A NEW COLORIMETRIC METHOD TO SHOW THE ACTIVITY OF EITHER 'PEPTIC' OR 'TRYPTIC' ENZYMES'

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Grützner, in 1874,² described a colorimetric method to estimate the amount of pepsin in a solution. He used fibrin which had been stained with carmine dissolved in ammonia. When the fibrin dissolved the carmine was set free and from the depth of colour the amount of fibrin which had been digested could be estimated. His method possesses one drawback in that it is applicable only to digestion in acid solution because when alkali is present the carmine is dissolved out of the fibrin without the latter being dissolved.

The stage of digestion which is shewn is the first step in hydrolysis, namely, that of the formation of acid-albumin. This stage, according to the present view of the action of proteoclastic enzymes in the body, is a very early one but the choice of it as an indicator of the rate of digestion is just as sound as many other methods which have been used. Almost any single stage might be taken when comparing the amount of the same enzyme in different solutions provided that it is regarded in the light of a means of indicating the rapidity of digestion and not as a final judgment on the normal mechanism of digestive processes.³

When one wishes to compare the relative activity of two enzymes, one of which acts better in an acid medium whilst the other only acts in an alkaline reaction, the stage of digestion chosen is of great importance. It is hardly fair to use a precipitant which precipitates

1. This method was shewn at a meeting of the Lancashire and Cheshire Branch of the British Medical Association, *British Medical Journal*, Vol. I, 1908, *Supplement*, p. 138.

2. *Arch. f. d. ges. Physiol.*, Vol. VIII, p. 452, 1874.

3. Cf. Bayliss, *Journ. Physiol.*, Vol. XXXVI, p. 225, 1907.

the end products of the one whilst the other, carrying the hydrolysis a step further, furnishes substances which remain in solution. A Kjeldahl nitrogen estimation of the filtrate after such a precipitation might shew entirely different results to a similar estimation made after the use of a different precipitant. There is, however, a certain amount of truth in saying that the further the hydrolytic splitting is carried the 'stronger' the digestive activity; but on the other hand there is something to be said for choosing, as a mark of the activity of a digestive enzyme, the rapidity with which an insoluble protein is dissolved.

The following method was devised to obviate the delay necessarily caused in carrying out total nitrogen estimations when a large number of comparative estimations are being made. It is brought forward in the belief that for most purposes it gives almost as much information as more elaborate procedures. The process is as simple as the 'Grützner' method, and possesses the advantage of acting just as well for digestions in alkaline solutions as for those in the presence of acid.

In seeking for some dye that would stain fibrin so that it would part with its colour neither to acid nor to alkali the one finally chosen was congo red. Bayliss¹ found that this dye is well absorbed and retained by filter paper, and that heating to 100° C. fixed the dye so that it cannot be removed by washing.

Fibrin stained by congo red was found to slowly part with its pigment when kept for some hours at 40° C. in the presence of one per cent. sodium carbonate. It is difficult to say whether this was due to slow solution of the fibrin or to the alkali removing the congo red from the fibrin, leaving the latter unchanged. This difficulty was surmounted by dropping the fibrin after staining into boiling water for a few minutes and then removing the excess of dye by washing in running water. Fibrin treated by this method did not cause any coloration of either 0.18 per cent. hydrochloric acid² or of

1. This Journal, Vol. I, p. 175, 1906.

2. The fibrin becomes changed from red to blue by the action of the acid.

1 per cent. sodium carbonate, when kept for one hour at 40° C. and then left at room temperature for three weeks.

As the heating to 100° C. renders fibrin less easily digested, the following experiment was performed to see if a lower temperature would suffice to prevent a red colour appearing in 1 per cent. sodium carbonate solution.

Experiment I.—20 gms. of moist fibrin, which had previously been minced and washed, was kept for 24 hours in 40 c.c. of 0.5 % solution of congo red. At the end of this time it was divided into six equal portions, and these were heated for five minutes to different temperatures. Each lot was then well washed and divided into two portions, one of which was placed in 0.18% HCl and the other in 1% Na₂CO₃ and they were all placed in the incubator at 40° C., toluol being used as a preservative.

Procedure	Kept at 40° C. in 0.18 % HCl		Kept at 40° C. in 1 % Na ₂ CO ₃	
	At end of 2 hours	At end of 24 hours	At end of 2 hours	At end of 24 hours
Heated for 5 mins. to 50° C.	No coloration	Bluish purple	Faint pink colour	Pink colour
Heated for 5 mins. to 60° C.	No coloration	Faint bluish purple	No coloration	Slight pink colour
Heated for 5 mins. to 70° C.	No coloration	Very faint bluish purple	No coloration	Slight pink colour
Heated for 5 mins. to 80° C.	No coloration	No coloration	No coloration	Slight pink colour
Heated for 5 mins. to 90° C.	No coloration	No coloration	No coloration	Slight pink colour
Heated for 5 mins. to 100° C.	No coloration	No coloration	No coloration	Slight pink colour

From this table it is seen that it is sufficient to keep the stained fibrin for five minutes at a temperature much lower than 100° C. in order to prevent the colour being removed by sodium carbonate. This appears to take place at 60° C., and is probably due to the destruction of an autolytic enzyme. As the final colour in all, except the first, was about the same tint, the slight colour observed is most probably due to a slight formation of alkali albumin by the long continued action of the carbonate at 40° C. In preparing the congo red fibrin it is a great advantage to use as low a temperature as possible because when the temperature has been raised to 100° C. for some time the fibrin is rendered much less easy to digest.

As the congo red turns blue with acid it is necessary to render the solution alkaline when it is desired to compare the depth of colour of an acid digest with that resulting from digestion in an alkaline solution. It was found that the easiest way to do this was to add solid anhydrous sodium carbonate until the precipitate, which forms

at the neutral point, redissolves. The depth of red colour of the product of peptic digestion can then be directly compared to that of the tryptic digest. Even when comparing peptic digestions with each other it is more convenient to render them alkaline before comparing them.

The method was next tested to compare whether the amount of colour given to the solution on digestion showed any relation to the amount of soluble nitrogen left after precipitation by trichloroacetic acid.

Experiment II.—Two enzyme solutions, one peptic and the other tryptic, were prepared, and the rate of digestion was compared as follows:—Twenty cubic centimetres of the peptic enzyme was digested, for one hour at 40° C., with 1 gr. minced fibrin. This was then precipitated by ten cubic centimetres of a ten per cent. solution of trichloroacetic acid and filtered hot; twenty-five cubic centimetres of the filtrate was then Kjeldahled. The number of cubic centimetres of decinormal acid given by a control, in which the enzyme had been destroyed by boiling before digestion, was subtracted from the figure obtained for the peptic digest, giving 7.4 c.c. The figure given for the tryptic enzyme under similar conditions was 8.0 c.c. The ratio of tryptic to peptic digestion was thus found to be 1.1.

Ten cubic centimetre portions of the same enzyme solutions were compared by the method described in this paper, namely by adding 0.5 gr. congo red fibrin and diluting the digest, obtained by allowing digestion to proceed for one hour at 40° C., until the solutions were of equal tint. The ratio obtained by this method was 1.0.

A second experiment, using different enzyme solutions, gave the following ratio of tryptic to peptic action. From total nitrogen determinations, 1.3. From congo red dilution, 1.5.

It is not claimed that the method of diluting the solutions until the colours appear to be the same is very accurate, but the results show a certain parallel between the two methods employed.

The relation, of the amount of enzyme added, to the rate of solution was also investigated.

Experiment III.—

Ratios of peptic enzyme in 10 c.c. of 0.18 % HCl with 0.5 gr. congo red fibrin	Ratios of enzyme action as shown by the amount of dilution to reach an equal tint	Ratios of tryptic enzyme in 10 c.c. of 1 % Na ₂ CO ₃ with 0.5 gr. congo red fibrin	Ratios of enzyme action as shown by the amount of dilution to reach an equal tint
4	2	4	4
9	2.9	9	10
16	3.6	16	18

This experiment shows that the enzyme action, as shewn by solution of congo red fibrin, for pepsin follows the Schütz¹ law as it is nearly the square root of the amount of enzyme present, whilst for trypsin it is roughly proportional to the amount of enzyme present.

It is thus shown that fibrin stained by congo red can be used to shew the amount of either peptic or tryptic enzyme present in a solution. The best method to prepare the fibrin is as follows. The fibrin should be minced and washed until free from blood. To each hundred cubic centimetres of one half per cent. solution of congo red in water, add fifty grammes of moist fibrin. Leave for twenty-four hours and then pour the pasty mass into a large volume of water heated to the required temperature and kept at that temperature for about five minutes. The temperature used can be varied according to the object required, but for most purposes 80° C. is the best, as it fixes the dye without interfering to any great extent with the ease of digestion of the resulting congo red fibrin. The fibrin can then be placed in a piece of cloth and washed in running water by tying the cloth on the nozzle of a tap and letting the water run slowly. After washing, the excess of water is squeezed out and the fibrin kept in equal parts of glycerine and water. When the fibrin is to be kept for a long time, a little toluol (or other preservative) can be added to prevent the growth of moulds.

Specimens of fibrin prepared in this manner have been found not to deteriorate on keeping, and one sample was found after an interval of five months to be just as good as when freshly prepared.

1. *Zeit. f. physiol. Chem.*, Vol. IX, p. 577. 1885.