APPENDIX

Notes on a Modification of the Neuman & Logan Method for the Determination of the Hydroxyproline

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The Neuman & Logan (1950) technique for the determination of hydroxyproline in protein hydrolysates consists of (i) oxidation of hydroxyproline with hydrogen peroxide in the presence of alkaline copper sulphate, (ii) destruction of excess of peroxide by heat and (iii) reaction of the oxidation product with *p*-dimethylaminobenzaldehyde by heating in the presence of dilute sulphuric acid to produce a red colour, the intensity of which is compared with a standard. The original method has been modified by a number of workers (Baker, Lampitt & Brown, 1953; Martin & Axelrod, 1953; Wierbicki & Deatherage, 1954; and Miyada & Tappel, 1956) in an attempt to improve (*a*) the removal of the hydrogen peroxide, which, if undestroyed, leads to low and variable colour yields, (*b*) the reproducibility amongst replicates and (*c*) the colour yield. The present modification has, in our Laboratories, given a greater reproducibility amongst replicates and colour yields reproducible over a prolonged period. In the modification to be described, stages (i) and (ii) of the original method are combined. The oxidation is carried out at 40° and is completed before the total destruction of the excess of peroxide has occurred. For ease of reference the parts of the original Neuman & Logan method used are described with the modification.

REAGENTS

0·05 M-*Copper sulphate (A.R.) in water.* This is the concentration recommended by Baker *et al.* (1953), and results in higher colour yields than the 0·01 M concentration recommended by Neuman & Logan (1950).

2·5 N *(approx.)-Sodium hydroxide.* Sodium hydroxide (100 g. of pellets; A.R.) was made up to 1 l. with water.

6% *(approx.) Hydrogen peroxide.* This was made by diluting A.R. 30% (w/v) hydrogen peroxide. The diluted solution is not reliable for more than a day and it is advisable to make up a fresh solution immediately before use. The parent solution is stable over a long period, but can be checked by the method of Vogel (1957).

3 N *(approx.)-Sulphuric acid.* The solution contained 83 ml. of A.R. conc. H_2SO_4/l.

5% p-*Dimethylaminobenzaldehyde.* The reagent (May and Baker Ltd.) dissolved in redistilled propan-1-ol (fraction 95·8–98·0°) has been found satisfactory. This solution should be stored in the dark.

Standard L-*hydroxyproline solutions.* Hydroxyproline (0·05 g.) was dissolved in water and diluted to about 400 ml. with water, 20 ml. of conc. HCl was added (J.E. Eastoe, personal communication) and the solution made up to 500 ml. with water. The acid prevents microbiological destruction; the solution will keep only for a short period if the HCl is omitted. The 100 μg./ml. solution is diluted to give concentrations of 5, 10 and 15 μg. of hydroxyproline/ml. The amino acid samples supplied by British Drug Houses Ltd. and L. Light and Co. Ltd. were found to be pure and dry. Drying at 105° for 2 hr. is, however, a safeguard against moisture.

PROCEDURE

The solution under test should contain preferably between 5 and 15 μg. of hydroxyproline/ml. For a single determination 13 1 in. × 6 in. rimless Pyrex test tubes are required. In each of tubes 1–3 is placed 1 ml. of 5 μg. standard, in tubes 4–6 1 ml. of 10 μg. standard, in tubes 7–9 1 ml. of 15 μg. standard, in tubes 10–12 1 ml. of the test solution and in tube 13 1 ml. of water. Into each tube is placed 1 ml. of 0·05 M-$CuSO_4$, followed by 1 ml. of 2·5 N-NaOH, and the tube contents are each mixed by gentle swirling of the liquid. The tubes are placed in a water bath at 40°. When the contents have reached 40° (3–5 min.), 1 ml. of 6% hydrogen peroxide is added, which is immediately mixed by swirling the contents of a tube before the addition is made to the next tube. During the swirling of the contents no solution should remain on the wall of the tube above the level reached by the water in the bath, otherwise the destruction of the peroxide is incomplete. The tubes are left in the bath for 10 min., but are occasionally removed from the bath and the contents swirled. The tubes are cooled with tap water, then 4 ml. of 3 N-H_2SO_4 and 2 ml. of 5% *p*-dimethylaminobenzaldehyde solution are added, the contents of the tubes being mixed by swirling after each addition. Caps (Oxoid) are placed on the tubes, which are kept in a water bath at 70° for 16 min.; the solutions are then cooled, mixed and their extinctions measured against the blank solution at a wavelength of 555 mμ in 4 cm. cells.

The average reading for each set of tubes is used in the calculation. In order to minimize the effect

of a slight negative deviation from Beer's law it is advisable to draw straight lines between each pair of standard values rather than a single line through the whole set.

RESULTS

Fig. 1 shows the variation of extinction with the temperature of the first bath with the modified technique. The values obtained for an average run by the original method are included for comparison. At first bath temperatures below 35° the colour yields are not reproducible and below 25° the normal reddish pink is sometimes replaced by an orange oxidation product, resulting from the presence of undestroyed hydrogen peroxide. The points below 25° are the average of a number of determinations where the colour did appear and are included to give an idea of the order of the decreased colour yield.

The scatter amongst replicates is less with the modified method; for example, carrying out both methods with the same solutions gave standard deviations, amongst replicates for the three standards and unknown, of 0·74 and 0·49 % by the original and modified methods respectively. The modified method has often yielded standard deviations amongst replicates of the order 0·1–0·2 %, an order of accuracy which was not achieved with the original method over a considerable period. The reproducibility of the colour yield from one series to another has been very high over a prolonged period. The colour yields are represented by the plateaus in Fig. 1, and the reproducibility of the method is such that it serves as a guide to reagent quality. A decrease in colour yield by 4 % or more from the values shown indicates inferior or decomposing reagents; or, if acid is omitted from the hydroxyproline solutions, that microbiological decomposition of the amino acid is taking place.

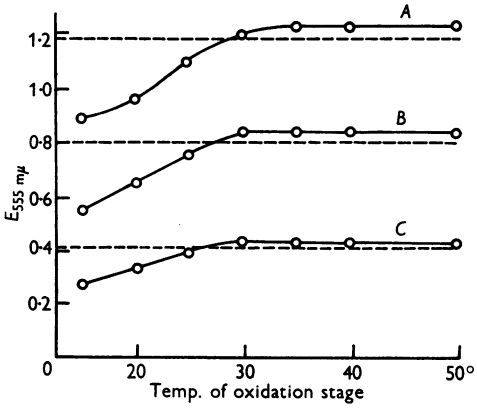

Fig. 1. Temperature of oxidation versus colour yield: *A*, 15 μg. of hydroxyproline/ml.; *B*, 10 μg. of hydroxyproline/ml.; *C*, 5 μg. of hydroxyproline/ml. Broken lines refer to average values obtained by the original Neuman & Logan method at the concentrations given above. A 4 cm. cell was used.

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Spectrophotometric Studies on the Combination of Formaldehyde with Tetrahydropteroylglutamic Acid and Other Hydropteridines

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A previous paper (Blakley, 1959) described studies on the reaction of formaldehyde with tetrahydropteroylglutamic acid and other hydropteridines, which were undertaken to elucidate the coenzyme function of tetrahydropteroylglutamate in the synthesis of serine from glycine and formaldehyde.

It was found that formaldehyde reacted with all the hydropteridines studied, but the complexes formed differed greatly in the extent to which they were dissociated. Formaldehyde was very firmly bound by tetrahydropteroylglutamate, the dissociation constant for the complex being at least 100 times lower than those for the complexes formed by simple hydropteridines. Since N^5-formyltetrahydropteroylglutamate (leucovorin) and N^{10}-formyltetrahydropteroylglutamate also formed