3 × 100 cm.) was poured in four lots as recommended by Moore & Stein (1951). When the pH of the effluent was the same as that of the inflowing buffer the partially hydrolysed casein (C_2, 69 mg. N) dissolved in 10 ml. of the formate buffer was added to the column and development started at a flow rate of 30 ml./hr. Gradient elution was begun after collection of 3·35 l. of effluent (25 ml. fractions) by allowing a second buffer, 0·2 M, pH 4·9 (250 ml. 2 N-NH_3, 70 ml. 98% formic acid, 825 ml. ethanol and water to 2·5 l.) to flow from a supply reservoir into 3 l. of the pH 3·0 buffer in a mixing chamber. When the total volume collected was 7·6 l., the reservoir was emptied and refilled with pH 6·1, 0·5 M buffer (970 ml. 2 N-NH_3, 70 ml. 98% formic acid, 1320 ml. ethanol and water to 4 l.) and at 10·5 l. effluent again emptied and refilled with pH 7·1, 0·5 M buffer (500 ml. 2 N-NH_3, 37 ml. 98% formic acid, 660 ml. ethanol and water to 2 l.). This buffer was continued to a total effluent volume of 15·1 l., when the column was stopped (605 fractions of 25 ml.).

Analysis of column (C_3) effluent. Each effluent fraction was concentrated to 5 ml. and 200 μl. of this concentrate analysed by descending single-dimension paper chromatography using Whatman no. 3 paper and, in the first instance, phenol–NH_3 as solvent. Peptides were detected by spraying with the collidine–ninhydrin reagent of Lewis (1952). Satisfactory resolution was usually obtained with this solvent in the presence of ammonium formate. Where resolution was unsatisfactory owing to high R_F values a second chromatogram was run in butanol–acetic acid, after sublimation of ammonium formate. Fractions were pooled on the basis of qualitative similarity in their chromatographic pattern. The buffer was removed from the bulked peptide fractions by sublimation *in vacuo* and the peptides converted into their dinitrophenyl (DNP) derivatives. While this method allowed us to isolate some peptides (Table 2) it was laborious and, in dealing with the partial hydrolysate of lactoglobulin, another method of effluent analysis was developed (see below). The isolation of DNP peptides from the pooled fractions is not described in detail since, although each was treated differently, the same general principle was followed in all cases. An example of such a fractionation is given later for β-lactoglobulin.

Peptides from β-lactoglobulin

Partial hydrolysis of β-lactoglobulin. The twice-recrystallized protein was freed from salts by dialysis and freeze-dried. A sample (1·68 g.) was suspended in 12 N-HCl (25 ml.) and hydrolysed as described for casein. The amino N in the hydrolysate was 45% of total N. After treatment with Deacidite E in the same way as casein, the unadsorbed fraction (L_1) contained 184 mg. N. The acid washings from the resin were discarded.

Fractionation of lactoglobulin hydrolysate (L_1) on charcoal. Fraction L_1 (184 mg. N) was passed through a column of 5 g. charcoal in the same way as C_1 to give an acetic acid eluate (L_2, 99 mg. N) and a phenol–acetic acid eluate (L_3, 50 mg. N).

Fractionation of amino acids on Zeo-Karb 225 WR 1·55. The resin used for the fractionation of lactoglobulin peptides, a sulphonated polystyrene, was specially made by the Permutit Co. as micro-beads (25–60 μ. diam.). Although essentially similar to the Dowex-50 × 12 used for casein it had a much lower degree of cross-linking. We do not consider it satisfactory to define resins by the percentage cross-linking agent since this is difficult to control accurately and this resin is defined as WR 1·55 using the water-regain method of Pepper (1951, Fig. 7). WR 1·55 corresponds to about 5% cross-linking agent.

We have already reported on the use of this resin for the quantitative estimation of amino acids in protein hydrolysates, using sodium citrate buffer (Campbell, Jacobs, Work & Kressman, 1955). For the separation of peptides we preferred to use ammonium formate buffer since it is readily sublimed from the effluent. In order to find suitable conditions for operation with this buffer, columns were run using different initial pH values and with a complete hydrolysate of lactoglobulin as test mixture. Satisfactory results were obtained starting with 0·2 M ammonium formate (pH 2·46) as first buffer and carrying out gradient elution with progressive rise in pH and molarity.

The resin (about 1·5 kg. wet wt.) was first washed as recommended by Hirs *et al.* (1952), suspended in 2 l. of 4 N-NH_3, left overnight, filtered and washed first with water and then with ammonium formate buffer (0·2 M, pH 2·46) (A.R. formic acid, 410 ml. of 85% (w/v); 400 ml. 2 N-NH_3 and water to 4 l.). The column (150 × 3 cm. diam.) was prepared by dividing the resin into four roughly equal portions suspended in buffer. Each portion was poured into the column and allowed to settle before adding the next. A sample of completely hydrolysed β-lactoglobulin (60 mg. N) was dissolved in formate buffer (7 ml.) and added to the column. Elution was commenced with the pH 2·46 buffer, the flow rate being 30 ml./hr. After collecting 14 l. of effluent, gradient elution was begun with pH 5·5 buffer (0·2 M; 38 ml. of 85% formic acid, 400 ml. 2 N-NH_3 made up to 4 l.). The second buffer was allowed to flow from a supply reservoir into 3 l. of pH 2·46 buffer in a mixing chamber. Conditions for further development of the column and the order of elution of amino acids are given in Fig. 2. The composition of the effluent was determined as described for the casein column C_3.

Fractionation of lactoglobulin peptides (L_2) on Zeo-Karb 225 WR 1·55. A portion of lactoglobulin partial hydrolysate L_2 (68 mg. N) was evaporated to dryness, redissolved in formate buffer (7 ml.) and added to a column prepared as above. Elution was commenced with the pH 2·46 buffer and 60 ml. fractions were collected (30 ml./hr.). When 14·5 l. of buffer had been collected (tube 243) gradient elution was begun with 0·2 M, pH 5·5 formate buffer. When the total volume collected was about 22 l. (tube 362) the supply reservoir was emptied and refilled with 2·0 M buffer pH 5·5 (895 ml. 4·47 N-NH_3, 190 ml. 85% formic acid and water to 2 l.). This was continued until the total volume of effluent was about 28 l. (tube 462) when the reservoir was again emptied and refilled with a similar 2·0 M buffer containing 25% ethanol. Elution with this buffer was continued to tube 595 (35·7 l.).

Detection of radioactivity in column (L_3) effluent. Each 60 ml. fraction was transferred to a Pyrex evaporating basin and the solvent and buffer were removed in a specially designed multiple-sample evaporator (Fig. 1). The Pyrex

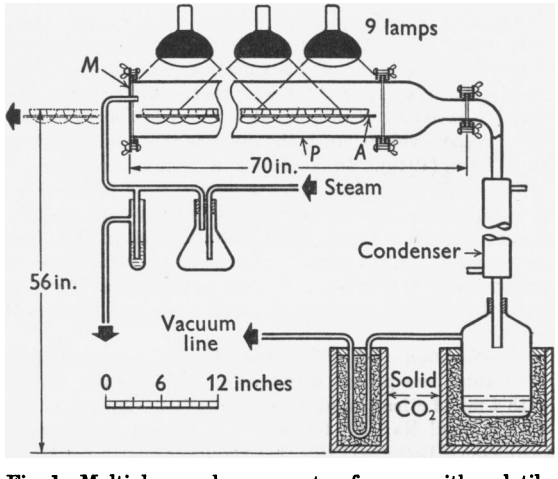

Fig. 1. Multiple-sample evaporator for use with volatile buffers. *M*, metal flange with controlled air leak; *P*, Pyrex pipe. *A*, perforated aluminium tray. For method of operation see text.

pipe line *P* (6 ft. × 6 in.) was closed at one end with a rubber-covered metal flange (*M*) carrying an adjustable air leak and connected at the other end through an adaptor to the vertical copper condenser and CO_2-cooled vapour trap. Samples were pushed into the pipe line on a perforated aluminium tray (*A*) and heated by a series of nine infrared lamps with rheostat control of heat input. After loading the tube with effluent samples it was evacuated to about 10 mm. Hg (oil pump) and when the initial bubbling had ceased the lamps were switched on and the power increased slowly. After about 4 hr. the buffer began to crystallize in the bottom of each dish and at this stage heat input was reduced to avoid spluttering. Final sublimation of the buffer could be accelerated by connecting a steam supply to the air leak. The vacuum of 10 mm. Hg could be maintained even with a rapid flow of steam which helped to carry the subliming buffer into the condenser.

When buffer had been made from A.R. formic acid and freshly distilled NH_3, dishes which contained buffer only were chemically clean when removed from the evaporator. All dishes containing effluent samples were washed with 0·5 ml. distilled water containing a trace of detergent, the washings transferred to a nickel planchet (5 sq.cm.), evaporated to dryness and assayed for radioactivity using an automatic recording Geiger counter with a thin end window. Fractions were pooled on the basis of a plot of radioactivity against fraction number (Fig. 3).

Isolation of DNP-peptides

The peptide-containing fractions from both ion-exchange columns (C_3 and L_2) were treated in the same way. Each group of tubes was a mixture of different peptides and amino acids and purification of each was an individual problem. A detailed account of the purification of the twenty-eight radioactive peptides isolated is not necessary; the same general procedure was followed in each case and the treatment of only one group of pooled fractions is outlined.

Preparation of silica. The method of Porter (1950) was used for the preparation of silica gel. Porter noted that the qualitative behaviour of different batches of gel varied even when the method of preparation was standardized. Advantage was taken of this variation; five batches of gel were prepared and tested using synthetic DNP-amino acids. Batch 2 gave high *R* values, batch 3 very low *R* values and batch 5 gave values much the same as those published by Blackburn (1949). In analysing a complex mixture of DNP-peptides the three silica gels were used in the order 2, 5, 3. Gel 2 was usually mixed initially with half its weight of water. Bands which travelled fast in this silica were transferred to a second or third column using buffer of progressively higher pH as stationary phase and changing from the non-adsorbent to the more adsorbent batches of silica.

Fractionation of L_2 tubes 444–449. The material from these tubes was pooled, dissolved in 2 ml. of water diluted with 4 ml. of ethanol and treated with fluorodinitrobenzene (20 mg.) and $NaHCO_3$ following the general directions of Schroeder & LeGette (1953). The resultant mixture of DNP-peptides was extracted into ethyl acetate and taken to dryness, redissolved at once in 2 drops of hot *n*-butanol and diluted with 10 ml. of washed chloroform. Owing to the sparing solubility of some DNP-peptides in chloroform there is a tendency for peptide to come out of solution at this stage; transfer of the solution to a silica gel column should, therefore, follow immediately. *Column* 1. The column (15 g. silica batch 2 mixed with 7·5 ml. water and packed in a 2 cm. diam. tube) was developed at once with washed chloroform followed by chloroform containing successively 2, 4, 7 and 16% *n*-butanol (v/v). Six separate yellow bands (L_2-444 *A* to L_2-444 *F*) were collected in the effluent, each was taken to dryness, transferred to a 2 sq.cm. planchet containing a 2 sq.cm. disk of lens tissue (Fager, 1947) and counted, using a thin-window Geiger counter. These counts had no absolute significance and were used only to decide which bands were worth further investigation. The counts were as follows (counts/min. above background): L_2-444 *A*, 384; L_2-444 *B*, 0; L_2-444 *C*, 227; L_2-444 *D*, 66; L_2-444 *E*, 74; L_2-444 *F*, 0. Bands *B*, *D*, *E* and *F* were discarded along with an inactive band *G* which had not moved in 16% butanol and was eluted with ethyl methyl ketone. *Column* 2. L_2-444 *A* was further fractionated on a second silica column (15 g. batch 5 + 7·5 ml. buffer; 0·5 M-NaH_2PO_4 7 vol. to 3 vol. 0·5 M-Na_2HPO_4), starting with chloroform and following with the chloroform–butanol mixtures used previously. L_2-444 *A* separated into two bands, L_2-444 A_1 (153 counts/min.) and L_2-444 A_2 (142 counts/min.). *Column* 3. Band L_2-444 A_1 was fractionated on batch 3 silica (15 g. silica, 7·5 ml. buffer; 0·5 M-NaH_2PO_4 4 vol. to 6 vol. 0·5 M-Na_2HPO_4) with chloroform–butanol as before. A single band was formed with no sign of separation into multiple components and his material was judged to be a pure DNP-peptide. This was confirmed by acid hydrolysis (0·2 ml. 12 N-HCl for 8 hr. at 100°) and paper-chromatographic analysis of the hydrolysate, when only one DNP-amino acid was found. After removal of HCl *in vacuo*, the hydrolysate was separated into an ethyl acetate-soluble and a water-soluble fraction, both yellow. The ethyl acetate-soluble fraction (not radioactive) was chromatographed on phthalate-buffered paper with *tert.*-amyl alcohol (Blackburn & Lowther, 1951) and the single spot identified as DNP-leucine. One-fifth of the water-soluble fraction was chromatographed in butanol–acetic acid, a single yellow component corresponding to N^{ϵ} DNP-lysine was detected, no additional spot showed after spraying with ninhydrin. Peptide L_2-444 A_1 was therefore leucyllysine (or isoleucyllysine; the methods used do not distinguish between leucine and isoleucine). The remaining four-fifths of the water-soluble hydrolysate was treated with fluorodinitrobenzene (cf. Schroeder & LeGette, 1953) and the resultant bis-DNP-lysine purified by chromatography on two silica columns, estimated colorimetrically and assayed for radioactivity (see below). *Column* 4. Band L_2-444 A_2 was fractionated on 15 g. of batch 5 silica using the same buffer and solvent mixture as for column 3. Two bands formed on the column, the faster (L_2-444 A_3) contained all the radioactivity and the slow band was discarded. The material from band A_3 was hydrolysed and analysed in the same way as L_2-444 A_1. (The results are given in Table 3.) *Column* 5. Band L_2-444 *C* was fractionated on 15 g. of batch 3 silica with 7·5 ml. buffer (4 vol. 0·5 M-NaH_2PO_4 to 6 vol. 0·5 M-Na_2HPO_4) starting with chloroform and developing with 2% butanol in chloroform. Two bands separated; the faster band was not radioactive and was discarded, the slower band L_2-444 C_1 was judged to be pure and was hydrolysed to give N^{α} DNP-lysine, proline and methionine. The lysine was converted into bis-DNP-lysine, purified and assayed for radioactivity (Table 3).

Isolation of DNP-amino acids and errors in determination of radioactivity

For determination of the radioactivity of DNP-amino acids from a complete hydrolysate of protein, or from purified and hydrolysed DNP-peptides, the methods already outlined were used (Askonas *et al.* 1954*a*). For the micro-scale purification of DNP-glycine, DNP-serine, DNP-valine and bis-DNP-lysine from peptides, silica and Celite columns were used. Where there was doubt about the purity of the band eluted from the first column the material was refractionated on a second column at a different pH and with a different solvent system.

Glycine. Suitable systems were: (*a*) 0·25 M-KH_2PO_4–0·25 M-Na_2HPO_4 (97·5:2·5 by vol.) with 1·2% (v/v) butanol in chloroform as moving phase (silica batch 3-buffer 2:1, w/v); (*b*) Celite as described previously (Krol, 1952); (*c*) as for (*e*) below.

Serine. Suitable systems were: (*d*) Celite as described previously (Campbell & Work, 1952). (It should be noted that there is an error in the description of the buffer system used, the vol. ratio 0·25 M-Na_2HPO_4 : 0·25 M-NaH_2PO_4 should be 2:8 not 1:9 as given earlier.); (*e*) silica batch 2 with a *cyclo*hexane–ether mixture (2:1, v/v) as moving phase and water as stationary phase (this mixture could be used also for glycine).

Valine. For DNP-valine, suitable systems were as follows: (*f*) buffer, equal vols. of 0·5 M-NaH_2PO_4 and 0·5 M-Na_2HPO_4, with 2% butanol in chloroform as moving phase (silica batch 5–buffer 2:1, w/v); (*g*) same salt solutions in the ratio 4:6 with 5% propanol in *cyclo*hexane (silica 5).

Lysine. For bis-DNP-lysine two suitable systems were: (*h*) buffer as in (*g*) with 10% butanol in chloroform (silica 5–buffer 2:1, w/v); (*i*) unbuffered batch 5 silica with water as stationary phase and chloroform as solvent, followed when the band was near the bottom of the column by 0·5% butanol in chloroform.

The determination of the specific activity of DNP-glycine and DNP-valine on a micro scale has been described previously. The error was ±8%, estimated by a parallel macro experiment in which the DNP-glycine and DNP-valine were crystallized and weighed (Askonas *et al.* 1954*a*; Campbell & Work, 1952). To test the reliability of the lysine method on a micro scale a sample of synthetic (^{14}C) radioactive bis-DNP-lysine was made and purified to constant radioactivity by crystallization from aqueous formic acid. Five small samples (range 75–514 μg.) of this material were pipetted from a standard solution, no. 2 was mixed with DNP-valine, no. 3 with DNP-proline, no. 4 with DNP-alanine, no. 5 with DNP-glycine and no. 1 was kept pure. Each sample was repurified on silica gel, the bis-DNP-lysine band collected, transferred to 0·5% $NaHCO_3$, estimated colorimetrically (cf. Krol, 1952), diluted with a weighed sample of bis-DNP-lysine (20–30 mg.) and counted at infinite thickness on a 1 sq.cm. disk. The relative activities of the samples were calculated as 38200, 39000, 38000, 36400, 37000 as compared with 39600 for the original synthetic material. Here, the specific activity of the lysine was high enough to permit counting to an error of ±2%. With the less active lysine from milk, the counting error was ±5%. The over-all error of the method is likely to be about ±15%.

RESULTS

Casein

Casein was obtained from a sample of goat milk (540 ml.) collected 4 hr. after injection of a mixture of glycine, DL-valine and DL-lysine (250, 25 and 40 μc; cf. Askonas *et al.* 1954*a*, Table 3). To determine whether any one amino acid had uniform radioactivity in different parts of the protein molecule, the casein was partially hydrolysed with acid as described in the experimental part of this paper and radioactive peptides were isolated.

The partial hydrolysate was fractionated, first by adsorption on charcoal (cf. Sanger & Thompson, 1953). This divided the partial hydrolysate into two main fractions C_2 and C_3, C_2 being the material which could be eluted by 5% acetic acid and C_3 the material which could not be desorbed with this solvent but was removed by a mixture of phenol

Table 1. *The distribution of radioactivity after fractionation of partial hydrolysates of casein (C_2 and C_3) on Dowex-50 × 12 resin*

Details of the preparations of C_2 and C_3 are given in the Experimental section. Radioactivity was determined on $\frac{1}{10}$ of each pooled sample. These values have no absolute significance.

C_2		C_3	
Tube no.	Radioactivity (counts/min.)	Tube no.	Radioactivity (counts/min.)
1– 53	0	1– 50	62
54– 63	16	51–100	90
64–102	12	101–123	15
103–107	60	124–154	18
108–115	40	155–172	13
116–119	14	173–184	5
120–132	8	185–193	5
133–140	0	194–222	12
141–147	10	223–228	16
148–162	14	229–238	13
163–180	30	239–246	8
181–192	160	247–268	50
193–205	48	269–279	6
206–216	54	280–288	37
217–234	32	289–297	4
235–252	103	298–310	6
253–273	22	311–320	6
274–284	22	321–330	10
285–299	14	331–340	7
300–320	0	341–350	5
321–330	6	351–360	15
331–350	6	361–370	13
351–360	12	371–380	8
361–370	18	381–401	4
371–380	46	402–411	20
381–390	62	412–426	24
391–406	66	427–446	35
407–414	100	447–466	49
415–428	—	467–479	24
429–445	46	480–489	27
446–465	160	490–531	24
466–605	204	532–559	52
		560–580	25

and acetic acid. Both C_2 and C_3 were fractionated by gradient elution from columns of Dowex-50 × 12 resin using ammonium formate buffer in aqueous ethanol (see Experimental).

As judged by paper chromatography of the effluents, fractionation of C_2 on Dowex was fairly effective, few of the ninhydrin-positive spots extending over more than twenty tubes (500 ml.) of effluent. The fractions were combined according to the appearance of paper chromatograms. As shown in Table 1 the resultant 32 fractions were nearly all radioactive but certain peaks of radioactivity were evident. The hydrolysate fraction C_3 was treated in exactly the same way as C_2 but chromatography of the column effluent indicated a less effective fractionation. A large amount of radioactive material came through in the first 100 tubes; the most likely interpretation of this is that C_3 contained peptides too large to be effectively held by Dowex-50 × 12.

two different pH values and with two different grades of silica gel; it is quite possible, however, that in some cases a band was a mixture of two peptides with the same end group; thus, for example, Lys.[Asp, Glu, Leu, Phe] (Table 2) may be a mixture of two tripeptides, but this possibility does not alter our general conclusions. Each radioactive peptide was hydrolysed, its composition determined by paper chromatography and the radioactive amino acid isolated and counted as the DNP-amino acid. The results are given in Table 2.

Some idea of the complexity of each mixture was obtained from the number of bands separated on silica. This was of course a minimum figure, since the material from each band was tested for radioactivity and bands which were inactive, or which gave few counts above background, were discarded as unlikely to give sufficient pure peptide for accurate assay of radioactivity; these discarded bands probably contained in most cases

Table 2. *Radioactivities of amino acids isolated from peptides of fraction C_2 and of amino acids isolated from a complete hydrolysate of casein*

Radioactivity is expressed as counts/min./sq.cm. at infinite thickness and is calculated for the pure amino acids. Under the conditions used 1×10^{-3} μc/mg. C gives 1000 counts/min. The sequence of amino acids enclosed by squared brackets has not been determined.

Radioactive amino acid	Peptides	Fraction numbers	Radioactivity*
Valine	Thr.[Asp, Val]	64–102	1120
	Val.[Pro, Leu, Val]	446–465	1040
	Val.[Pro, Leu, *Val*]	446–465	1680
	Val.[?]	141–162	1100
Complete hydrolysate	—	—	1008
Glycine	Thr.[Gly, Glu]	206–234	10200
	Leu.Gly	235–252	11200
Complete hydrolysate	—	—	11400
Serine	Ser.Pro	253–284	1475
	Ser.Leu	253–284	1334
Complete hydrolysate	—	—	1510
Lysine	Lys [Asp, Glu, Leu, Phe]†	381–406	2780
	Lys [Glu, Phe, Lys]	407–428	3040
	Lys [Glu, Phe, *Lys*]	407–428	2950
Complete hydrolysate	—	—	3170

* Where two radioactive residues are present in one peptide the value refers to the residue which is italicized.
† May be a mixture of two tripeptides.

Isolation of DNP-peptides from casein hydrolysate C_2. The radioactive fractions listed in Table 1 (C_2) were treated with fluorodinitrobenzene. The resultant mixtures of DNP-peptides and DNP-amino acids were partitioned on silica-gel columns by the general methods described in the Experimental section. All the DNP-peptide bands eluted from the columns were tested for radioactivity and inactive or slightly active bands were discarded, A band was generally judged to be due to a single peptide when it showed no sign of fractionation at

several different peptides. The number of bands separated from each fraction were as follows: 64–102, 5; 141–162, 10; 206–234, 15; 235–252, 12; 253–284, 11; 381–406, 12; 446–465, 11.

It might be supposed at first sight that by discarding the less active bands we reduced the chance of finding peptides containing amino acids of low radioactivity. To eliminate any possibility that we were being misled in this way, another sample of the same radioactive casein was completely hydrolysed and radioactive DNP-amino acids isolated

(Askonas *et al.* 1954*a*). The mean specific radioactivity for each amino acid at all points in the molecule was thus determined and found (Table 2) to be close to that calculated for the same amino acid isolated from peptides. Had we discarded from our peptide mixtures amino acids of low specific activity and isolated those of high specific activity, then the mean radioactivity for any amino acid from a complete hydrolysate would have been considerably below that of amino acid isolated from a purified peptide.

Isolation of DNP-peptides from casein hydrolysate C_3. Fractions C_3 (247–268), C_3 (280–288) and C_3 (447–466) were converted into DNP-peptides and fractionated on silica-gel partition columns. Each contained a large number of peptides, but in no case could sufficient of a radioactive DNP-peptide be obtained to permit characterization and

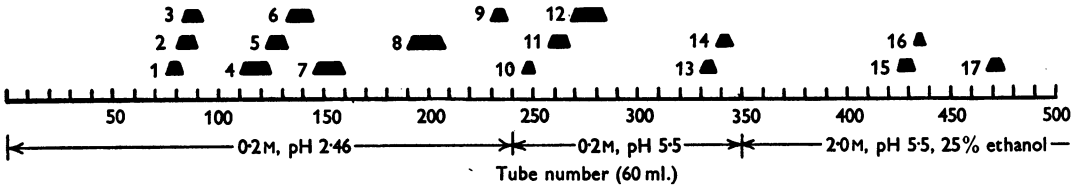

Fig. 2. Fractionation of a complete hydrolysate of β-lactoglobulin on Zeo-Karb 225 WR 1·55 ion-exchange resin. Column 3 × 150 cm., tube volume 60 ml. Gradient elution from tube 240 using ammonium formate buffer. The shaded areas represent the tubes over which individual amino acids were spread. 1, Asp; 2, Thr; 3, Ser; 4, Glu; 5, Pro; 6, Gly; 7, Ala; 8, Val; 9, Met; 10, Cys; 11, Ileu; 12, Leu; 13, Tyr; 14, Phe; 15, Lys; 16, His; 17, Arg.

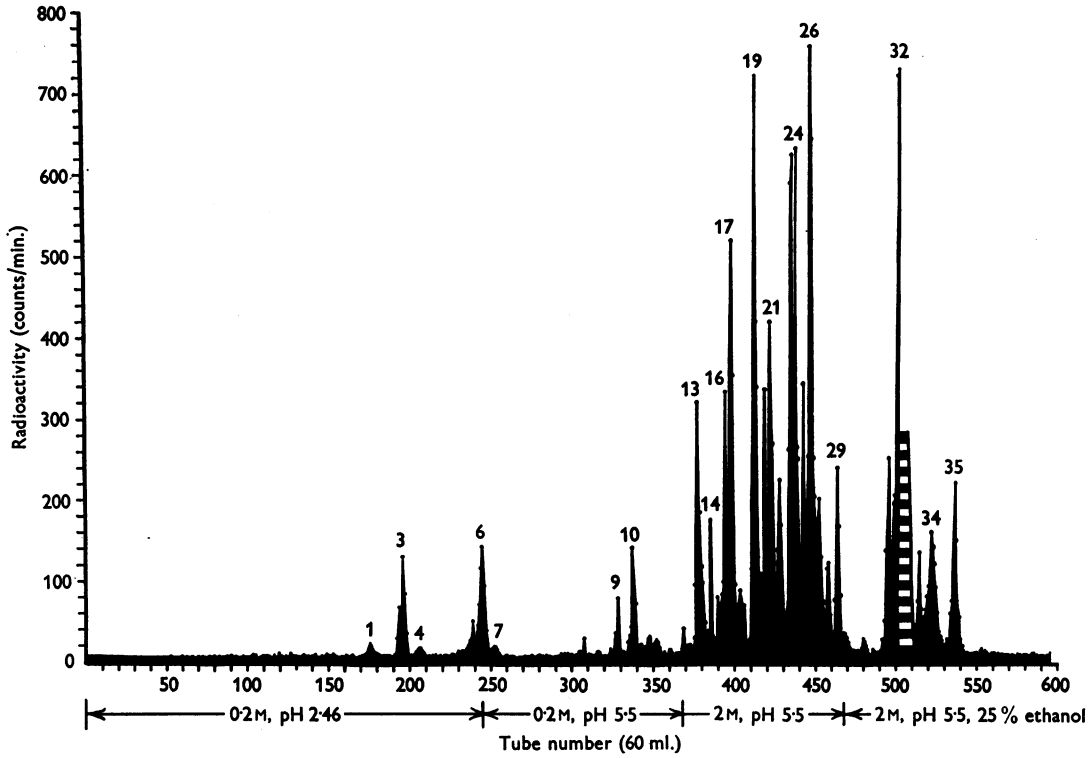

Fig. 3. Fractionation on Zeo-Karb 225 WR 1·55 ion-exchange resin of a partial acid hydrolysate of radioactive β-lactoglobulin. Valine (peak 3) and lysine (peak 19) were the only radioactive amino acids present. The radioactive peptides are listed in Table 3. For positions of amino acids on a similar column see Fig. 2. Tube volume 60 ml. Column 3 × 150 cm. The shaded area below peak 32 represents a peak of unknown contour where 10 tubes were grouped.

accurate assay of radioactivity. As these were the most promising groups of tubes from C_3 (Table 1) it was clear that fractionation of C_3 on Dowex-50 × 12 had not been effective and the remainder of the material was discarded.

β-Lactoglobulin

Goat milk (660 ml.) was collected 3 hr. after injection of DL-valine (160 μc, 112 mg.) and DL-lysine (110 μc, 204 mg.) and diluted to 2 l. with milk collected immediately before injection. β-Lactoglobulin, isolated and crystallized by the method of Askonas (1954), was partially hydrolysed with acid (see Experimental). The partial hydrolysate was fractionated by adsorption on charcoal and elution with acetic acid [L_2] followed by a mixture of phenol and acetic acid [L_3]. Since the casein experiments had shown that the phenol eluate was difficult to fractionate, L_3 was discarded.

As the fractionation of casein hydrolysates on Dowex-50 × 12 was not entirely satisfactory, a new sulphonated polystyrene resin Zeo-Karb 225 WR 1·55 with lower cross-linking (see Experimental) was used to fractionate the peptides in L_2, gradient elution being carried out with aqueous ammonium formate buffer. Solvent and buffer were removed from the effluent in a multiple-sample evaporator (Fig. 1) and the distribution of radioactivity was found to be as shown in Fig. 3. A complete hydrolysate of β-lactoglobulin was also fractionated on this resin (Fig. 2). Since neither valine nor lysine is likely to be transformed in the animal into other amino acids, all the peaks of radioactivity shown in Fig. 3, except for two peaks due to free valine and free lysine, must be due to valine and lysine peptides. It is clear from these figures that fractionation on Zeo-Karb 225 WR 1·55 was extremely effective. Samples of all the effluent fractions were also examined by single-dimension paper chromatography. Each effluent fraction gave a complex chromatographic pattern with from four to twelve components, but few components extended over more than five fractions. On the basis of these chromatograms it was estimated that L_2 was a mixture of at least 500 peptides.

DNP-Peptides from β-lactoglobulin. The material from each radioactive peak (Fig. 2) was treated with fluorodinitrobenzene, and the resultant mixtures of DNP-peptides and DNP-amino acids were fractionated in the same way as the casein hydrolysate C_2. The radioactive peptides isolated are listed in Table 3. Each peptide was hydrolysed and the radioactive amino acid isolated and assayed as the DNP-amino acid; results are given in Table 3.

Table 3. *Radioactivities of amino acids isolated from peptides of fraction L_2 and of amino acids isolated from a complete hydrolysate of β-lactoglobulin (cf. Fig. 3)*

Radioactivity is expressed as in Table 2. Where no value for radioactivity is given the peptide was in too small yield for assay. The sequences of amino acids within squared brackets have not been determined.

Radioactive amino acid	Peptides	Peak no. (Fig. 3)	Radioactivity
Valine	Val.[Ala, Gly]	1	2190
	Val.[Ala, Leu]	4	2115
	Val.Glu	5	2100
	Val.[?]	6	2060
	Val.Leu	13	2155
	Lys.[Val, Ala]	21	1030
Valine, complete hydrolysate	—	—	2124
Valine (released during partial hydrolysis)	—	3	2057
Lysine	Asp.[Asp, Lys]	10	—
	Asp.Lys	14	2460
	Glu.Lys	17	2960
	Lys.[Val, Ala]	21	2620
	Thr.Lys	21	2480
	Lys.Gly	22	2120
	Pro.Lys	23	2100
	Lys.Pro	23	—
	Ala.Lys	24	2480
	Leu.Lys	26	2080
	Pro.[Ala, Lys]	26	2215
	Pro.[Met, Lys]	26	2220
	Phe.Lys	35	2290
Lysine, complete hydrolysate	—	—	2300
Lysine (released during partial hydrolysis)	—	19	2250

DISCUSSION

Arterio-venous changes during lactation have been measured by various workers (cf. Kay, 1947). There seems to have been, as a result of this work, rather general agreement that the drop in free amino acid nitrogen across the mammary gland was insufficient to account for more than one-half of the total milk nitrogen. Graham, Peterson, Houchin & Turner (1938) and Reineke, Williamson & Turner (1941) produced evidence suggesting that peptides derived from plasma-protein globulin could be used for milk-protein synthesis, but Folley (1949) pointed out the dangers of drawing conclusions from arterio-venous differences and suggested that the evidence was inconclusive.

In the first paper of this series (Campbell & Work, 1952) we showed that, in lactating rabbits, plasma peptides must be of relatively minor importance compared to the free amino acids. The possibility remained, however, that certain peptide sequences were common to plasma proteins and to milk proteins so that plasma protein could be partially degraded in the mammary gland and the resultant peptides used immediately for synthesis of new milk protein.

When a lactating goat is given a single intravenous injection of radioactive amino acid, the milk protein collected between 2 and 4 hr. after the injection is about 50 times as active as plasma protein collected at the same time (Askonas *et al.* 1954*a*). Thus, if plasma protein were degraded to peptides and these peptides were used for milk protein synthesis together with free amino acids drawn from the blood, milk protein synthesized immediately after injection would have to contain some amino acids with the high radioactivity of the amino acid pool and some with the low radioactivity of the plasma.

The results reported in this paper (Table 3) show that β-lactoglobulin synthesized during 3 hr. following intravenous injection of [^{14}C]valine and [^{14}C]lysine is uniformly labelled. In other words, the valine and lysine used for lactoglobulin synthesis during this period have been drawn from the free amino acid pool and there is no evidence that any valine or lysine peptide produced by partial degradation of plasma protein was used for β-lactoglobulin synthesis. There is one valine peptide (Table 3) from β-lactoglobulin in which the radioactivity of the valine residue was significantly below that of the other peptides and lower than the mean value for valine from a complete hydrolysate. The radioactivity was, however, much higher than would be expected had this valine residue come from a plasma protein and we believe that this low value is due to some experimental error, e.g. failure to obtain a completely pure valine.

The error in determination of radioactivity of lysine on a micro scale was rather large, probably because bis-DNP-lysine was difficult to purify, but all the lysine radioactivity values except one were within ±15% of the value obtained on lysine isolated from a complete hydrolysate of β-lactoglobulin. The one exceptional value (Table 3) is only 30% higher than the mean value and we do not regard this as significant, having regard to the micro scale of the purification procedure and the impossibility of crystallizing to constant radioactivity.

For casein, our evidence is not quite so extensive since fewer peptides were isolated from a partial hydrolysate (Table 2). One valine peptide from casein did appear to contain valine of significantly higher specific activity than the other valine residues but this again is probably due to a defect in the purification, which in this case had to be carried out on only 80 μg. of DNP-valine. The difference observed was of the opposite sign (+) from what we should have expected had this peptide been produced by breakdown of plasma protein and used directly for milk-protein synthesis. We cannot be certain that there is no degradation of plasma protein in the mammary gland but if there is any such degradation it is probably complete. Our earlier results with lactating rabbits (Campbell & Work, 1952) indicated that such complete degradation of plasma protein to amino acids was quantitatively unimportant as a source of milk nitrogen. We can only conclude that previous measurements of arterio-venous changes in lactating animals were misleading, and that casein and β-lactoglobulin are synthesized entirely from free amino acids.

Our results have also wider implications with respect to the general problem of the mechanism of protein synthesis. The proteins of an animal may become radioactive after administration of radioactive amino acids by two general mechanisms (cf. Schoenheimer, Ratner & Rittenberg, 1939). In the first, the radioactive amino acid is incorporated into newly synthesized protein which is built up from free amino acids; in the second, an exchange mechanism is postulated whereby two peptide bonds in a polypeptide chain are labilized so that the amino acid held at this point is able to exchange with an identical amino acid from the medium. In this second mechanism, the incorporation of radioactive amino acid may not indicate net synthesis of protein. There is no conclusive evidence as to whether such an exchange does occur in the intact animal, but the work of Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg (1942) and of Humphrey & McFarlane (1954) on the stability of antibody globulin suggests that an exchange of this type does not take place between complete

protein and the amino acid pool. In these experiments, however, the antibody protein was present in the extracellular space and may have been excluded from the sites of protein synthesis. Further evidence is provided by Simpson & Velick (1954) and by Heimberg & Velick (1954), who gave a mixture of radioactive amino acids to rabbits and compared the activity of these amino acids in three muscle enzymes isolated at various times after injection. Although the amino acid compositions of these proteins differed, the ratios of radioactivities of the amino acids isolated from different proteins was the same. These results indicated, in agreement with our own, that protein had become radioactive by a process of net synthesis from free amino acids rather than by an exchange mechanism.

Various authors have suggested that tissues contain pools of incomplete protein or 'storage' peptide which can be converted into specific proteins without degradation to amino acids. Steinberg & Anfinsen (1952) gave repeated injections of $NaH^{14}CO_3$ to laying hens over a period of 16 hr. and afterwards isolated radioactive ovalbumin from the oviduct. The distribution of radioactivity in the aspartic and glutamic acid residues of this protein was non-uniform and the results seemed to indicate the existence of an exchange mechanism of the type indicated above or the existence of substantial peptide pools available for protein synthesis. Before commencing the present investigation, we examined extracts of various cells for peptides but found only insignificant quantities of peptides other than glutathione. Christensen & Riggs (1953) have also sought for peptides in oviduct extracts but have failed to find significant quantities.

The present results, demonstrating uniform labelling of milk proteins, are contrary to those obtained *in vivo* by Steinberg & Anfinsen and suggest that any intermediate peptides must be in rapid reversible equilibrium with the free amino acid pool. A similar conclusion was reached by Muir, Neuberger & Perrone (1952), who showed that after injection of [^{14}C]valine the *N*-terminal valine of haemoglobin had the same specific activity as valine from the rest of the molecule.

On the whole, then, the evidence obtained using intact animals favours the view that protein synthesis takes place through the amino acid pool, that any peptides formed during synthesis have a transitory existence and that exchange reactions between pre-existing protein and the amino acid pool play little, if any, part in the process. The uniformity of labelling of the milk proteins in the present experiments implies also that the time required to synthesize an individual protein molecule is short. Richardson & Folley (1954) calculated the rates of synthesis of lactose and fats in the mammary gland and arrived at a figure of the order of 4×10^6 molecules/cell/sec. It seems likely that casein and β-lactoglobulin molecules are synthesized from amino acids at similarly high speeds so that peptide intermediates will have extremely short half-lives.

The results obtained with whole animals provide no direct evidence as to the nature of the intermediate steps in protein synthesis but results obtained in isolated tissues differ substantially from those already quoted, and suggest either that exchange between protein-bound amino acid and the amino acid pool can take place, or that there are appreciable quantities of intermediates not in equilibrium with the amino acid pool. Thus, for example, Steinberg & Anfinsen (1952) found, using oviduct minces, that incubation with radioactive amino acids resulted in non-uniform labelling of ovalbumin. More recently, Vaughan & Anfinsen (1954) have produced convincing evidence that incubation of pancreatic slices with radioactive glycine results in non-uniform incorporation of radioactivity into the insulin present in the slices and that incubation of pancreas slices with radioactive phenylalanine similarly results in non-uniform labelling of ribonuclease. The conditions used were such that there was probably very little if any net increase in specific protein during the experiment and it may be that with this extremely limited synthesis the amount of peptide intermediate available in the tissue was sufficient to influence the distribution of radioactivity. It seems equally likely, however, that under some conditions an exchange reaction can take place between formed protein and the amino acid pool. Gale & Folkes (1953) have accumulated much evidence that such an exchange mechanism can operate when cells of *Staphylococcus aureus* are incubated in a medium containing radioactive glutamic acid but not permitting net increase in protein. Similarly, Rabinovitz, Olson & Greenberg (1954), in a study of the effect of analogues of phenylalanine on the incorporation of amino acids into Ehrlich ascites cells, have shown that such exchange reactions are probable.

The only conclusion that can be reached from all these investigations, including our own, is that there is still insufficient evidence to allow any firm conclusions to be drawn as to the nature of the intermediate steps in protein synthesis. The apparent contradiction between experiments on whole animals and on isolated systems may well be due to the ability of cells to incorporate radioactive amino acids into protein by more than one mechanism.

On the technical problem of the fractionation of a complex mixture of peptides, the present investigation illustrates the advantage of applying

a different physical method at each stage; in this case adsorption chromatography on charcoal followed by ion-exchange and partition methods. It is clear also that a lightly cross-linked sulphonated polystyrene resin gives better fractionation of peptides than a more highly cross-linked resin (cf. Dowmont & Fruton, 1952; Moore & Stein, 1954).

SUMMARY

1. Methods have been developed for the isolation of peptides from partial acid hydrolysates of casein and β-lactoglobulin.

2. Radioactive casein and β-lactoglobulin were obtained from milk collected 4 and 3 hr. after injection of radioactive amino acids.

3. Partial acid hydrolysis of these proteins and isolation of radioactive peptides permitted the determination of specific radioactivity of the same amino acid at different points in the protein chain.

4. It was found that radioactivity was uniformly distributed within the protein molecule.

5. The results indicate that casein and β-lactoglobulin are synthesized from the free amino acids of the blood and that peptides for milk protein synthesis are not supplied by partial hydrolysis of plasma protein.

6. Intermediate peptides cannot be stored in the mammary gland in significant quantity. It is suggested that peptides as intermediates in protein synthesis have only transitory existence.

7. An apparatus is described which simplifies the recovery of amino acids and peptides from ion-exchange column effluents.

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