The Estimation of Tryptic Activity in Duodenal Contents

By A. H. GOWENLOCK

Department of Physiology, University of Manchester

(Received 16 April 1952)

A rapid reproducible method for the assay of tryptic activity in duodenal juice has been developed in connexion with investigations in this department on the hormonal stimulation of pancreatic function. Titrimetric methods (see, for example, Lagerlöf, 1942) for estimation of such juices were found to be difficult as some samples contain high concentrations of bile pigments which obscure the titration end point. This same difficulty has recently been commented on by Hartiala (1951). Colorimetric methods have also been elaborated in the past. Coloured proteins (e.g. dyed fibrin, azocasein) have been employed, the degree of proteolysis being assessed by determination of the non-precipitable coloured digestion products (see, for example, Smorodinzew & Adowa, 1924; Charney & Tomarelli, 1947). Another approach has been the colorimetric estimation of the substrate itself (White & Bowman, 1947) or alternatively of non-precipitable tyrosine (and tryptophan) both before and after enzyme action.

The present method follows the latter course and is based on Anson & Mirsky's (1933) estimation using denatured haemoglobin as substrate. This substrate has been replaced by a more readily available one, namely denatured human serum proteins, and is therefore analogous to Hunt's (1948) modification of Anson & Mirsky's method for the estimation of pepsin.

METHOD

Reagents. 0.35N-Trichloroacetic acid; 0.25N-NaOH, Folin and Ciocalteu's (1927) phenol reagent; buffer solution, pH 9.5, prepared from $\text{m-NH}_4\text{Cl}$ solution and NH_3 solution (sp.gr. 0.880); solutions of phenol in 0.001N-HCl (10, 20, 30, 40, 50, 60 and 70 mg./l.).

Substrate solution. Dehydrated human serum which was regarded as unfit for transfusion purposes has been used. The undenatured substrate solution is made up to contain 4 g. of dried serum in 100 ml. of 0.01N-NaOH solution and its pH is about 10. This solution, contained in a flask plugged with cotton wool, is immersed in a boiling-water bath for 12 min. After this time, the flask is transferred to a bath of running cold water to cool to room temperature and the resultant solution is filtered through cotton wool. To the filtrate is added 0.25 vol. of the $\text{NH}_4\text{Cl-NH}_4\text{OH}$ buffer solution. The pH of the substrate solution should be 9.5 and any necessary adjustment is made with the aid of the glass electrode.

Duodenal juice. This is obtained from human subjects by duodenal intubation. Contamination by gastric contents is avoided as far as possible by simultaneous gastric intubation and suction. The samples are immediately mixed with an equal volume of glycerol and stored at 2° (Lagerlöf, 1942). The technique has recently been outlined by Howat (1952). Activation by addition of enterokinase is not necessary, duodenal juice being fully activated (Agren and Christiansen 1933, quoted by Lagerlöf, 1942).

Estimation of tryptic activity

A series of pairs of test tubes is made up, two pairs being necessary for each assay which involves 'test' and 'control' determinations. One tube of each pair contains 5.0 ml. of substrate solution, the other contains 10.0 ml. of 0.35N-trichloroacetic acid solution. The tubes are placed in a water bath at 37° for 15 min. prior to the start of the determination. The duodenal juice-glycerol mixtures (approx. 2.5 ml.) are placed in the bath about 5 min. before the start of the determination. If a duodenal juice-glycerol mixture is suspected to have a very high activity, as after pancreozymin stimulation, it is diluted with an equal volume of 50% (v/v) glycerol.

At zero time, 1.0 ml. of the juice is added to the substrate tube of the first pair (the 'test' determination), followed 30 sec. later by the addition of 1.0 ml. of the same juice to the trichloroacetic tube of the second pair (the 'control' determination). The process is repeated at intervals of 30 sec. for the different samples of juice to be assayed. After each addition of juice the tubes are agitated to ensure adequate mixing.

Exactly 15 min. after the addition of a juice to a pair of tubes, any reaction is brought to a standstill by mixing the contents of the paired tubes by pouring back and forth a few times. This precipitates any residual protein and the bile pigment is adsorbed on to the precipitate. After allowing the tubes to remain at 37° for 4–5 min., the contents are filtered through a 12.5 cm. Whatman no. 1 filter paper. (Occasionally the filtrate from a control determination on a very concentrated active juice gives a slightly cloudy filtrate. This is cleared by filtration again through a Whatman no. 30 paper.) When no free liquid is apparent in the filter paper, the colourless clear filtrate is mixed to ensure homogeneity and 2.0 ml. are transferred to a 100 ml. conical flask containing 20.0 ml. of 0.25N-NaOH solution. The filtrate is run in down the side of the flask, mixed by a gentle swirling motion and immediately followed in the same way by 1.0 ml. of Folin and Ciocalteu's phenol reagent. In this way aerial oxidation of the phenolic substances is reduced to a minimum.

A blue colour develops rapidly, and 15–30 min. later its intensity is measured by means of a Hilger Spekker absorption spectrometer using an Ilford 607 filter and a 1 cm. absorption

cell. (Any colorimeter may be used provided it is standardized as described below. An EEL Photoelectric Colorimeter and Ilford 607 filter have proved satisfactory.)

The difference in colorimeter readings between the test and control determinations is a measure of the tryptic activity of the juice, and use is made of a standardization curve to convert this figure into units of tryptic activity.

Preparation of the standard curve. It was considered that human duodenal juice rather than commercial trypsin preparations should be used for the construction of this curve, as the method was to be used in conjunction with investigations on the effects of gastro-intestinal hormones on human pancreatic secretion. Furthermore, it was necessary to ensure that the method was reliable both for juices obtained from normal and pathological subjects and for

Definition of the unit of tryptic activity. As the estimation involves the colorimetric estimation of soluble phenolic substances, the unit of activity was defined by comparison of the chromogenic activity of the protein-free filtrate described above with that of a phenol solution of known strength.

The composite activity curve in Fig. 1 does not differ significantly from that obtained by using 2.0 ml. of solutions of phenol (concn. 10, 20, 30, 40, 50, 60 and 70 mg./l.) in place of 2.0 ml. of the protein-free filtrate in the method of estimation described above. These phenol solutions are made up in 0.001 N-HCl to reduce any aerial oxidation of the phenol. By this means therefore a standardization curve can be prepared for any colorimeter and a convenient unit of tryptic activity is available.

Fig. 1. The standard curve relating tryptic activity to the colour difference between test and control determinations as determined by the Hilger Spekker absorptiometer. ●, points used to construct the composite activity curve. ○, points obtained by using phenol solutions to standardize the colorimeter.

juices obtained after different methods of stimulation or under resting conditions. This has been investigated as follows:

An activity curve was prepared for a given juice by determining the colour differences between test and control for a juice diluted to 50, 40, 30, 20 and 10% (v/v) of its original concentration (50% = juice as stored with glycerol). Dilutions were carried out with 50% (v/v) aqueous glycerol. The colour differences were plotted as ordinates against the percentage concentrations of the juice as abscissae to give an activity curve for a single juice.

These activity curves were determined on juices obtained from six subjects. In each case the juices obtained under resting conditions and after secretin and pancreozymin stimulation were investigated. Three of the subjects were normal medical students (two female, one male). The fourth was a woman with a gastroenterostomy and suspected pancreatitis, the fifth, a man with suspected pancreatitis and the sixth, a man with a disease of the central nervous system and achlorhydria. The eighteen curves so obtained have been made to overlap by a suitable choice of units along the horizontal axis, to provide a composite activity curve (Fig. 1).

A duodenal juice is said to have x units/ml. of tryptic activity when, under the conditions of the above estimation, 1 ml. of the 50% (v/v) juice-glycerol mixture gives a protein-free filtrate with a chromogenic activity equal to that of a phenol solution of concentration x mg./l.

Development of the method

The method described above is based on a series of investigations outlined below.

Preparation of the substrate. It was necessary to find a suitable way of denaturing the serum proteins whilst keeping the protein concentration as high as possible and avoiding coagulation. Preliminary investigations were carried out with solutions of varying concentration, adjusted to various pH values by addition of Na_2CO_3 solution and heated to 100° for different periods of time. The results given in Table 1 show that a 4% serum protein solution of pH 10 heated at 100° for 10–15 min. is satisfactory. (Plasma proteins cannot be used as the fibrinogen coagulates under these conditions.) Later experiments demonstrated the need for a $\text{NH}_4\text{Cl-NH}_4\text{OH}$ buffer during the actual estimation. This cannot be added during the initial denaturation owing to the loss of NH_3 and consequent change of pH. It is

Table 1. *The effect of different methods of denaturation of serum protein solutions*

(- denotes no coagulation; + denotes coagulation.)

Protein concn. (% w/v)	...	3	4	4	4	4	5	5.6
pH		10	9	10	10	10	10	10
Time of heating at 100° (min.)		15	15	10	15	20	15	8
Result		-	+	-	-	+	+	+

therefore added after denaturation has occurred. Finally, it has been found most convenient to dissolve the dried serum protein in 0.01N-NaOH to give a 4% solution of suitable pH without any need for further pH adjustment. The period of heating has been standardized at 12 min.

Fig. 2. The rate of formation and stability of the blue colour.

Optimum pH for enzyme action. Preliminary tests suggested that this lay in the pH range 9-10. The decision as to the exact pH to be used was made by carrying out the estimation on substrates prepared by adding 1.0 ml. of $\text{NH}_4\text{Cl-NH}_4\text{OH}$ buffer of suitable pH to 4.0 ml. of the denatured protein solution. The pH values of the substrates were checked electrometrically and the results of a typical experiment are: pH 9.00 (0.360), pH 9.45 (0.395), pH 9.80 (0.358). The figures given in parentheses are the differences in absorptiometer reading between test and control determinations. A pH of 9.5 has therefore been adopted for the standard method outlined above.

Table 2. *Reproducibility of the substrate*

(The results are given as the difference in the EEL colorimeter readings between test and control. Substrates prepared from sera 2 hr. previously, except for D, prepared 22 hr. previously.)

Substrate	Results of triplicate estimations		
A	42.2	42.2	42.7
B	42.7	43.2	43.7
C	42.8	41.6	40.0
D	44.3	42.3	44.3

Stability of the pH during the reaction. Since the reaction is sensitive to changes in pH it was necessary to check that the pH was constant during the reaction time. This was done by electrometric determination using a glass electrode. Under the standard conditions of the estimation, the addition of a duodenal juice (35 units/ml.) and subsequent incubation

did not alter the pH by more than 0.02 unit. In the absence of the buffering agent the change may be as much as 0.50 of a pH unit. It has therefore been the practice to carry out the estimation in the presence of a buffer.

Influence of bile. The bile pigment does not interfere with the estimation. The greater part of the pigment is adsorbed on to the precipitated protein and does not appear in the protein-free filtrate. Traces of pigment in this filtrate are further diluted tenfold during the colour development and thus have little effect on the colorimeter reading.

Fig. 3. The relationship between the Lagerlöf and Gowenlock methods for the estimation of tryptic activity in twenty-nine duodenal juices.

The influence of bile on the enzyme activity was investigated by adding cat bile (1 vol.) to duodenal juice (4 vol.) relatively free from bile. Two samples of juice treated in this way showed activities of 11.6 and 27.4 units/ml. Control samples to which water had been added instead of bile had activities of 11.8 and 28.0 units/ml. It is concluded that bile is without serious effect on tryptic activity. Similar conclusions were reached by Lagerlöf (1942).

Colour development. The protein-free filtrates have been treated in the manner described by Hunt (1948) whose observations regarding procedure have been confirmed. The rate of formation and stability of the blue colour are shown in Fig. 2.

Reproducibility of the substrate. This has been investigated by carrying out, in triplicate, estimations of the activity of a duodenal juice using four different substrate preparations. The results given in Table 2 show that there are only minor variations in the results obtained with the different substrates. Analysis shows that the variation between substrates is not significant ($P > 0.05$).

Accuracy of the estimation. From the experiment indicated in the last section, the twelve estimations on the given duodenal juice gave a result of 42.7 ± 0.4 (s.e.m.), expressing the result in terms of the colorimeter reading. This is accurate enough for the purposes for which the method was designed.

Correlation with Lagerlöf's method. The results of the estimations of tryptic activity of twenty-nine different duodenal juices by the present method and by that of Lagerlöf (1942) are shown in Fig. 3. The correlation coefficient for the two sets of results is 0.82, a figure which statistically is highly significant ($P < 0.001$). Fig. 3 enables the two sets of units to be interconverted; one Lagerlöf unit is equal to 70.5 units as defined in this paper.

RESULTS

The concentration of enzyme in juices obtained under different conditions is very variable. This is in part due to the variation in the output of juice per minute. The relationship between enzyme concentration, minute output and hormonal stimulation is to be reported in detail elsewhere, but the following are some typical values.

In seventeen determinations carried out on resting juice the range of activity was 1–75 units/ml., but twelve of these results lay in the range 8–38

units/ml. In the case of juice obtained after secretin administration the range for nineteen estimations was 19–93 units/ml. and fourteen of these lay in the range 25–75 units/ml. Juice obtained after pancreozymin stimulation showed a range of 18–90 units/ml. for sixteen juices, but eleven of the results lay in the range 50–80 units/ml.

SUMMARY

1. A method is described for the assay of tryptic activity in duodenal juices using a denatured preparation of dried human serum as the substrate and measuring the phenolic substances liberated by trypsin.

2. A unit of tryptic activity is defined, and a method for the calibration of any colorimeter is suggested.

3. The experimental basis of the method is presented and the overall accuracy is assessed.

4. The results from this method have been correlated with those from Lagerlöf's (1942) method.

5. The range of activity of varying types of juice is discussed briefly.

I wish to thank Prof. A. A. Harper and Dr H. T. Howat for their interest and for allowing me to use the duodenal juices obtained in their experiments. I am indebted to Dr S. Oleesky for the estimations by the Lagerlöf method and to Dr F. Stratton for the dried serum used in this work.

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The Action of Mould Enzymes on Sucrose

By F. J. BEALING AND J. S. D. BACON

Department of Biochemistry, The University, Sheffield 10

(Received 30 April 1952)

The hydrolysis of sucrose by mould invertase preparations has been considered to result from α -glucosidase (glucosaccharase) activity (Kuhn, 1923), on the basis of observations that the reaction rate is decreased by the presence of free α -glucose, presumed to act as a competitive inhibitor. Modification of this view became necessary when mould extracts were found to hydrolyse part of the ' γ '-

methyl fructoside mixture (Schlubach & Rauchalles, 1925; Purves & Hudson, 1934), and, in the absence of melibiase activity, to attack raffinose (Rohdewald, reported by Kuhn & Münch, 1927; Hestrin, 1940). These results indicated the presence of a β -fructosidase similar to that found in yeast invertase preparations; accordingly, it has been suggested that sucrose breakdown by moulds may