CIV. INVESTIGATIONS OF SOURCES OF ERROR IN THE ESTIMATION OF TYROSINE AND TRYPTOPHAN IN COMPLEX MATERIALS, WHICH ARE ASSOCIATED WITH HYDROLYSIS[1]

By JOSEPH WILLIAM HENRY LUGG

Department of Biochemistry, Imperial College of Science and Technology, South Kensington, London, S.W. 7

(*Received 8 February 1938*)

It is the purpose of the present article to discuss such errors in the estimation of tyrosine and tryptophan in complex materials, as may be associated with the liberation of the amino-acids by acid and alkali hydrolyses. From general considerations these errors may be regarded as falling into three classes, namely, (1) failure to liberate the amino-acids completely from combinations originally existing, (2) destruction or modification of the amino-acids by the hydrolysing reagent or by other substances during liberation and (3) destruction or modification of the amino-acids subsequent to their liberation. Provided that satisfactory methods of analysing hydrolysates are available, these possible sources of error can all be investigated but the third by far the most easily. The first two can be examined properly only if suitable synthetic complex materials of known composition are accessible: failing these it may be conjectured from indirect evidence that errors of the first class have been eliminated in the case of certain complex materials, and the effects of various added impurities may be tested.

Fragmentary data have been available for many years. Herzfeld [1913] showed that tryptophan could be boiled in aqueous solution without serious destruction, whereas in the presence of added NaOH and $CuSO_4$ there was very serious decomposition indeed. He claimed that the amino-acid is reasonably stable to boiling in $Ba(OH)_2$ solution. The claim was supported by Homer [1915], who recommended the employment of $Ba(OH)_2$ as an alkali for the hydrolysis of proteins (25 ml. 0·45M $Ba(OH)_2$ solution per g. protein at about 100° for 48 hr.). She found that tryptophan is by no means readily decomposed by heating in dilute H_2SO_4 solution for long periods, unless oxidizers like $Fe_2(SO_4)_3$ or $CuSO_4$ are present, and attributed the virtual non-appearance of tryptophan in acid hydrolysates of proteins to the presence of oxygen carriers in the protein or to a putative instability of "nascent" tryptophan in acid solutions.

Kraus [1925] found some 20 % decomposition of tryptophan in the hydrolysis procedure recommended by Homer, the destruction of the indole nucleus itself being perhaps 7 %.

Folin & Ciocalteu [1927] stated that acid hydrolysis is more destructive of tryptophan, and alkali hydrolysis less destructive, than had formerly been supposed, but gave no data. Their method of analysing hydrolysates does not

[1] Commenced in the Nutrition Laboratory of the Commonwealth Council for Scientific and Industrial Research, University of Adelaide, South Australia; and completed at the Imperial College during the tenure of a Leverhulme Fellowship and a grant from the Agricultural Research Council.

differentiate between tryptophan and other indoles or between indoles and a variety of reducing substances, and they assumed that each mol. of tryptophan decomposed in their recommended procedure (25 ml. 5*N* NaOH solution per g. protein at the boiling point for 18–20 hr.) would appear as indole or as an indole derivative. Von Deseö [1934] prefers to conduct the hydrolysis under pressure at high temperature for relatively short periods, claiming that the amounts of tyrosine and trytophan estimated in the hydrolysates are thereby increased.

Gortner & Blish [1915] showed that when tryptophan is heated with carbohydrate in acid solution much of the tryptophan-nitrogen is to be found in the resultant insoluble "humin". Roxas [1916] confirmed this observation and demonstrated a smaller, but still serious, loss with tyrosine.

Experimental

The work comprises a fairly thorough investigation of point (3) (mentioned in the opening paragraph) in regard to acid and alkali "hydrolyses" under conditions approximating to those necessary for hydrolysis of proteins, in the absence and presence of added substances, and a partial investigation of points (2) and (1).

All digestions were done in sealed tubes at 100° (immersion in boiling water bath), for, as will appear later, the use of higher temperatures for the alkali hydrolysis of proteins, such as the boiling points of the mixtures, is quite unnecessary. In the schedules given below the "*" and "†" signs indicate that the mixture was sealed in the tube with about 8 or with about 0·2 vol. of air, respectively. Precipitates and suspensions were separated from liquids sometimes by filtration but generally by centrifuging, particularly when working on a small scale.

Acid hydrolysates were first diluted somewhat and the liquid separated from any solid residue. They were then adjusted to *p*H 1–2 by addition of NaOH solution, separated from any insoluble material, and adjusted to a suitable volume. Alkali hydrolysates were likewise diluted, cleared, adjusted to *p*H 1–2 with H_2SO_4, cleared again and brought to a suitable volume. The solutions were sometimes extracted with pure (peroxide-free) ether or pure toluene at *p*H 1–2, 7 or 14, before analysis; and as a rule, aliquots, suitably adjusted to appropriate *p*H, were taken for the purpose. At *p*H 7, the concentration of tyrosine was kept below 0·5 mg. per ml. to avoid risk of precipitation. After extraction, the solutions were freed from traces of solvents by warming for some minutes at 45° in a current of air, cooled and adjusted to volume.

It became of interest at one stage to examine the effects of hydrolysing with alkali under strongly reducing conditions. Stannite was selected as the reducer, because the metal is readily removed subsequently from either acid or alkaline solution by zinc dust. Minimal amounts of zinc are dissolved if the substitution is carried out in alkaline solution. Gelatinous zinc hydroxide appears if the solutions are adjusted later to *p*H 7, making extraction with solvents very troublesome. Whilst extractions in this region were intended to remove substances like indole or skatole (if present), it was found that indole is extracted from aqueous solution at *p*H 14 by toluene or ether just as effectively as by toluene at *p*H 7 (an equal volume of solvent removes at least 95% of the indole), and at *p*H 14 the zinc hydroxide is not precipitated. Indole is extracted also from aqueous solution at *p*H 1–2 by ether, but less readily, especially in the more acid regions where the imino group can function more pronouncedly as a base.

The methods of estimation employed here are freely applicable to solutions containing small amounts of silica and have been described elsewhere in some

detail [Lugg, 1937], and the satisfactory results of reliability trials have been given. It has also been explained there, in reference to these colorimetric methods, that variations in tint or in development or fading rates will serve as criteria for the presence of phenols and indoles other than tyrosine and tryptophan. The method recommended for general purposes was used almost exclusively, but a few estimations were made by modifications of it. In no instance in the present investigation was there any indication of a partial conversion of tyrosine into some other phenol during digestion with hydrolysing reagents. Tryptophan, however, invariably appeared to undergo some (usually slight) conversion into one or more other indoles, as well as suffering, perhaps, a more extensive (but still slight) degradation or loss. The losses recorded in such cases are therefore inaccurate when these indole-like degradation products have been left in solution, and are based upon colorimetric readings made, before much fading had occurred, by a normal eye under approximately "daylight" filament illumination. Extraction of the solutions with solvents before analysis sometimes effected virtually complete removal of these degradation products. Results which are unquestionably vitiated by their presence are indicated in the following schedules by a " > " sign placed before the stated losses, because they must be greater than colorimetric comparison suggests; results obtained from readings which revealed only a very uncertain change in fading rates are prefixed by the sign, " ? > ", and those which appeared not to be vitiated are not prefixed. Tyrosine losses of less than 1 % and tryptophan losses of less than 2 % are to be regarded as lying within the errors of analysis.

The edestin used in some of the later experiments was an air-dried, recrystallized sample containing 10·6 % moisture and 18·5 % N (on a dry basis). The analyses given in the schedules are quoted as percentages of the original protein-N appearing as tyrosine- and tryptophan-N. The amounts actually present in this sample were probably 1·79–1·80 %, and 1·09–1·10 % (corrected), respectively. In recording the results of tryptophan estimations the signs " < " and " ? < " are used where necessary. The cystine content of edestin is not such as to interfere detectably with the estimation of tyrosine or tryptophan in the hydrolysates.

A. *Stability of tyrosine and tryptophan solutions at room temperatures.* Over the *p*H range 1–14, tyrosine solutions appear to be very stable. At about 20° there was no detectable destruction of tyrosine at *p*H 14 in 60 days, at *p*H 13 in 100 days or at *p*H 1 in 200 days. Tryptophan solutions are relatively unstable. At *p*H 7, even when bacteria are excluded, there is a loss of ? > 5 % in 30 days at about 20° (by direct analysis), and a loss of 6 % after extraction with an equal volume of toluene. At *p*H 13 the losses are perhaps slightly greater (> 12 % in 60 days by direct analysis), and the solution develops a faint odour of indole and a very pale yellow coloration. At *p*H 1 degradation is more pronounced and follows a different course. There is, in the early stages at any rate, a fictitious "gain" in tryptophan content (by direct analysis) but the pronounced difference in tint from that of the tryptophan colour standard and the change in fading rate, show that considerable degradation has occurred even in 15 days at 20°. For this reason, solutions which have been made acid and are intended for tryptophan analyses must be used without much delay.

B. *Tyrosine and tryptophan heated in H_2SO_4 solution.* (1) 50 mg. tyrosine in 7 ml. 7*N* H_2SO_4 at 100° for 20 hr.* Direct analysis: no loss.

(2) 25 mg. tryptophan in 15 ml. 7*N* H_2SO_4 at 100° for 30 hr.* A small amount of a very fine brownish black suspension was produced. Direct analysis: > 6 % loss (tint too grey and fading rate too low).

(3) 10 mg. tyrosine and 5 mg. tryptophan in 2 ml. 7*N* H_2SO_4 at 100° for 24 hr.† Direct analysis: 0·5% "gain" in tyrosine, >5% loss of tryptophan. After extraction at *p*H 1 with equal volume of ether: 0% loss of tyrosine, >4% loss of tryptophan. After extraction at *p*H 7 with an equal volume of ether: 0·5% loss of tyrosine, >5% loss of tryptophan. After extraction at *p*H 7 with an equal volume of toluene: 0% loss of tyrosine, >5% loss of tryptophan. (Tint too grey and fading rate too low in all the tryptophan estimations.)

C. *Tyrosine and tryptophan heated in NaOH solution.* (1) 50 mg. tyrosine in 10 ml. 5*N* NaOH at 100° for 20 hr.* Direct analysis: no loss of tyrosine.

(2) 25 mg. tryptophan in 10 ml. 5*N* NaOH at 100° for 30 hr.* The solution became very pale yellow. Direct analysis: >4% loss of tryptophan.

(3) 10 mg. tyrosine and 5 mg. tryptophan in 2 ml. 5*N* NaOH at 100° for 24 hr.† A very faint odour of indole was detected on opening the tube. Direct analysis: 0% loss of tyrosine, >5% loss of tryptophan. After extracting at *p*H 1 with an equal volume of ether: 1% loss of tyrosine, >4% loss of tryptophan. After extracting at *p*H 7 with an equal volume of ether: 0% loss of tyrosine, ?>5% loss of tryptophan. After extracting at *p*H 7 with an equal volume of toluene: 0% loss of tyrosine, ?>6·5% loss of tryptophan.

D. *Tyrosine and tryptophan heated in alkali-stannite solution.* The alkali-stannite reagent used in these and later experiments was prepared by adding solid $SnCl_2, 2H_2O$ at the rate of 5 g. per 100 ml. 5·5*N* NaOH solution and shaking in the presence of nitrogen or of a very small quantity of air until the solid had dissolved. The reagent rapidly absorbs oxygen upon exposure to air and adequate precautions must be taken in manipulations with it. On heating for some time in the absence of oxidizers, crystals of metallic tin are deposited in accordance with the requirements of (stannite)/(stannate) = K (solid Sn), but equilibrium is reached very slowly.

(1) 25 mg. tyrosine and 12·5 mg. tryptophan in 10 ml. of NaOH-stannite reagent at 100° for 24 hr.† A very faint odour of indole was detected upon opening the tube. Direct analysis: 1% loss of tyrosine, >1% loss of tryptophan.

(2) 10 mg. tyrosine and 5 mg. tryptophan in 2 ml. of NaOH-stannite reagent at 100° for 25 hr.† Direct analysis: 0% loss of tyrosine, >1% loss of tryptophan. After extraction at *p*H 1 with an equal volume of ether: 0·5% "gain" in tyrosine, ?>2% loss of tryptophan. After extracting at *p*H 7 with an equal volume of toluene: 0·5% loss of tyrosine, 3% loss of tryptophan.

(3) 10 mg. tyrosine and 5 mg. tryptophan in 2 ml. of NaOH-stannite reagent at 100° for 30 hr.† Direct analysis: 0·5% loss of tyrosine, ?>2% loss of tryptophan. After extraction at *p*H 14 with twice the volume of toluene: 0% loss of tyrosine, ?>3% loss of tryptophan.

E. *Tyrosine and tryptophan heated in H_2SO_4 solution with amino-acid and carbohydrates.* (1) 50 mg. tyrosine and 1 g. sucrose in 7 ml. 7*N* H_2SO_4 at 100° for 20 hr.* Considerable brownish black insoluble "humin" separated. Direct analysis: 54% loss of tyrosine.

(2) 50 mg. tyrosine and 0·25 g. arabinose in 7 ml. 7*N* H_2SO_4 at 100° for 20 hr.* Considerable black humin separated. Direct analysis: 22% loss of tyrosine.

(3) 50 mg. tyrosine, 0·25 g. arabinose, 0·25 g. sucrose and 1 g. glycine in 7 ml. 7*N* H_2SO_4 at 100° for 20 hr.* Much insoluble humin appeared. Direct analysis: 16% loss of tyrosine.

(4) 25 mg. tryptophan and 0·5 g. sucrose in 7 ml. 7*N* H_2SO_4 at 100° for 24 hr.* Considerable insoluble humin was produced. Direct analysis: a loss of at least 98% tryptophan.

F. *Tyrosine and tryptophan heated in NaOH solution with amino-acids and carbohydrates.* (1) 50 mg. tyrosine and 1 g. sucrose in 10 ml. 5*N* NaOH at 100° for 20 hr.* Direct analysis: 0·5% loss of tyrosine.

(2) 50 mg. tyrosine and 0·25 g. arabinose in 10 ml. 5*N* NaOH at 100° for 20 hr.† Direct analysis: 0% loss of tyrosine.

(3) 50 mg. tyrosine, 0·25 g. arabinose, 0·25 g. glucose and 1 g. glycine in 10 ml. 5*N* NaOH at 100° for 20 hr.* Direct analysis: 1·5% loss of tyrosine.

(4) 50 mg. tyrosine, 25 mg. tryptophan, 50 mg. cystine, 0·6 g. glycine, 0·1 g. arabinose and 0·3 g. sucrose, in 18 ml. 5*N* NaOH at 100° for 24 hr.* Direct analysis: 1·5% loss of tyrosine >7·5% loss of tryptophan.

G. *Hydrolysis of* O-*β-glucosido*-l-*tyrosine by heating with alkali-stannite reagent.* (1) 10·40 mg. *O-β*-glucosidotyrosine in 2 ml. NaOH-stannite reagent at 100° for 20 hr.† Direct analysis: 5·55 mg. tyrosine. (Theoretical for complete hydrolysis: 5·49 mg. tyrosine.) As *O-β*-glucosidotyrosine itself gave a coloration corresponding with less than 3% hydrolysis in the procedure used in analysis and the actual extent of hydrolysis could not have been much greater, "complete" hydrolysis must have been effected by the alkali-stannite reagent.

H. *Hydrolysis of edestin by heating with NaOH solution.* (1) Approx. 1 g. edestin in 10 ml. 5*N* NaOH at 100° for 24 hr.* Direct analysis: 1·81% tyrosine-N, ? <1·06% tryptophan-N.

I. *Hydrolysis of edestin in the presence of carbohydrate by heating with NaOH solution.* (1) Approx. 1 g. edestin and 0·2 g. arabinose in 10 ml. 5*N* NaOH at 100° for 20 hr.* Direct analysis: 1·78% tyrosine-N, ? <1·05% tryptophan-N.

J. *Hydrolysis of edestin by heating with alkali-stannite reagent.* (1) Approx. 1 g. edestin in 10 ml. NaOH-stannite reagent at 100° for 20 hr.† Direct analysis: 1·79% tyrosine-N, ? <1·10% tryptophan-N.

(2) Approx. 0·2 g. edestin, which had first been dried by heating in air at 105° for 18 hr., in 2 ml. NaOH-stannite reagent at 100° for 50 hr.† Direct analysis: 1·80% tyrosine-N, <1·07% tryptophan-N. After extraction at *p*H 1·5 with 1·5 volumes of ether: 1·78% tyrosine-N. After extraction at *p*H 14 with an equal volume of toluene: 1·80% tyrosine-N, 1·02% tryptophan-N.

K. *Hydrolysis of edestin in the presence of carbohydrate by heating with alkali-stannite reagent.* (1) Approx. 1 g. edestin and 0·2 g. arabinose in 10 ml. NaOH-stannite reagent at 100° for 24 hr.† Direct analysis: 1·78% tyrosine-N, ? <1·06% tryptophan-N. After extraction at *p*H 1·5 with an equal volume of ether: 1·77% tyrosine-N, >1·07% tryptophan-N. After extraction at *p*H 14 with an equal volume of toluene: 1·78% tyrosine-N, ? <1·05% tryptophan-N.

L. *Digestion of 3:5-diiodo*-l-*tyrosine with alkali (alone and in presence of cystine) and with alkali-stannite reagent.* Small quantities of KI, exceeding the amount of iodide which could be present in any aliquot of solution taken for analysis in L (1), L (2) and L (3), were found not to affect the estimation of tyrosine.

The diiodotyrosine gave virtually no colour reaction with the reagent and under the conditions normally employed in the tyrosine estimations, and none was expected, for the substance (in accordance with Vaubel's [1900] generalization) is reputed not to give an ordinary Millon reaction. 2 mg. of the substance did not interfere with the reaction given by 1 mg. of tyrosine. The substance was almost quantitatively reduced to tyrosine by exhaustive reduction with zinc dust in alkaline solution.

(1) 22·5 mg. diiodotyrosine in 2 ml. 5*N* NaOH at 100° for 24 hr.† The solution became yellow coloured. Direct analysis: doubtful 0·2 mg. tyrosine. Exhaustive reduction with zinc dust in alkaline solution showed that some 17% of the diiodotyrosine had been destroyed during the digestion with alkali.

(2) 22·4 mg. diiodotyrosine and 15·0 mg. cystine in 2 ml. 5*N* NaOH at 100° for 24 hr.† The solution developed a pale yellow coloration and smelled faintly of ammonia. On acidifying, it smelled faintly of H_2S. Direct analysis: 0·5 mg. tyrosine.

(3) 22·4 mg. diiodotyrosine in 2 ml. of the NaOH-stannite reagent at 100° for 24 hr.† The solution remained colourless and metallic tin was deposited. Direct analysis: 9·3 mg. tyrosine. (Theoretical for complete reduction: 9·37 mg. tyrosine.)

Discussion

Pure samples of *l*-tyrosine and *l*-tryptophan were used in the experiments and in the preparation of standard solutions. It is not known to what extents these substances were racemized during digestion with the reagents, but some degree of racemization presumably occurred and it seems that the *d*- and *l*-forms behave similarly in the reactions upon which the methods of analysis are based. As a modification which the substances might undergo in the digestions, racemization has been ignored in the foregoing descriptions and in the following discussion.

Tyrosine is practically unaffected by digestion for 20–30 hr. at 100° in 7*N* H_2SO_4, 5*N* NaOH and NaOH-stannite solutions. With the addition of carbohydrate there is considerable loss of tyrosine in acid digests (attributable perhaps to aldehyde-phenol condensation, as in "bakelite"), but if there is any loss in NaOH and NaOH-stannite digests it is too small to be certain.

Whilst the tryptophan estimations in B (2) and B (3) were vitiated by the presence of what were probably other indoles, the changes in fading rate were not such as to suggest that these were present in large proportions. Using a large quantity of tryptophan in the same sort of experiment, Homer [1915] actually isolated some 50 % of it from the digest. We must conclude that tryptophan is much more stable when digested with acid than is commonly believed, not less as asserted by Folin & Ciocalteu [1927]. With alkali there is less extensive modification and the indoles produced can be extracted apparently completely with ether or toluene at *p*H 7–14. The losses are about only one-third as great as those recorded by Kraus [1925] for the digestion of tryptophan with comparatively dilute $Ba(OH)_2$ solution at 100° for 40 hr. Part of the destruction is doubtless due to oxidation, the losses being reduced when stannite is present (compare C (2) and C (3) with D (1), D (2) and D (3)).

There is practically complete loss of tryptophan when it is heated in acid solution with much carbohydrate, a result which was only to be anticipated from the well-known reactions between indoles and aldehydes in acid solution. Protein preparations usually contain at least sufficient carbohydrate to give a Molisch reaction, and the customary failure to find tryptophan in acid hydrolysates might be attributed more reasonably to the presence of original carbohydrate than to the causes advanced by Homer [1915]. Over and above the destruction which occurs when tryptophan is digested with alkali or with alkali-stannite, there may be a slight loss in the presence of carbohydrate, but it is so small as to be uncertain. Apparently the slow absorption of atmospheric oxygen by sugars in alkaline solution will not serve as stannite does in protecting tryptophan. The strongly reducing decomposition products of cystine (sulphydryl bodies and inorganic sulphides) might afford some protection if insufficient oxygen were admitted to oxidize them.

The only phenol and indole residues present in the edestin molecule appear to be those of tyrosine and tryptophan respectively, and they suffer no appreciable destruction when the protein is heated in the dry state at 105° for 18 hr.

Hydrolysis must have been virtually complete when the protein was heated with 5*N* NaOH or with the NaOH-stannite reagent at 100° for 20–24 hr., a conclusion supported by the failure of the hydrolysates to give definite biuret reactions. Tyrosine and tryptophan peptides appear to be no more susceptible to destruction than the free amino-acids are, during digestion with alkali or alkali-stannite in the presence of carbohydrate.

The tryptophan estimations in J (2) as compared with those in J (1), show a loss which is only to be expected in prolonged hydrolyses. The small loss of tyrosine encountered by Bailey [1937] in prolonged alkali hydrolysis of edestin, may, in his opinion (private communication), not exceed his errors of estimation with his colorimeter.

From G (1) it is concluded provisionally that prosthetic carbohydrate in *O*-glycoside linkage with tyrosine residues in a protein will be hydrolysed off in the course of alkali hydrolysis of peptide links.

Whilst diiodotyrosine is partially decomposed by digestion in hot alkaline solution, the products do not give the Millon (and associated) reactions. In presence of the strongly reducing decomposition products of cystine, however, there is a slight reduction, whereas in the presence of stannite the reduction to tyrosine is quantitative.

In regard to recommendations for the hydrolysis of complex materials as a preliminary step in the estimation of their tyrosine and tryptophan contents, the supremacy of alkali over acid hydrolysis needs no stressing. The matter is one of deciding the conditions under which the alkali hydrolysis may be conducted most satisfactorily, and in certain of its aspects the methods employed for the subsequent analyses will have some bearing. Where these particular aspects are involved in the following recommendations, provision is made for the use of the methods which have been used throughout this work.

Hydrolysis with plain alkali and with free access to oxygen (air) is recommended, (*a*) for the estimation of tyrosine if diiodotyrosine be present, and (*b*) for the estimation of tyrosine and tryptophan if much cystine be present. Otherwise, hydrolysis with alkali-stannite is recommended, for not only is a diminished destruction of tryptophan assured, but the finally prepared hydrolysates, particularly of plant tissues and of the rather impure protein preparations obtained therefrom, are generally much less strongly coloured and corrections for adventitious coloration in colorimetry are correspondingly reduced. There is incidental protection against strongly oxidizing impurities.

It will be clear that under favourable circumstances, hydrolysis with alkali-stannite will provide for the estimation of "total tyrosine" (tyrosine plus diiodotyrosine), whereas hydrolysis with plain alkali in the presence of oxygen will permit the estimation of the tyrosine moiety only, and the diiodotyrosine content can then be computed from the difference.

The alkali-stannite reagent described in D is unlikely to require modification. It must be remembered that it contains chloride. An adequate criterion for the presence of sufficient stannite is the appearance, during hydrolysis, of metallic tin, either in the form of glistening crystals or a fine grey deposit.

For hydrolysis with plain NaOH as a reagent the vessel may be sealed with plenty of air enclosed, or left open (and preferably with a CO_2 trap). The use of stannite, however, necessitates sealing from oxygen, and exceptional circumstances may demand some type of safety-valve and a preliminary flushing out with nitrogen, but the ammonia ordinarily evolved from proteins develops only a very slight pressure. Glass vessels would normally be used, and whilst an alkali-resistant type is preferable, ordinary soft glass serves quite well, very little

solution occurring in 30 hr. at 100°. Test-tubes make very satisfactory vessels, and if intended for subsequent sealing-off in a flame they should first be constricted at about the proper length. The hydrolysis mixture should be reasonably fluid and its alkalinity must not fall seriously during hydrolysis. 10ml. of 5*N* NaOH or of the NaOH-stannite reagent per g. of protein preparation will generally be found satisfactory, and the corresponding air-space above the liquid after sealing-off should not exceed 2 ml. if stannite is used. Finely divided materials are easily wetted if the interstitial air is first removed at the pump and the reagent admitted at low pressure.

It is recommended that the vessel be immersed in boiling water for 30 hr., a period longer than has been found necessary for the hydrolysis of any protein thus far examined, and which is probably long enough for any protein, and the vessel should be vigorously shaken occasionally, particularly during the early stages, to ensure that no solid is left for long out of contact with the reagent.

After removing the liquid from the vessel and rubbing up and washing out any deposit adhering to the walls, the volume is made up to three or four times the original volume. If stannite has been included the mixture is now thoroughly agitated for a minute or so each time with three successive portions each of about 0·2 g. Zn dust per 10 ml. of reagent used, and then centrifuged. In the absence of stannite the treatment with Zn dust is omitted. After pouring off the clear liquid and washing the residue with successive small portions of 0·1*N* NaOH solution, the solution is extracted with 1–2 volumes of ether or toluene, toluene being the less troublesome. The solvent and the interior of the separating funnel are washed, preferably with the last few washings from the centrifuge tube. The solution is made acid by stirring in 5 ml. of 15*N* H_2SO_4 per 10 ml. of reagent used, and clear liquid is separated from silica and other precipitated material by centrifuging, the residue requiring thorough washing with successive small volumes of 0·1*N* H_2SO_4 solution. The solution is extracted with 1–2 vol. of ether, and once again the solvent is most satisfactorily washed with the final few centrifuge washings. Solvents are removed from the solution, either by evaporating off at reduced pressure at up to 45° (a convenient method if the solution needs concentrating before analysis) or by exposing the surface to a current of air. The temperature may be raised to aid the process, but it should not exceed 45°, and foaming, with consequent loss by spraying, should be avoided. On cooling, the solution is centrifuged if necessary and diluted to a suitable volume for analysis. Tyrosine estimations require no corrections for loss but tryptophan estimations require correction for 3 % loss if stannite has been included or 6 % if plain alkali has been used.

The extractions with toluene and ether may frequently be omitted when "pure" proteins are being analysed, but individual operators must then decide for themselves upon the matching of the tryptophan colour standard with the test solution. Intensity matchings to the normal eye, with the colorimeter under approximately daylight filament illumination, will then require correction for a loss of tryptophan of about 1–2 % if stannite has been used, or of about 4–5 % if omitted. On the other hand, the hydrolysates of some preparations may require an additional extraction with ether at *p*H 7–8 to remove phenols which contain amino but no carboxyl groups.

Test-tubes of 7·5 cm. length, 0·8 cm. internal diameter and about 2·5 g. in weight, have been found eminently suitable for the hydrolysis of 0·2 g. protein with 2 ml. of the alkali-stannite reagent; and the final volume of solution has generally been 25 ml., 3 ml. aliquots of which have been taken for analysis.

SUMMARY

Satisfactory means of hydrolysing complex materials for the subsequent estimations of tyrosine and tryptophan, have been sought. Certain procedures have been recommended and detailed instructions are provided. Errors are discussed and corrections are indicated.

The effects of the presence of carbohydrate upon the estimations of tyrosine and tryptophan are so small as to be uncertain, when the hydrolysis is done with alkali or alkali-stannite reagent, and may ordinarily be ignored.

Evidence is adduced to show that alkali hydrolysis will liberate tyrosine from *O*-glycoside linkage.

3 : 5-Diiodotyrosine is quantitatively reduced to tyrosine by digestion with alkali-stannite reagent.

For their interest and the provision of materials used in this work I am much indebted to Prof. A. C. Chibnall, Dr K. Bailey, and Mr H. R. Marston. Thanks are due also to Prof. C. R. Harington and Mr R. F. Clutton, who very kindly provided the samples of *O*-β-glucosidotyrosine and 3 : 5-diiodotyrosine.

REFERENCES

Bailey (1937). *Biochem. J.* **31**, 1406.
Folin & Ciocalteu (1927). *J. biol. Chem.* **73**, 627.
Gortner & Blish (1915). *J. Amer. chem. Soc.* **37**, 1630.
Herzfeld (1913). *Biochem. Z.* **56**, 82.
Homer (1915). *J. biol. Chem.* **22**, 369.
Kraus (1925). *J. biol. Chem.* **63**, 157.
Lugg (1937). *Biochem. J.* **31**, 1422.
Roxas (1916). *J. biol. Chem.* **27**, 71.
Vaubel (1900). *Z. angew. Chem.* **13**, 1125.
von Deseö (1934). *Biochem. Z.* **271**, 142.