Biochemical

Qualitative Test for Ascorbic Acid. R. S. Tipson (*J. Amer. Pharm. Assoc., Sci. Ed.*, 1945, **34**, 190–192)—It has been observed that dehydroascorbic acid reacts with pyrroles in aqueous trichloroacetic acid at 50° C. to give an intense blue colour with a band at 630–635 *mμ*. The advantages of using trichloroacetic acid are that it precipitates proteins from biological extracts, gives a *p*H at which dehydroascorbic acid is relatively stable and facilitates oxidation of ascorbic acid to dehydroascorbic acid in presence of activated charcoal.

To 15 ml of a soln. containing about 100 mg of ascorbic acid in 5% trichloroacetic acid add a little activated charcoal, shake and filter through a fluted filter. To each of two 5-ml portions of the filtrate add 0·02 ml of pyrrole and shake gently until dissolved. Heat one portion for 5 min. in a bath at 50° C., and at the same time a blank consisting of 5 ml of 5% trichloroacetic acid and 0·02 ml of pyrrole; leave the other portion of the filtrate at room temperature as a control. The formation in the heated tube of a blue colour absorbing at 630–635 *mμ* indicates the presence of ascorbic acid. It is believed that the reaction could be made the basis of a quantitative colorimetric method.

A large number of substances, including the water-soluble vitamins, were tested but only alloxan hydrate gave a similar blue colour under the conditions described. F. A. R.

Microbiological Estimation of Amino Acids. II. Assay and Utilisation of Glutamic Acid and Glutamine by *Lactobacillus arabinosus*. L. R. Hac, E. E. Snell and R. J. Williams (*J. Biol. Chem.*, 1945, **159**, 273–289)—Prepare stab-cultures of *Lactobacillus arabinosus* 17–5 on a medium containing 1% of glucose, 1% of yeast extract and 1·5% of agar. Prepare a medium containing per litre: Bacto-peptone, 5 g; Bacto yeast extract, 1 g; sodium acetate, 10 g; glucose, 10 g; salt solns. A and B (see below), 5 ml each. Inoculate from the stab-culture and incubate for 18–24 hr. Centrifuge, wash once with saline and resuspend in 10 ml of saline. For a "heavy" inoculum use one drop of this suspension per 2·5 ml of assay medium (or 1·25 ml of double-strength medium). When a "light" inoculum is preferred, add 3 to 7 drops of the above suspension to 10 ml of saline and use one drop of the diluted suspension per 2·5 ml of assay medium. Prepare the basal medium for assay as follows (the values record the concentration per ml of final medium): *l*(+)-arginine monohydrochloride, 0·1 mg; *l*(−)-cystine, 0·1 mg; glycine, 0·1 mg; *l*(−)-histidine monohydrochloride, 0·1 mg; *l*(−)-hydroxyproline, 0·1 mg; *l*(−)-leucine, 0·1 mg; *l*(+)-lysine monohydrochloride, 0·1 mg; *l*(−)-proline, 0·1 mg; *l*(−)-tryptophan,* 0·1 mg; *l*(−)-tyrosine, 0·1 mg; *l*(+)-aspartic acid, 0·4 mg; *l*(+)-glutamic acid,† 1·0 mg; *dl*-alanine, 0·2 mg; *dl*-isoleucine, 0·2 mg; *dl*-methionine,* 0·2 mg; *dl*-norleucine,* 0·2 mg; *dl*-norvaline,* 0·2 mg; *dl*-phenylalanine,* 0·2 mg; *dl*-serine,* 0·2 mg; *dl*-threonine, 0·2 mg; *dl*-valine, 0·2 mg; biotin, 0·001 μg; folic acid, 0·01 μg; calcium pantothenate, 0·2 μg; nicotinic acid, 0·2 μg; riboflavin, 0·2 μg; *p*-aminobenzoic acid, 0·3 μg; aneurine hydrochloride, 0·5 μg; choline chloride, 2·5 μg; inositol, 2·5 μg; pyridoxine hydrochloride,‡ 10·0 μg; adenine sulphate, 0·01 mg; guanine hydrochloride, 0·01 mg; uracil, 0·01 mg; xanthine, 0·01 mg. Salts A, KH_2PO_4 1·0 mg, K_2HPO_4 1·0 mg; Salts B, $MgSO_4.7H_2O$ 0·2 mg; $FeSO_4.7H_2O$ 0·01 mg, $MnSO_4.4H_2O$ 0·01 mg, NaCl 0·01 mg, $(NH_4)_2SO_4$ 3·0 mg; sodium acetate (anhydrous), 6·0 mg; glucose, 10·0 mg.

Use pure *l*(+)-glutamic acid for preparing the standard curves. Hydrolyse samples of the protein to be analysed by sealing 100 mg in thick-walled Pyrex test tubes with 2 ml of 10% hydrochloric acid and autoclaving at 15 lb. pressure for 4 to 36 hr. Adjust the *p*H to 6·1–6·3 with sodium hydroxide and dilute to 50 or 100 ml. When the amount of growth is to be measured turbidimetrically pipette a suitable aliquot into a test tube, make up to 1·2 ml with water and add 1·2 ml of the double-strength basal medium (omitting glutamic acid). Double these quantities when the amount of growth is to be estimated by titration, close the tubes with metal caps or glass vials and autoclave at 15 lb. pressure for 15 min. Inoculate with a "heavy" or "light" inoculum and incubate at 34° C. for 72 hr. For turbidimetric readings, dilute the contents of the tubes with 5 ml of water and measure the turbidity in a thermocouple turbidimeter or Klett-Summerson colorimeter. For titration of the acid produced, use 0·05 *N* sodium hydroxide with a glass electrode or with bromothymol blue as indicator. Use an assay range of 0·01 to 0·2 mg of *l*(+)-glutamic acid per 2·5 ml of (single strength) medium. Calculate the results from a standard curve, using the average of duplicate analyses at three or four concentration levels.

The standard curve for glutamic acid proved to be anomalous, since it showed an initial plateau at which no growth occurred, followed by a curve of the conventional shape. This observation suggests that *L. arabinosus* (and certain other organisms) is unable to assimilate glutamic acid as such. Further investigation showed that the assay curve for glutamine had no plateau, so that glutamic acid apparently has to be converted into glutamine or a similar substance before it can be utilised. Conversion of glutamic acid to glutamine appeared to be optimal in acidic soln. in presence of ammonium salts. It took place somewhat more readily with the "heavy" than with the "light" inoculum. After 72 hours incubation, however, the glutamine and glutamic acid curves were identical, except at the lowest concentration of glutamic acid. Results obtained turbidimetrically were usually slightly higher than those obtained by titration, but as the method was easier and less time-consuming than titration, it was preferred unless the sample was too highly coloured or developed a turbidity during incubation. The use of a "heavy" inoculum gave in general slightly higher results than a "light" inoculum. The recovery of glutamic acid added to various protein hydrolysates was quantitative, whilst the results obtained for the glutamic acid content of a number

* Half the indicated amounts have been found satisfactory for assays of glutaminc acid.

† Omitted in medium for assay of glutamic acid.

‡ One-tenth the amount indicated is adequate for assays with *L. arabinosus* 17-5.

of pure proteins showed in the main satisfactory agreement with previously reported results obtained by chemical methods. F. A. R.

Microbiological Estimation of Amino Acids. III. Assay of Aspartic Acid with *Leuconostoc mesenteroides*. L. R. Hac and E. E. Snell (*J. Biol. Chem.*, 1945, **159**, 291–294)—Maintain stock cultures of *Leuconostoc mesenteroides* P-60 and prepare the inoculum as previously described by Dunn *et al.* (*cf.* ANALYST, 1945, **70**, 182). The basal medium was identical with that used for *l*(+)-glutamic acid (*cf.* preceding abstract), but aspartic acid was omitted. The phosphate content of the medium was increased four-fold and the final *p*H of the medium was adjusted to 6·8. Use pure *l*(+)-aspartic acid for the standard curve. The assay range is 0·001 to 0·04 mg per 2·5 ml. Prepare the protein hydrolysates as in the assay of glutamic acid and analyse the sample in duplicate at three or four concentration levels. Measure the growth turbidimetrically.

The standard curve for aspartic acid was similar to those reported for most other amino acids, whereas asparagine gave an anomalous curve similar to that of glutamic acid, as that whilst glutamic acid is less readily available for growth than glutamine, asparagine appears to be less readily available than aspartic acid. The recovery of aspartic acid added to protein hydrolysates was within 4% of the theoretical, and the results obtained with a number of proteins were in fairly good agreement with previously reported results obtained by chemical methods. F. A. R.

Spectrophotometric Estimation of Small Amounts of Choline. R. J. Winzler and E. R. Meserve (*J. Biol. Chem.*, 1945, **159**, 395–397)—Choline is usually estimated by precipitation with Reinecke salt and colorimetric estimation of choline reineckate in acetone. An increase in the sensitivity of this method can be obtained by taking advantage of the intense absorption of ultra-violet light by choline reineckate. In addition to the broad adsorption band at 525 $m\mu$ ($\epsilon = 120$) choline reineckate gives an intense peak at 327 $m\mu$ ($\epsilon = 5820$).

Extract the sample continuously with methanol for 24 hr., evaporate off the methanol and heat the extracted lipoids with 15 ml of saturated barium hydroxide soln. under reflux for 2 hr. Adjust the *p*H to 4–5 with glacial acetic acid, make up to a specific volume and filter. Treat an aliquot of the filtrate, containing 50 to 400 μg of choline, with an equal volume of a saturated soln. of reineckate salt and leave the mixture for at least 3 hr. in an ice-bath. Filter off the precipitated choline reineckate on a sintered glass filter cooled in an ice-bath and wash the precipitate in the ice-bath with three 2-ml portions of ice-cold water saturated with choline reineckate at 0° C. Aspirate the precipitate as dry as possible and dissolve in acetone by passing 15 ml through the filter. Measure the optical density at 327 $m\mu$ and calculate the choline content from the value given above for the molecular extinction coefficient. The error of the method, in the range of 50 to 400 μg of choline hydrochloride, is about ±5%. F. A. R.

Micro-Kjeldahl Determination of the Nitrogen Content of Amino Acids and Proteins. L. Miller and J. A. Houghton (*J. Biol. Chem.*, 1945, **159**, 373–383)—The micro-Kjeldahl method gives low results for the nitrogen content of lysine, tyrosine and tryptophan, especially when selenium is used as catalyst. An examination of the method showed that the conditions of heating during distillation should be rigidly standardised and maintained and that a mixture of potassium sulphate, mercuric oxide and sulphuric acid is the most satisfactory catalyst for use in the digestion. A 6-hour period of digestion is recommended, counted from the time the charred material clears or, if no charring occurs, from the time that fumes of sulphur trioxide appear.

Digest the soln., containing 0·4 to 1·4 mg of nitrogen, in aliquots of 1 or 2 ml with potassium sulphate (500 mg), mercuric oxide (50 mg) and conc. sulphuric acid (1·5 ml) with gentle heat until the water has evaporated and then with the full flame of the micro-burners. At the end of 5 hr., cool the flasks, add one drop of ethyl alcohol and heat for a further hour. Cool, add 5 ml of water and stopper the flasks until distillation can be carried out. The distillation is best carried out with a steam generator into the bottom of which is sealed a coil of fine platinum wire 2¾ in. long, projecting through the outside about 1/16 in. This permits of more rapid generation of steam. Steam out the apparatus for at least 10 mins. and then, with the tip of the delivery tube dipping under the surface of 2% boric acid soln. (10 ml) containing a little methyl red-bromocresol green, transfer the digests to the apparatus with about 8 ml of water divided into three portions. Rinse the funnel and add 5 ml of a soln. made by dissolving 40 g of sodium hydroxide and 5 g of $Na_2S_2O_3.5H_2O$ in 60 g of water. Rinse the last traces of alkali into the flask and make up the volume to about 32 ml. Close the stop-cocks, mix thoroughly and heat the steam generator with one micro-burner until the first trap is warm, then with both burners. Set a stop-watch going when the first drop of condensate appears between the traps and the condenser. Distil for 2 mins. and then lower the receiver so that the tip of the condenser is about 3 cm above the level of the acid. At the end of 3 mins. lower the receiver again so as to bring the tip of the delivery tube into the neck of the flask and after a further ½ min. detach the receiver. After making each of these changes rinse the tip of the condenser. Make up the distillate to 40–50 ml and titrate with 0·01 *N* hydrochloric acid. Carry blanks identical with the experimental solns. through the whole procedure. Theoretical nitrogen contents for all the common amino acids were obtained by this procedure. The values obtained for proteins were in good agreement with the most reliable values previously recorded in the literature.

F. A. R.