XXXIV.—*The Measurement of Tryptic Protein Hydrolysis by Determination of the Tyrosine Liberated.*

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OF the numerous methods described for following the course of protein hydrolysis, that described by Brown and Millar (T., 1906, 89, 145) apparently offers many advantages, since it is volumetric in character, easy of manipulation, and in the hands of these authors gave good results. The method, in brief, is based on the estimation, by absorption of bromine, of the tyrosine formed during tryptic digestion, and is carried out along the lines suggested by J. H. Millar (*Trans. Guinness Research Laboratory*, 1903, Part I.). The tyrosine-containing liquid is acidified with hydrochloric acid to which sodium bromide is added, and the solution thus obtained is titrated with standard sodium bromate solution. The product of reaction is dibromotyrosine.

We had occasion to study this method during the preliminary work attending another investigation, and found that, as originally described, the method is difficultly workable, but can be made useful by the introduction of several modifications.

Use of Starch and Potassium Iodide as Indicator.

J. H. Millar originally used the persistence of the yellow colour of bromine to mark the end-point of the reaction, but also states that starch and potassium iodide may be used as indicator of free bromine. The latter method was adopted by Brown and Millar.

In colourless solutions the yellow colour induced by excess of bromine may certainly be used to show the completion of the reaction, but this is impossible in ordinary protein digests, and the use of starch and iodine is also impossible, as a little consideration might show, since hydrochloric acid itself will liberate sufficient iodine from potassium iodide to cause coloration of the starch. We tried the effect of acidifying the solution with other weaker acids (such as phosphoric, citric, and acetic acids), but on keeping for a period equivalent to the length of time occupied by a titration they also liberate iodine and mask the end-point.

Use of Methyl-violet as Indicator.

Attempts were made to utilise the oxidising power of bromine in discharging the colour of organic dyestuffs, but with only partial success. Good results were, however, obtained by using the well-known stain, Gentian-violet, which shows marked colour changes. Gentian-violet is the commercial name given to a mixture of Methyl-violet, Crystal-violet, and dextrin, and the colour changes were found to be due to the Methyl-violet (pentamethylpararosaniline hydrochloride). In the acid solution used for titration this dyestuff becomes olive-green, and the addition of sodium bromate or traces of free bromine causes a sharp change to a deep bluish-violet. The change is very marked and exceedingly sharp, although the colour fades after several minutes. Excess of bromine or sodium bromate solution discharges the blue colour, changing it to a dirty yellow.

Rate of Bromination of Tyrosine.

Tyrosine rapidly takes up bromine to form dibromotyrosine, and the absorption at first is very rapid, but falls off considerably, under the conditions of titration, towards the end of the reaction, as is shown by the following case.

Twenty-five c.c. of $N/25$ -tyrosine solution in 5 per cent. hydrochloric acid and 20 c.c. of 20 per cent. sodium bromide solution were titrated with $N/20$ -sodium bromate; 0.5 c.c. of the latter was added at a time as long as the bromine was absorbed at once, and then two drops at a time until the reaction was complete.

	<i>N</i> /20-Sodium bromate used. C.c.	Tyrosine brominated. Per cent.	Time taken for absorption of bromine.
Up to	7.0	55.1	3.0 sec. (normal shaking)
	8.0	62.9	3.5 "
	9.0	70.8	6.0 "
	11.0	86.6	9.0 "
	12.0	94.5	25.0 "
	12.4	97.6	2 min.
	12.6	99.2	permanent after 30 min.

The falling off of the rate of bromination shows the impossibility, in any case, of the use of starch and potassium iodide as indicator, and precludes the use of any indicator within the experimental liquid, since free bromine may be present for a short time in the solution, and yet be absorbed by the tyrosine on keeping.

Method Used.

Indicator.—Methyl-violet or Gentian-violet may, however, be satisfactorily used as an outside indicator. The colouring matter is made of 1 per cent. strength in 70 per cent. alcohol, and about ten drops of the solution are added to 10 c.c. of 5 per cent. hydrochloric acid. This liquid is dotted over a white tile in the usual manner, and tested with the experimental liquid from time to time. With a little practice and after doing a preliminary titration the end of the reaction, as indicated by the formation of the blue colour, may be detected with accuracy.

On the tile, water alone produces a very similar colour, but dilute acid effects no change. Free bromine water of all strengths will not give the colour change, but does so with practically the same delicacy when dissolved in sodium bromide solution. This may be due to the formation of an unstable perbromide or similar compound with the Methyl-violet, or possibly to its action as nascent bromine (as in the actual titration of acid bromide solution with sodium bromate), the sodium bromide solution acting as NaBr, α Br.

Procedure.—The solution to be titrated is made of not more than 5 per cent. acidity with hydrochloric acid. A lower concentration of acid than 2 per cent. turns the indicator blue, whilst a higher concentration than 5 per cent. turns it yellow. To the acid solution is added 15–20 c.c. of 20 per cent. sodium bromide, and the liquid is then titrated with *N*/20-sodium bromate. Brown and Millar used *N*/5-sodium bromate solution for titration, but since it is seldom that more than 1 c.c. of solution of such strength is required, more accurate results are obtained by using the more dilute liquid.

Towards the end of the reaction at least thirty seconds should

elapse between successive additions of the sodium bromate, and the reaction is best carried out in a stoppered bottle, which can be vigorously shaken after each addition.

Under these conditions sufficiently accurate, comparable results can be obtained to make the method extremely useful. Dibromotyrosine only is apparently formed. Sodium bromate corresponding with 6 molecules of bromine were added to a known weight of tyrosine dissolved in dilute hydrochloric acid, and mixed with sodium bromide, and the mixture allowed to remain for twenty-four hours. At the end of that time the excess of bromine was estimated, and the amount absorbed was found to be practically only 2 molecules.

The following results were obtained with tyrosine and tyrosine-containing mixtures of strengths unknown at the time:

Weight of tyrosine used. Gram.	N/20-Bromate solution used. C.c.	Weight of tyrosine found. Gram.	Error. Per cent.
0.1274	9.80	0.1274	nil.
0.1460	11.45	0.1495	+2.3
0.1559	11.80	0.1576	1.09
*0.1000	7.60	0.1010	1.0
*0.0481	3.65	0.0483	0.4

* In solutions containing tyrosine, leucine, asparagine, and ammonium chloride.

Experiments carried out with edestin digested in dilute sodium carbonate solution with trypsin, and corrected for the bromine absorbed by the protein, confirmed Brown and Millar's contention that the method can be used for determining tyrosine in presence of proteins and other early cleavage products, and also that practically the whole of the tyrosine is liberated in the first stages of tryptic digestion.

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