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A Method for Estimating Peptic Activity in Gastric Contents

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An accurate simple method of estimating the activity of proteolytic enzymes is clearly desirable. To achieve this end, not only must a precise method of measuring proteolysis be devised, but a reproducible substrate must also be found. These two requirements were first met in the method of estimating pepsin described by Anson & Mirsky (1932) and Anson (1938). Their method, in which the substrate is carboxyhaemoglobin, has been widely used for estimating pure enzymes on account of its accuracy. In modified forms it has proved valuable in the determination of the activity of digestive juices (Beazell, Schmidt, Ivy & Monaghan, 1938; Bucher, Grossman & Ivy, 1945). However, without large scale apparatus, the preparation of sufficient quantities of the substrate of carboxyhaemoglobin is time-consuming and the method is correspondingly restricted in its applicability.

This paper describes the use of another reproducible but more readily available substrate. The modified reagents and procedure are given in full. A method of standardization is described and the adoption of a new pepsin unit is suggested.

METHOD

The substrate

General remarks. Dehydrated human plasma and serum, rejected as unfit for transfusion purposes, have been used exclusively for this work. The dried plasma or serum is of variable colour and is freely soluble in distilled water. Two samples have been encountered which immediately formed a gel on addition to water. These were discarded.

The substrate solution. Substrate solution is made up to contain 5.6 g. dried citrated plasma or dried serum in 100 ml. of distilled water with sufficient HCl to give an acidity of pH 2.1 as determined with a glass electrode at 18–22°. The substrate solution is stirred and then filtered through a Green's 309½ Agar filter paper or cotton wool. Considerable variations in the rate of filtration have been encountered but this had no apparent significance in the method.

Other reagents are: 0.350 N-trichloroacetic acid (approximately 6 g./100 ml.), 0.250 N-NaOH, HCl solution, pH 2.1, Folin & Ciocalteu's (1927) phenol reagent, phenol solution (5.0 mg./100 ml.).

Estimation of proteolytic activity

The sample of gastric juice under examination is mixed with an equal volume of dilute HCl of pH 2.1. A series of pairs of test tubes, one tube containing 5.0 ml. of substrate and the other 10.0 ml. trichloroacetic acid, are placed in a water bath at 35°. Stock tubes of gastric juice mixtures are also warmed in the water bath. After 20 min. 1.0 ml. of gastric juice mixture is added to the substrate tubes of pairs 1, 3, 5 *et seq.* and to the trichloroacetic acid tubes of pairs 2, 4, 6 *et seq.* Each addition is made at regular intervals of 30 sec. so that a series of up to 15 estimations can be handled in 14 min. Mixing is ensured by agitation of the tubes outside the water bath. Thus peptic digestion proceeds in the substrate tubes of the odd-numbered pairs whilst the even pairs provide controls.

Fig. 1. The relationship between the difference in colour between control and test and the peptic activity of the gastric juice.

Exactly 15 min. after the addition of gastric juice to any pair of tubes, their contents are mixed by pouring back and forth. This stops any digestion in the odd-numbered pairs. After this mixing the tubes are allowed to stand in the water bath for approximately 4 min. The mixture is filtered through a 15 cm. paper (Whatman no. 1). 15–30 min. after the beginning of filtration 2.0 ml. of the filtrate are pipetted into a 50 ml. flask containing 20 ml. 0.25 N-NaOH and thorough mixing is ensured by swirling (vigorous shaking must be avoided as it tends to reduce the development of colour). Forthwith 1.0 ml. of Folin & Ciocalteu's phenol reagent is run down the side of the flask and mixed by immediate swirling.

A blue colour gradually develops and 15–40 min. later it is estimated in a photoelectric colorimeter with a suitable filter. A single cell colorimeter (King, 1946) with an Ilford 204 filter has been found satisfactory.

The difference in colour between the test and the control is used to evaluate the peptic activity from a curve which relates activity to colour difference (Fig. 1). If a more accurate evaluation is required

Fig. 2. The relationship between the difference in colour between control and test and the peptic activity of the gastric juice, plotted on a logarithmic scale.

Table 1. Relation of peptic activity of gastric juice to difference in colour between control and test

Pepsin units	Optical density ($E \times 100$) (Difference between control and test)
10	15.0
15	21.0
20	27.0
25	32.0
30	36.8
35	41.0
40	44.5
45	48.0
50	51.0
60	56.0
70	60.5
80	64.5
90	68.0
100	71.4
110	75.0
120	78.0
130	81.0
140	83.5
150	86.2
160	89.0
180	94.0
200	98.7
220	103.0
240	107.2
260	111.5
280	115.0
300	118.0

and the peptic activity falls between 25 and 60 pepsin units/ml. (see below), the straight-line relationship, in which the peptic activity is plotted on a logarithmic scale can be used (Fig. 2). These curves can be plotted from the values shown in Table 1.

A gastric juice is said to have 50 units of peptic activity/ml. when under the standard conditions of estimation (viz. after dilution with an equal part of HCl solution of pH 2.1) it gives a colour equivalent to that produced by 2.0 ml. of a standard phenol solution (5.0 mg./100 ml.).

The standard colour is developed by mixing 2.0 ml. of freshly prepared phenol solution (5.0 mg./100 ml.) with 20.0 ml. 0.25N-NaOH and promptly adding 1.0 ml. Folin & Ciocalteu's phenol reagent. Between 5–10 min. later the colour is read in the colorimeter. A blank estimation is made omitting the phenol; in this way ten consecutive estimations gave values, after subtracting the blanks, of 0.51 with no measurable deviations.

The relation of peptic activity to the colour produced

Since the method was devised to determine the proteolytic activity of gastric juice, this was used in preference to a solution of pure pepsin in establishing the relationship between proteolytic activity and colour differences between control and test. A series of known dilutions of a gastric juice were made. After 15 min. digestion, the value of the difference in colour between control and test for each of these dilutions was recorded. With these values as ordinates and percentage concentration of gastric juice as abscissae, a curve was plotted. In this way twelve curves relating peptic activity to the colour produced were made, two for juice obtained after the injection of histamine in known hypersecretory subjects and the remainder for fresh resting and post-prandial juice. The percentage concentration of gastric juice, corresponding to an ordinate value of 0.51 optical density, was then found from each curve and this percentage concentration was taken as equivalent to 50 pepsin units. The figures for percentage concentration of gastric juice were then converted into values in pepsin units/ml. for each curve. Thus if a concentration of 32% contained 50 pepsin units/ml. all the abscissal percentage concentrations for that particular curve were multiplied by $\frac{50}{32}$ to give values in pepsin units/ml. From the 12 recalculated curves a composite curve, based on 138 determinations, was plotted. The values of Table 1 are taken from the curve so produced.

The experimental points shown in Fig. 1 have been selected to include those furthest from the

composite mean curve whilst all the determinations made over the range 25–60 pepsin units are shown in Fig. 2.

The method may be used in any laboratory by determining the colour given by the standard reagents with 2 ml. phenol solution (5.0 g./100 ml.). If this optical density be C , then before reading the peptic activity from the curve, all colorimeter readings must be corrected by a factor $C/0.51$, since the graph is plotted for our equivalent of the standard, viz. 0.51 optical density.

One of the disadvantages of using gastric juice as a source of proteolytic enzyme, for establishing the relationship between colour produced and peptic activity, is that gastric juice of very high activity is difficult to obtain. With a solution of pepsin, concentrations greater than those found in gastric juice can be used to produce a curve whose range embraces all peptic activities likely to be encountered in gastric juices. In determining the relationship shown in Table 1, the experiments for values of above 150 pepsin units/ml. was made by increasing the digestion time. The validity of this step is supported by the fact that, provided the product of digestion time and dilution is constant, the colour produced is constant. No samples of gastric juice have been encountered with an activity falling outside the range of Table 1.

EXPERIMENTAL BASIS OF METHOD

The details of the preceding method are based upon a series of experiments and the following is a summary of their more important results. The gastric juice in the experiments described was obtained from pooled filtered fractional-test-meal samples adjusted to pH 2.1.

Concentration of substrate. Table 2 shows the effect of variation of concentration of the substrate on the values obtained for peptic activity for a single specimen of gastric juice. A concentration of 5.6 g./100 ml. has been chosen as being the highest concentration conveniently prepared from fresh citrated plasma which can be used in place of the dried product. If untreated plasma is used a different curve relating activity to colour is needed. A high concentration of plasma is desirable to ensure maximal buffering and to increase the range of peptic activity over which the method can be employed (see Beazell *et al.* 1938). If it is desired to husband the substrate, the method has been found to be quite satisfactory for juices of activity

Table 2. *Effect of variations of substrate concentration*

(Optical density ($E \times 100$) corresponding to peptic activity at different concentrations of substrate.)

		Conc. of substrate (g./100 ml.)				
		4	5	5.6	6	7
Activity of dilutions of a gastric juice in pepsin units	9.0	13.4	13.6	13.3	13.0	8.9
	31.5	33.0	35.5	37.8	35.3	35.5
	45.0	44.0	48.0	48.0	48.0	44.3
	63.0	52.0	59.5	57.5	59.5	55.9

Table 3. *Relation between the pH of substrate and enzyme activity*

pH	1.4	1.6	1.8	1.9	2.0	2.1	2.2	2.3	2.6	3.5
Optical density ($E \times 100$) corresponding to peptic activity with 3% substrate. Gastric juice 'P'	22.0	26.0	31.0	34.5	36.0	37.0	37.0	34.0	31.0	18.5
Optical density ($E \times 100$) corresponding to peptic activity with 5.6% substrate. Gastric juice 'Q'	—	—	—	48.5	51.0	51.2	51.0	48.8	—	—

up to 100 units/ml., with concentrations of 2.4 g. substrate/100 ml. and 4 g./100 ml. trichloroacetic acid. A new curve relating peptic activity to the colour produced is required for each such variation in substrate.

pH of substrate. Table 3 shows the relation between the pH of the substrate and rate of digestion for two specimens of gastric juice. The optimum pH lies between 2.0 and 2.2 and it can also be seen that this does not change with the concentration of the substrate.

Influence of variations of pH of gastric juice. Table 4 shows the effect of variations of the pH of a single sample of the gastric juice upon the results for the estimation of its peptic activity. From a 'pooled' gastric juice of pH 4.1 three dilutions were made to contain equal concentrations of the original juice at pH of 1.6, 2.1 and 4.1. These diluted

Table 4. *Effect of variations of pH of gastric juice on the estimation of its activity*

pH of gastric juice	1.6	2.1	4.1
Optical density ($E \times 100$) corresponding to peptic activity	8.7	8.6	8.8
pH of substrate (3 g./100 ml.) after adding diluted gastric juice	2.05	2.10	2.12

samples were then estimated by the routine method, with a substrate containing 3 g. plasma proteins/100 ml. A similar result was obtained with all these specimens. Further the pH of the substrate after the addition of the gastric juices was determined. It may be seen that this pH was not shifted outside the limits for the maximal rate of digestion. These experiments are of great practical importance because they show that it is not necessary to adjust the pH of the gastric juice to that of the substrate even when the latter is used in a dilute solution.

Physiological variables. It has been found that additions of chloride, bile or mucus do not influence the estimation of the activity of gastric juice.

Reproducibility of substrate. To test the reproducibility of the substrate, samples of protein were obtained from various sources (e.g. America and Britain) and used in the estimation of the activity of three samples of gastric juice. Table 5 shows that, despite the difference of origin of the materials employed, the results obtained were surprisingly consistent. Sample CP199/330, which gave a high result, had been shipped to Italy and back where conditions of storage may not have been favourable.

Stability of the substrate solution. The results of experiments made to test the stability of the substrate solution are shown in Table 6. Substrate solutions were made on the

Table 5. *The estimation of the activity of samples of gastric juice with proteins from various sources*

Protein substrate	Gastric juice	Optical density ($E \times 100$) corresponding to peptic activity	
		Individual values	Means
2 May			
Slough serum protein ₁	A	31.5 31.0	31.3
Slough serum protein ₂	A	28.4 29.4	28.9
British plasma protein	A	30.0 29.4	29.7
American plasma protein	A	30.2 29.4	29.8
11 July			
Plasma proteins LP119/6	B	39.2 39.2 39.8 39.8	39.5
Plasma proteins LP119/122	B	41.2 40.0 40.0 38.5	39.9
Plasma proteins LP119/4	B	39.0 40.0 39.0 40.0	39.5
Plasma proteins LP119/228	B	39.0 37.7 40.0 41.0	39.4
Plasma proteins CP199/330	B	41.8 41.0 42.0 41.5	41.6
Plasma proteins LP119/50	B	38.5 39.5 40.1 39.3	39.4
26 Sept.			
Plasma LP119/50	C	69.5 70.0 69.0 70.0	69.6
Serum 1050-46	C	69.0 69.0 70.0 69.0	69.3

dates shown and kept under the conditions shown. They were later concurrently used to estimate the peptic activity of a gastric juice. It is apparent that the substrate is stable for at least 24 hr. at 20° and for 48 hr. at 6°. The gastric juice has been found to be stable for approximately 6 hr. at 20° after dilution.

Table 6. *Tests of the stability of substrate*

Substrate solution	Date of test	Optical density ($E \times 100$) corresponding to peptic activity	
		Individual values	Mean
Made up immediately before test. 23 Mar.	23 Mar.	58.9 59.5	59.2
Made up 22 Mar. Kept at 20° for 24 hr.	23 Mar.	59.9 60.3	60.1
Made up 21 Mar. Kept 4 hr. at 20° followed by 48 hr. at 6° in refrigerator	23 Mar.	60.5 60.3	60.4

Trichloroacetic acid. The strength of the trichloroacetic acid has been chosen by trial and error to give a clear filtrate. It should be standardized by titration to 0.350 N.

The filtration. A very small amount of the substance producing blue colour is retained by the filter paper, but this is of little consequence as the error is minimized by the use of the curve relating activity to colour. The filtrate is not uniform, and therefore filtration should be allowed to proceed almost to completion as it does in the 15 min. allotted, and the filtrate must be mixed before the 2.0 ml. are pipetted out for analysis. The colour-producing property of the filtrate is only constant up to 30 min. after the beginning of filtration.

The colour-developing reaction. This is influenced by hydrogen ion concentration and by the amount of Folin & Ciocalteu's reagent used. If less alkali than the standard 20.0 ml. 0.25 N-NaOH is used the colour develops more slowly to a higher intensity, while with more than 20 ml. the colour develops more quickly but begins to fade sooner. Thus 20 ml. ensures a useful balance between the rate of development and the fading of the colour, which is maintained at a constant intensity for 25 min. under the standard conditions at pH 12.5. The rate of development and persistence of the colour is not influenced by the concentration of the filtrate.

If the colour produced is too dark to be read in the colorimeter, it can be diluted with a convenient volume of water before reading. The true colour can then be calculated.

1.0 ml. of undiluted Folin & Ciocalteu's reagent has been found to be convenient and, further, it appears that when this amount is used errors in measuring produce minimal changes in the final colour developed. The reagent should be admitted uniformly for all tests.

To test the reproducibility of the phenol reagent four samples were collected from various laboratories, where they were kept in refrigerators, and tested against the phenol standard and the Folin & Ciocalteu reagent in use for this work. In spite of the different degrees of yellowness of the samples they gave consistent results with the phenol standard (Table 7).

Table 7. *Test of the reproducibility of Folin & Ciocalteu's phenol reagent from different sources*

(2.0 ml. standard phenol (5.0 ml./100 ml.) were used in each test.)

Source of sample	Optical density ($E \times 100$)		
	Phenol reagent and NaOH	Phenol reagent, NaOH and standard phenol	Difference corresponding to phenol standard
British Drug Houses Ltd.	2.0	52.5	50.5
646256/460909	2.0	52.5	50.5
Unknown	4.5 4.6	55.0 55.0	50.5 50.4
Hopkins and Williams Ltd.	2.0	53.0	51.0
1 Nov. 1946	2.0	53.0	51.0
British Drug Houses Ltd.	2.0	53.0	51.0
650319/470219	2.0	53.0	51.0
British Drug Houses Ltd.	2.0	53.0	51.0
643791/460424	2.0	53.0	51.0

Experiments have shown that it is essential to add the phenol reagent with the minimum of delay to obtain consistent results. The consistency obtainable by these procedures was tested by estimating ten samples from a pooled filtrate. The readings of these ten estimations fell between 22.8 and 23.0. It is particularly important that the 50 ml. flask used should be free from contamination with any colour-producing agent. As commercial ethanol contains such substances, it is advised that the final washing of these flasks should be with water.

Differences between duplicates may be due to such contamination, and this can be checked by analysis of another sample of the same filtrate. Substances which reduce the amount of colour produced have not been detected, therefore, if the duplicates differ, the lower reading is probably better.

In the original method of Folin & Ciocalteu (1927), Na_2CO_3 was used as the alkali in the final system. Anson & Mirsky (1932) used NaOH in place of Na_2CO_3 and this has been found the more reliable agent.

The accuracy of the method. Fourteen measurements of the activity of a single sample of gastric juice with one substrate gave a mean reading of 44.0 pepsin units, with a coefficient of variation for individual readings of $\pm 2.64\%$. The coefficient of variation of an individual reading in pepsin units for twenty-eight determinations with seven samples of substrate shown in Table 5 for the experiments of 11 July and 26 September is $\pm 2.5\%$. Results for sample CP199/330 have been omitted (cf. p. 107).

DISCUSSION

The main principles of the method of Anson & Mirsky (1932) have been followed, but several significant details have been altered. They used tyrosine as a standard, but the appropriateness of this substance is perhaps doubtful since the digestive products giving a blue colour are probably a mixture of tyrosine and tryptophan. What is needed is a stable substance which can be readily obtained in

a pure state, and which will produce a consistent colour with the Folin & Ciocalteu reagent. Phenol is such a substance and it has been used to provide the colour standard in this work. Thus the preparation of a pure sample of tyrosine is avoided.

Since the relationship between concentration of phenol and the colour produced in the reaction with the phenol reagent is not linear over a wide range, peptic activity has been related to a standard phenol at one point only. A small divergence from true proportionality is also found for the mixture of tyrosine and tryptophan estimated in the digestion products. Herbert (1946) has confirmed this point for the estimation of phenol with Folin & Ciocalteu's phenol reagent when NaCO_3 is used as the alkaline medium for colour development.

The method has proved quite practicable with ordinary citrated plasma so that, in the absence of a supply of dried plasma or serum, it could be used with citrated or whipped ox blood. However, it is unlikely that the supply of plasma or serum will fail, since occasional batches will always be rejected as unfit for transfusion because they are icterogenic. A new relationship between peptic activity and colour would, however, have to be established for fresh plasma. Any alteration of the concentration

of the substrate beyond the range 5–6 g./100 ml. would also require a simultaneous modification of the strength of the trichloroacetic acid to ensure that the optimal pH was reached in the colour development reaction.

The method described estimates the total proteolytic activity of gastric juice at pH 2.1 with one special substrate. The practice is to attribute all this activity to pepsin, and the method has therefore been described as a method for measuring pepsin. However, the work of Northrop (1939) suggests that it may later prove necessary to apportion the activity of gastric juice to pepsin, gelatinase and possibly other enzymes. The uncertainty surrounding the source of the activity of the gastric juice seemed in part to justify the completely arbitrary measure of proteolytic activity used in this work.

SUMMARY

1. A method is described for measuring the proteolytic activity of gastric juice with a substrate of dried plasma or serum protein which has been found to be reproducible and convenient.

2. The method allows of the comparison of peptic activity in different laboratories without reference to a standard pepsin.

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The Persistence in the Blood Stream of some Compounds Related to Suramin

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Suramin (Antrypol, Germanin, Bayer 205, Moranyl, F. 309, Belganyl, Naganol, see formula I) displays an extraordinarily marked persistence in the blood stream of man and experimental animals. This property was recognized soon after suramin was first discovered, first, from the long duration of its prophylactic action (Mayer & Zeiss, 1920; Levaditi & Klarenbeek, 1927); secondly, from measurements of the trypanocidal activity of serum and urine of treated animals (Mayer, 1922; Mayer & Menk, 1922).

The development of adequate chemical methods of determining it (Steppuhn & Utkin-Ljubovzov, 1924; Lang, 1931; Dangerfield, Gaunt & Wormall, 1938*a*; Bournsnell, Dangerfield & Wormall, 1939; Vierthaler & Boselli, 1939), and their use in studying the persistence quantitatively (Lang, 1931; Dangerfield, Gaunt & Wormall, 1938*b*; Bournsnell, *et al.* 1939; Vierthaler & Boselli, 1939; Hawking, 1940*a, b*) showed that in man, dogs and rabbits, it may persist in the blood stream for as long as 3 months. The