Isolation of L-Threonine from Proteins

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L-Threonine was first isolated from fibrin by McCoy, Meyer & Rose (1935), after a series of careful investigations extending over a number of years. It was shown to be an essential growth factor for rats. In spite of the obvious biological importance of threonine, its metabolism in animals has not been studied by the modern isotope techniques which have yielded such valuable results in the amino-acid field. This is probably due to the difficulties involved in its isolation in a pure state. McCoy *et al.* (1935)

perties of the hydroxyamino-acids which have been briefly described previously (Elliott, 1948) and are now discussed in detail.

Separation of serine and threonine from protein hydrolysates by oxazoline derivatives

It has been shown by Elliott (1948, 1949) that serine and threonine can be converted into cyclic oxazoline derivatives by reaction of the ester hydrochloride with benziminoethyl ether in the cold. Under

worked on a very large scale and obtained only a minute yield of pure substance. Subsequently, Woolley & Peterson (1937) isolated threonine from rabbit myosin by fractional distillation of the esters of the 'monoamino-acid fraction', but oxidation with periodate revealed that the preparation was not pure (Martin & Synge, 1941). The latter authors were able to isolate a hydroxyamino-acid fraction from wool hydrolysate by their acetylation-benzoylation procedure. Pure serine was isolated from this fraction in fairly good yield, but the isolation of threonine was not attempted. Freudenberg, Walch & Molter (1942) isolated threonine from human blood-group A substance. This publication has been seen only in abstract form without details.

For adequate isotope analyses and determinations of purity about 25 mg. of pure threonine would be required. Owing to the small amount of threonine generally present, at least 0.5 g. of protein would be required to obtain the above quantity. Since the substance sought was a minor constituent of a complex mixture considerable loss seemed inevitable; to allow for this experiments were conducted with 5 g. of protein. Some large-scale experiments were also carried out. The method of isolation which has been worked out makes use of certain novel pro-

these conditions amino-acid esters lacking the β -hydroxyl group also react to give *N*-substituted imino ethers (Cornforth & Cornforth, 1947):

When this reaction was applied to the mixture of ester hydrochlorides resulting from the esterification of a protein hydrolysate a complex mixture resulted; this contained the oxazolines derived from serine and threonine and the substituted imino ethers from the remaining amino-acids.

In this work both ethyl and isopropyl alcohols have been used for esterifying the protein hydrolysates. Synthetic studies (Elliott, 1949) have shown that the isopropyl esters give somewhat higher yields in the reaction with benziminoethyl ether.

By means of a rough fractional distillation at low pressure considerable enrichment of the oxazoline fraction was effected without loss, the main contaminants being the imino ethers derived from glycine, alanine and valine. In contrast to amino-acid esters, both classes of derivatives obtained by the reaction with benziminoethyl ether are quite stable to distillation and can be kept in closed vessels for several weeks without appreciable decomposition. At an early stage in the investigation it was realized that the separation of the oxazolines in a state of purity by fractional distillation would be impossible except in a fractionating column with a high theoretical plate number. Alternative methods of

purification were sought. It was shown by Abraham, Baker, Chain, Cornforth, Cornforth & Robinson (1945) that a substituted imino ether of the type formulated above was hydrolysed by alkali to the corresponding acylamino-acid:

Some preliminary experiments with the compound $\text{C}_6\text{H}_5\cdot\text{C}(\text{OEt})\text{:N}\cdot\text{CH}_2\cdot\text{CO}_2\text{Et}$, obtained from glycine ethyl ester hydrochloride and benziminoethyl ether, showed that the above hydrolysis was almost quantitative under suitable conditions. Although oxazolines can also be considered as substituted imino ethers it was expected that they would be more stable to alkali by virtue of the cyclic system. Studies with synthetic compounds showed that this was the case; when the oxazolines derived from serine or threonine were boiled with dilute aqueous alkali the ring system remained intact and only the carbethoxyl group was hydrolysed. This alkali treatment, when applied to the oxazoline fraction from a protein hydrolysate, was expected to produce in solution the sodium salts of the oxazoline carboxylic acids, together with the sodium salts of hippuric acid, benzoylalanine, etc. This was the case only when hydrolysis was carried out as rapidly as possible using relatively strong aqueous-ethanolic alkali; milder conditions of hydrolysis led to almost complete destruction of the threonine. The reason for this is not clear, but it is probable that under alkaline conditions the esters condense with one another, the process being immediately arrested once the carbethoxyl groups have been hydrolysed.

After hydrolysis of the oxazoline fraction, the solution was acidified with hydrochloric acid to pH 1. This caused an immediate precipitation of an oily mixture of hippuric acid, etc., but the oxazoline carboxylic acids remained in solution as the hydrochlorides. Synthetic studies had established that the oxazoline ring was rapidly opened at pH 1 to give the corresponding *O*-benzoyl derivative (Elliott, 1949).

The acidified solution described above was then thoroughly extracted with ethyl acetate and the organic extract rejected. The aqueous layer contained only the *O*-benzoyl derivatives of serine and threonine as their hydrochlorides; when this solution

was made alkaline to phenolphthalein the benzoyl group moved instantaneously on to the nitrogen atom. A mixture of benzoylserine and benzoylthreonine free from impurities was then obtained by a second acidification and solvent extraction. The above series of transformations can be carried out quite easily in a few hours. The remaining problem was the separation of serine from threonine, but before discussing this it is necessary to consider the stereochemical aspects of this problem.

Separation of L-benzoylthreonine from DL-benzoylserine

As a result of experiments, which will be described later, convincing proof has been found that benziminoethyl ether reacts with serine and threonine ester hydrochlorides to produce oxazolines with complete retention of configuration at the α - (and in the case of threonine also at the β -) carbon atom. It has been shown (Elliott, 1948, 1949) that oxazolines of the type under discussion most probably possess an active α -hydrogen which is mobilized by the action of hot alkali. The discovery that the serine, isolated from the serine-threonine mixture as described below, was completely racemized was therefore in accordance with expectations. The configuration of the threonine derivative, however, appeared to be completely preserved. The reason for this becomes obvious when the following facts are considered. Racemization at the α -carbon atom in the threonine derivative would produce not the DL-oxazoline, but a mixture of oxazolines derived from L-threonine and D-allothreonine. These can be assigned *trans* and *cis* structures respectively, on the basis of the configurational relationship between L-threonine and D-threose established by Meyer & Rose (1936). These two forms must be in equilibrium in highly alkaline

solution, because of the mobile α -hydrogen atom, but it has been shown (Elliott, 1948, 1949) that the equilibrium favours the *trans* form to such an extent that the *cis* form cannot be isolated.

The separation of DL-benzoylserine from L-benzoylthreonine was carried out in two ways. In the first method the mixture of benzoyl compounds was hydrolysed and the amino-acids separated from one another by partition chromatography on a column of potato starch. The use of potato starch in partition

chromatography was first described by Synge (1944). Although development of the chromatogram required several days, very satisfactory results were obtained on a small scale. Larger quantities of material were more easily handled by countercurrent distribution after a preliminary step in which most of the benzoylserine was removed as the sparingly soluble β -phenylethylamine salt. In contrast to DL-benzoylthreonine, which also forms a sparingly soluble β -phenylethylamine salt (Carter & Risser, 1941), L-benzoylthreonine forms a very soluble salt which is not crystalline. When a high yield of pure material is desirable in the separation of two substances it is advantageous to use the principle derived by Bush & Densen (1948) in which $V_x/V_y = \sqrt{(1/K_a K_b)}$, where V_x and V_y are the volumes of solvent used in each equilibration, and K_a, K_b are the partition coefficients of the two substances to be separated. It is also advantageous to carry the procedure through a 'diagonal stage' (see Bush & Densen, 1948, p. 123). The distribution curves of the two substances are then mirror images of each other and, providing K_a/K_b is sufficiently large, the major part of one solute will accumulate in one solvent, while the second solute will accumulate in the other solvent. The upper and lower layers can then be separately pooled and the pure components isolated from them.

In initial experiments ether and water were found to give the largest ratio K_a/K_b . The ratio of solvents to be used in each equilibration was then calculated by applying the above formula as follows: K_a (benzoylthreonine) = 0.218; K_b (benzoylserine) = 0.100; V_x = volume of ether; V_y = volume of water; $V_x/V_y = \sqrt{(1/0.218)} = 1/0.148$. Let p_a = the fraction of benzoylthreonine which remains in the upper phase at each equilibration. Then

$$p_a/1 - p_a = K_a V_x/V_y,$$

$$p_a = K_a V_x/(K_a V_x + V_y) = 0.218/(0.218 + 0.148) = 0.60.$$

In the paper by Bush & Densen (1948), already referred to, a series of curves are given from which the fraction of solute in the pooled upper or lower layers can be found for all values of p and values of n (number of equilibrations) up to 100. Reference to these curves showed that a twelve-funnel process should yield 80% of the original amount of benzoylthreonine from the pooled ether layers. If the bulk of the benzoylserine were removed previously as the β -phenylethylamine salt this benzoylthreonine should be more than 80% pure. It was found that recrystallization from water removed the remaining impurities, but losses were considerable.

The advantage in using buffer solutions, particularly concentrated ones, in countercurrent distribution methods has been pointed out by Craig, Golumbic, Mighton & Titus (1945). After a series of experiments it was found that ethyl acetate and

a 2M-phosphate-citrate buffer at pH 3.6 gave the considerably increased value of 2.9 for K_a/K_b . In this case, by calculation as above, a ten-funnel process should raise the purity of the benzoylthreonine to 96%, and by repetition of this process on the purified material the purity of the benzoylthreonine should be raised almost to 100% with a loss of only 20%. Citric acid is not completely insoluble in ethyl acetate, and it was found preferable to remove this by a third countercurrent distribution of the benzoylthreonine between ethyl acetate and water. In this case K_a/K_b ($K_b = K$ for citric acid) was very high (39.5), so that the removal of citric acid was a simple matter. In practice it was found that the degree of purification was somewhat less than that calculated and the final product had a purity of 96.3%. This figure was obtained by periodate analysis of the L-threonine resulting from acid hydrolysis of the benzoyl derivative. The amino-acid had the correct specific rotation, but this did not exclude the possibility that a very small amount of D-allothreonine was present.

Fortunately, for the purpose for which this method was worked out, the optical purity of the threonine was of no consequence; it was necessary only to ensure that the product was free from other amino-acids, particularly serine. Although the available evidence suggests that the threonine isolated was optically pure, this must await confirmation by a more sensitive method.

Isolation of serine from the oxazoline fraction without racemization

In order to prove that no racemization occurred when benziminoethyl ether reacted with serine and threonine ester hydrochlorides it was necessary to isolate L-serine from the oxazoline fraction by some method which avoided treatment with alkali at any stage. It has already been stated that the oxazoline ring in the compounds under discussion is readily opened in aqueous solution at pH 1. At pH 4, however, no ring opening could be detected, at least for short periods of time. Model experiments with the imino ether derived from glycine, on the other hand, showed that it was rapidly hydrolysed at pH 4 into ethyl benzoate and glycine ester. This is exactly analogous to the fission of the oxazoline ring to give the corresponding O-benzoyl compound:

Oxazolines, being weak bases, should be extracted by ether from a buffer at pH 4, whereas the strongly basic amino-acid esters should remain in the aqueous phase.

To test out these ideas experiments were conducted on silk sericin, which contains a very high

proportion of hydroxyamino-acids (Nicolet & Seidel, 1941). This protein was obtained in solution, in partly degraded form, by a very short treatment of raw silk with hot dilute acid, the fibroin being practically unaffected. The oxazoline fraction from silk sericin was shaken with an acetate buffer at pH 4 for 3 hr., the insoluble oil extracted into ether, the combined extracts evaporated and the residue hydrolysed. Analyses showed that the hydroxyamino-acid nitrogen content of the solution, determined by periodate ammonia estimation, was only slightly less than the amino nitrogen, indicating almost complete purification of the hydroxyamino-acid fraction. L-Serine was isolated from the hydrolysate as the *p*-hydroxyazobenzene-*p*'-sulphonic acid salt by the method of Stein, Moore, Stamm, Chou & Bergmann (1942) and had the correct specific rotation. Unfortunately, this alternative method, which seemed promising for the isolation of threonine as well as serine, gave unsatisfactory results with a casein hydrolysate and cannot be of general application.

EXPERIMENTAL

Methods of analysis. Serine and threonine were determined by periodate oxidation according to the method of Rees (1946). Periodate NH_3 was determined by the method of Van Slyke, Hiller & MacFadyen (1941). The same apparatus was used for both these determinations. Amino-N determinations were carried out in the Van Slyke gasometric micro-apparatus. Partition coefficients were measured by equilibrating the substance between equal volumes of the two solvents and determining the amount in each layer by titration, or by Kjeldahl determinations when buffer solutions were used.

Hydrolysis of the proteins. A good brand of commercial casein was used for most experiments. A preparation of mixed rabbit-muscle proteins was also used. These were hydrolysed by refluxing 16–24 hr. with 5 parts (w/v) of 6*N*-HCl and were filtered from acid-insoluble humin. A preparation of raw silk, known commercially as 'Turkish Knubbs', was stirred with 20 parts (w/v) of boiling *N*-HCl for a minute or so until the sericin had been removed. This was easily judged by microscopic examination of the fibres. The fibroin was then squeezed out, steeped in distilled water and squeezed out again. The two solutions were combined. Hydrolysis of the protein was accomplished by evaporating the solution at ordinary pressure until the acid in the distillate was about 5*N*. The solution was then refluxed for 4 hr. Amino-N determinations revealed that hydrolysis was complete in this time. In this brand of raw silk, the sericin content, as determined roughly by the weight loss on acid treatment, was 15–20%.

Preliminary treatment of the protein hydrolysates. Silk sericin hydrolysate required no preliminary treatment. Hydrolysates from about 5 g. of casein were diluted with about 5 parts of water, heated to 60° and a small amount of a concentrated solution of phosphotungstic acid stirred in. This removed the acid-soluble humin as a precipitate which settled rapidly. Sufficient phosphotungstic acid was added to render the supernatant liquid almost colourless, but with a comparatively pure protein such as casein it was not

necessary to precipitate more than a small amount of the basic amino-acids. When crude proteins were used it was considered advisable to precipitate the basic amino-acids almost completely, as described below. After cooling, the solution was filtered through a Ford Sterilmat F.C.B. grade, extracted twice with a mixture of amyl alcohol 20 parts, ether 25 parts, ethanol 1 part (see Chibnall, Rees & Williams, 1943) and evaporated to dryness under reduced pressure.

On a larger scale it was preferable to remove as much as possible of the neutral, acidic and basic amino-acids. The following is an example of the method used. The hydrolysate from 300 g. of casein was evaporated under reduced pressure to about 540 ml. The solution was saturated with HCl at 0° and allowed to stand 4 days at 0°. To this solution was then added, with stirring, 900 ml. ethanol which had previously been cooled to about -30°; the purpose of this cooling was to prevent a rise in temperature above 0° when the ethanol and the acid solution were mixed. The glutamic acid hydrochloride was filtered through a sintered glass funnel and washed with 300 ml. of ice-cold ethanol. A yield of 67 g. of the hydrochloride was obtained, representing about 80% of the amount present in the protein. It should be emphasized here that quantitative removal of the various fractions was not necessary. The filtrate and washings from the glutamic acid were evaporated to dryness under reduced pressure, redissolved in water and evaporated again. The residue was dissolved in 5.25 l. of *N*-HCl, the solution heated to boiling and phosphotungstic acid (920 g. dissolved in 1.8 l. water) added fairly rapidly with stirring. After standing at room temperature for 2 days the crystalline precipitate was filtered at the pump, stirred with 750 ml. of 2.5% (w/v) phosphotungstic acid in *N*-HCl, filtered again, washed with 375 ml. of the same solution on the funnel and finally sucked as dry as possible on the pump. The combined filtrate and washings were saturated with ether, extracted three times with the amyl alcohol-ether-ethanol mixture using 2250 ml. in all and then evaporated to dryness under reduced pressure. This method of precipitating the basic amino-acids is a simplification of the method of Chibnall *et al.* (1943). The residue of amino-acid hydrochlorides was then dissolved in water (750 ml.), the solution cooled and 5*N*-NaOH added dropwise with stirring, the temperature being kept below 15°, until the pH of the solution was about 6. Crystallization began almost immediately, and after keeping overnight at 0° the crop was filtered and washed with a small quantity of saturated NaCl solution. The combined filtrates were evaporated to obtain a second crop, and so on until the crop of solid consisted almost entirely of NaCl. This procedure served to remove most of the leucine and tyrosine and probably some phenylalanine and methionine. The solution was then strongly acidified with conc. HCl and evaporated to dryness under diminished pressure. This was best carried out in a large flask because of bumping. The residue was extracted thoroughly with boiling ethanol, the NaCl filtered and thoroughly washed with boiling ethanol. The ethanolic extract was then evaporated to dryness under reduced pressure. Analyses showed that about 20% of the threonine was lost during the above operations.

Esterification of the protein hydrolysates

(a) *Ethyl esters.* The syrupy residue of amino-acid hydrochlorides was finally dried by evaporation twice under reduced pressure with dry ethanol. The amount of ethanol used for esterification was not critical and 5–20 parts (based

on the weight of protein) were used, the amount depending on the scale. On the small scale it was more convenient to use dilute solutions. A rapid stream of dry HCl was passed through a dry ethanolic solution of the amino-acid hydrochlorides until the solution boiled. It was then cooled and resaturated with HCl. After standing overnight the solution was evaporated to dryness under reduced pressure. To ensure complete esterification the process was repeated, but this may not have been necessary.

(b) *isoPropyl esters*. The residue of amino-acid hydrochlorides was dried as before by evaporating with ethanol and then evaporated once with isopropanol. The residue was suspended in 10–20 parts of dry isopropanol and a rapid stream of dry HCl passed in until the solution boiled. A steady stream of dry HCl was then passed through the solution for 1 hr. and boiling was maintained by application of heat at such a rate that about one-third of the alcohol was allowed to escape through a 15 in. air condenser fitted to the flask. After about 30 min. fresh isopropanol was added to compensate for the loss through the condenser. The hydrochlorides slowly dissolved. The solution was then evaporated to dryness under reduced pressure. In the case of both (a) and (b) the esters were finally dried in a vacuum desiccator over NaOH and H_2SO_4 .

Preparation of benziminoethyl ether. Dry HCl was passed into dry ethanol (46 g.) at 0° until 36.5 g. had been absorbed. The solution was then added fairly rapidly to freshly distilled benzonitrile (103 g.) which had also been cooled to 0°. The mixture was allowed to stand 4 days at 0° with careful exclusion of moisture. The solid mass of imino ether hydrochloride was broken up under dry ether, filtered and washed thoroughly with dry ether. A small amount of hydrochloride separated from the ether washings on standing at 0°. The total yield was 141 g. (76 %); m.p. 132–134° (decomp.). The hydrochloride (173 g.) was added to a sludge of K_2CO_3 (129 g.), and water (90 ml.) which was covered with a layer of ether and cooled to –5°. After thorough shaking the ether layer was decanted and the sludge washed twice more with fresh ether. The combined extracts were dried over anhydrous Na_2SO_4 , evaporated and the residue distilled. It had b.p. 112°/22 mm. Yield 124 g. (89 %). The product was stable for considerable periods of time if kept in a sealed vessel.

Reaction of the ester hydrochlorides with benziminoethyl ether

The ester hydrochloride mixture from 5 g. of protein was dissolved in 3 ml. of water, and the solution adjusted to pH 3–4 by addition of a drop or two of conc. NH_4OH . A solution of benziminoethyl ether (10 g.) in ether (30 ml.) was then added and the mixture thoroughly shaken for 16–24 hr. Griffin and Tatlock's 'Microid' flask shaker was found to be very suitable for this purpose. The shaking was generally carried out in 250 ml. conical flasks. On a larger scale it was preferable to divide the ester hydrochloride mixture into several portions, each not greater than about 10 g. The shaker held four flasks at a time. At the end of the shaking sufficient water was added to dissolve the NH_4Cl which had crystallized out, the upper layer was separated and washed several times with fresh portions of water. It was often necessary to filter the reaction mixture after addition of water to remove amorphous material which prevented a clean separation of the layers. The ethereal layer was then dried over anhydrous Na_2SO_4 , the ether evaporated and the

residue distilled, first at about 20 mm. to remove excess of benziminoethyl ether and finally at 0.01 mm., the fraction of b.p. 90–135°/0.01 mm. being collected. This is termed the oxazoline fraction. Periodate analysis showed that it contained about 80 % of the threonine originally present in the protein hydrolysate before esterification.

Hydrolysis of the oxazoline fraction: isolation of a mixture of benzoylserine and benzoylthreonine

The oxazoline fraction was refluxed for 30 min. with 8 parts (w/v) of aqueous-ethanolic $N-NaOH$, prepared by mixing equal parts of 2N- $NaOH$ and ethanol. The solution was then cooled, diluted with about half its volume of water and the ethanol removed under reduced pressure. The solution was acidified to pH 1 with 5N-HCl and allowed to stand 4 hr. at room temperature. A pale yellow oil separated. The solution was then extracted six times with about an equal volume each time of ethyl acetate, and the extracts rejected. To the aqueous solution, 5N- $NaOH$ was added until alkaline to phenolphthalein. The solution was then made weakly acid, evaporated to a small bulk and acidified (blue to congo paper) with conc. HCl. The benzoyl derivatives were obtained from the solution by six extractions with ethyl acetate. The organic extracts were combined and evaporated to dryness under reduced pressure. The yield of mixed benzoyl derivatives was about 0.35 g. from 5 g. of protein, a yield of approximately 35 % (assuming a threonine content of 4 % and a serine content of 6 %). On a larger scale the yield was about 30 %; for example, 11.84 g. of mixed benzoyl derivatives were obtained from 200 g. of casein.

Isolation of pure L-threonine and DL-serine

(a) Using a starch column

About 300 mg. of the mixture of benzoyl derivatives were refluxed 4 hr. with 3N-HBr (2 ml.) and evaporated to dryness under reduced pressure. The residue was redissolved in water (20 ml.), extracted with ether to remove benzoic acid and the aqueous solution evaporated again to dryness. The residue was dissolved in 95 % ethanol (2 ml.) and a slight excess of conc. NH_4OH added to the hot solution. After standing overnight the amino-acid was collected and washed with ethanol. The yield from 5 g. of protein was about 80 mg. It was recrystallized from a minimum of aqueous ethanol to remove traces of NH_4Br .

Syngé (1944) used unfractionated potato starch in his experiments with *n*-butanol as solvent. In the experiments about to be described phenol saturated with water was used as the solvent. It was found necessary to remove small particles from the starch by sedimentation in water until the granules, when viewed under the microscope, appeared to be of approximately equal size. This represented only about 20 % of the original amount of starch. The dried product was then washed several times by stirring with aqueous phenol and separating the liquid by centrifugation. It was then allowed to soak in aqueous phenol for several days. The starch was finally made into a chromatogram 25 cm. long and 1.1 cm. in diameter. The experiment was carried out in a room with the temperature controlled to about 70° F. The column was washed by allowing aqueous phenol to percolate through it for 4 days.

About 50 mg. of the serine-threonine mixture, dissolved in aqueous phenol, were added to the column, and when the solution had run through the column was developed

with aqueous phenol. The progress of the separation was followed by ninhydrin tests on a few drops of effluent. Threonine appeared in the effluent after 2-5 days and came through in a relatively small amount of solvent (12 ml.; flow rate of column about 1 ml./hr.). When the ninhydrin reaction became blank or very weak the receiver was changed and washing continued to recover the serine. The amino-acid was isolated from the phenol solution by adding it to an equal bulk of water and extracting the phenol twice with several volumes of ether. The aqueous solution was then evaporated to dryness and the residue crystallized from 80% ethanol. It was found that 100 mg. of amino-acid mixture could be used on a column 25×1.8 cm. The yield of L-threonine from 5 g. of protein was 40-45 mg., representing about 20% of the amount present in the hydrolysate. Samples of amino-acid isolated in this way were found to be 98-100% pure threonine by periodate analysis. No other amino-acid could be detected by paper chromatography. DL-Serine isolated from the column was also pure.

(b) Using countercurrent distribution

Most of the benzoylserine was first removed from the mixture of benzoyl derivatives by dissolving 4 g. in ethanol (10 ml.) and dry ether (30 ml.), and adding freshly distilled β -phenylethylamine (2.37 ml.). After standing at 0° for 2 days the crystalline DL-benzoylserine β -phenylethylamine salt (2.27 g.) was collected. The filtrate from the salt was evaporated to dryness under reduced pressure, the residue was dissolved in a small quantity of water and the solution acidified to congo red. The solution was extracted six times with ethyl acetate and the combined extracts evaporated to dryness under reduced pressure to recover the crude benzoylthreonine.

Isolation of DL-benzoylserine. The β -phenylethylamine salt was recrystallized several times from ethanol and then had m.p. 151-152°. (Found: N, 8.7. $C_{18}H_{22}O_4N_2$ requires N, 8.5%.) It gave no depression in melting point on admixture with a synthetic sample of the salt. DL-Benzoylserine was obtained in good yield from the salt by dissolving it in hot water and acidifying the solution (congo red paper). The acid crystallized on cooling and had m.p. 166-167°. (Found: N, 6.5. $C_{10}H_{11}O_4N$ requires N, 6.7%.) A 5% solution in ethanol was without effect on polarized light. About 7 g. of the β -phenylethylamine salt were obtained from 200 g. of casein.

Isolation of L-threonine

Method (i). Ether/water system. About 500 mg. of the crude benzoylthreonine were submitted to a 'diagonal stage' countercurrent distribution in twelve separating funnels using 50 ml. of ether and 7.5 ml. of water in each equilibration ($K_a=0.218$, $K_b=0.100$). The pooled ether layers were then evaporated to dryness and the residue washed with light petroleum (b.p. 60-80°). The solid was recrystallized several times from a small quantity of hot water to give 150 mg. of pure L-benzoylthreonine, m.p. 143-144°, $[\alpha]_D^{22} + 26.8^\circ$ in water (c., 0.7). (Found: N, 6.2. Calc. for $C_{11}H_{13}O_4N$: N, 6.3%.) A synthetic specimen of L-benzoylthreonine, prepared by the method of West & Carter (1937), also had the same melting point and no depression in melting point was produced on mixing the two samples. West & Carter gave $[\alpha]_D^{26} + 25.1$ for their product, whereas the synthetic sample prepared in this laboratory had $[\alpha]_D^{21} + 27.4^\circ$, $+ 26.9^\circ$ in water (c, 0.7, 0.8). An additional amount of benzoylserine was isolated from the pooled aqueous layers

as the β -phenylethylamine salt. Hydrolysis of the benzoylthreonine yielded pure L-threonine (found: threonine 100% by periodate analysis), which crystallized from 80% ethanol in hexagonal plates, characteristic of the pure amino-acid (see McCoy *et al.* 1935; Woolley & Peterson, 1937).

Method (ii). Ethyl acetate/buffer system. Phosphate-citrate buffer (2M) was prepared by adding solid Na_2HPO_4 to 2M-citric acid until pH 3.6 (glass electrode) was reached. The mixture was then diluted with sufficient water to make the solution 2M with respect to phosphate plus citrate. About 4 g. of crude benzoylthreonine were submitted to a 10-funnel 'diagonal stage' countercurrent distribution between ethyl acetate and the buffer, using 200 ml. of the organic solvent and 164 ml. of the buffer for each equilibration ($K_a=1.39$, $K_b=0.48$). Except for the contents of the first funnel, in which most of the coloured impurities and some benzoic acid were found, the ethyl acetate layers were pooled, evaporated and the residue submitted to a second distribution as before. Citric acid was removed from the final product by a 4-funnel process using 200 ml. of ethyl acetate and 50 ml. of water for each equilibration. The purified benzoylthreonine was crystallized once from ethyl acetate giving a product m.p. 137-141°, which was hydrolysed with HBr to give L-threonine. The yield from 200 g. of casein was about 1.3 g. (16.5% of the amount present in the casein). The amino-acid was recrystallized once from aqueous ethanol to give about 0.9 g. of amino-acid, $[\alpha]_D^{20} = -27.8^\circ$ in water (c, 5.0), purity by periodate analysis 96.3%. Another experiment, in which the benzoylthreonine was crystallized once from ethyl acetate before the second ethyl acetate-buffer distribution, gave a sample of amino-acid with $[\alpha]_D^{19} - 28.2^\circ$ in water (c, 2.0), purity by periodate analysis 98.3%. West & Carter (1937) gave $[\alpha]_D^{26} - 28.3^\circ$ for their synthetic sample; McCoy *et al.* (1935) found that the rotations of various isolated specimens varied from -27.5 to -28.2° .

Isolation of L-serine from silk sericin by an alternative method

A hydrolysate of silk sericin prepared from 50 g. of raw silk was esterified with isopropanol and the oxazoline fraction obtained in the usual way. The yield was 11.8 g. The main bulk (10.8 g.) of this fraction was shaken 3 hr. with 110 ml. of an acetate buffer prepared by adding solid sodium acetate to 20% acetic acid until pH 4 was reached (glass electrode). The oil which remained was extracted into ether and the extract thoroughly washed with water and $NaHCO_3$ solution. After evaporation of the ether the oil was refluxed 4 hr. with 5N- H_2SO_4 (50 ml.); the solution was then extracted with ether to remove benzoic acid, diluted several times and the sulphate ions removed with $Ba(OH)_2$ in the usual way. Experiments on a small scale had revealed that the hydroxy-amino-acid N content of the solution as found by periodate NH_2 estimations was only 8% lower than the total amino N determined by nitrous acid. The filtrate and washings from the $BaSO_4$ were combined, evaporated to 60 ml. and *p*-hydroxyazobenzene-*p'*-sulphonic acid (4 g.) added to the hot solution. On cooling the serine salt separated in the form of orange plates. It was recrystallized from hot water giving 3.2 g. of pure salt. On decomposition of the salt by the method of Stein *et al.* (1942) 0.7 g. of pure L-serine was obtained. This crystallized in heavy hexagonal prisms from aqueous ethanol. It had $[\alpha]_D^{20} - 7.15^\circ$ in water (c, 3.0), and was found to be 100% pure by periodate analysis.

SUMMARY

1. Reaction of an esterified protein hydrolysate with benziminoethyl ether gave a mixture of products containing the oxazolines derived from serine and threonine, and substituted imino ethers derived from amino-acid esters lacking the hydroxyl group in the β -position.

2. Complete separation of the oxazolines from the substituted imino ethers was not attempted because of the close proximity of the various boiling points. Alkaline hydrolysis of the oxazoline fraction, followed by a series of pH changes on the resulting solution gave a mixture of DL-benzoylserine and L-benzoylthreonine free from other amino-acid derivatives. The chemical and stereochemical changes involved are discussed.

3. Serine and threonine were separated from one another on a small scale by partition chromatography on a starch column and on a larger scale, as their benzoyl derivatives, by countercurrent distribution.

4. The yield of L-threonine obtained on a small scale was about 20% of the amount originally present in the protein.

5. L-Serine was isolated from silk sericin by an alternative method which was found not to be of general application.

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The Distribution of Ascorbic Acid in Developing Salmon (*Salmo salar* L.)

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Although attention has been focused upon physical and chemical changes that take place in the yolk and growing tissues of the developing salmon (and trout) egg, there are practically no references to vitamin content of the egg during these changes. The present investigation was limited to assays of ascorbic acid in two early embryonic stages, and one later, larval, stage of development in the salmon, and to its histological distribution. No estimations were made of the amounts of dehydroascorbic acid that might be present.

METHOD

The eggs were fertilized on 1 December 1947, and kept in running water at the following temperatures: 1–15 December 1948, 9–11°, average 9.9°. This covers the period for sample 1 (9 December) and sample 2 (15 December). 1 December–9 March 1948, 7–11°, average 9.0°. This covers the period from fertilization to the date of sample 3.

Fifty eggs or larvae were taken for each sample. The method followed was that worked out by Mapson (1943). Formaldehyde was used, as first recommended by Lugg (1942), to differentiate ascorbic acid from other substances