Estimation of serum pepsinogen

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EDITORIAL SYNOPSIS A modified method for estimating serum pepsinogen using haemoglobin as substrate offers some advantages over those previously published.

Measurement of serum pepsinogen, the proteolytic activity of serum at about pH 2.0, has been used as one of a number of indirect tests of gastric function. Patients with pernicious anaemia and related conditions show lower levels of serum pepsinogen than normal subjects (Chinn, 1953; Hoar and Browning, 1956; Mirsky, Futterman, and Kaplan, 1952; Nolan, 1958; Singh and Shinton, 1965). Bock, Arapakis, Witts, and Richards (1963), using a modification of the method of Edwards, Jepson, and Wood (1960) with freeze-dried human plasma as the substrate, found a correlation between the serum pepsinogen level and the histological appearance of gastric biopsy specimens.

As part of an investigation being made in this Department of the incidence of 'latent pernicious anaemia' (Callender and Spray, 1962) among the relatives of patients with pernicious anaemia, we wished to measure pepsinogen in the serum of a large number of subjects. A study of the method used by Bock *et al.* (1963) showed it to be both too laborious and unsuitable in other respects for use in a survey requiring several hundred estimations. A modified method has therefore been developed, using haemoglobin as substrate, and incorporating a buffer system to control the pH of the mixtures of substrate and serum. Both substrate and serum are treated with charcoal to reduce the blank readings, and the use of the Folin-Ciocalteu reagent to measure tyrosine has been eliminated. This method appears to offer some advantages over those previously published, and it is described in detail in this paper, together with the results obtained in normal subjects and patients with pernicious anaemia.

METHODS

SUBSTRATE Haemoglobin type II powder (Sigma London Chemical Co., Ltd.) was used throughout as the substrate. Haemoglobin (2 g.) was dissolved in 25 ml. water containing 3-4 drops of 0.5 N NaOH solution, using a magnetic stirrer. The volume was made up to 100 ml. with sodium acetate-HCl buffer (16.4 g. anhydrous

sodium acetate + 260 ml. N HCl per litre). The solution was treated with 1 g. charcoal (Norit NK, Hopkin and Williams Ltd.) for 15 minutes, and the charcoal was removed by centrifuging for 20 minutes at 2,400 r.p.m.

SERUM Blood was obtained by venepuncture and was allowed to clot in glass vessels. The clots were broken up with glass rods, the samples were centrifuged, and the serum was stored at -15°C until required for estimation. After thawing at room temperature and mixing, serum (3 ml.) was treated with about 5 mg. charcoal for 15 minutes and the charcoal was removed by centrifugation.

ESTIMATION OF PROTEOLYTIC ACTIVITY Serum (0.5 ml.), after treatment with charcoal, was mixed with 2.5 ml. substrate solution in a 6 in. $\times$ $\frac{3}{4}$ in. Pyrex test tube. Four identical tubes were prepared for each sample of serum. Immediately after mixing, 5 ml. of 5% (w/v) trichloroacetic acid was added to two tubes to precipitate the proteins, which were removed by filtering through Whatman no. 41 paper. The other two tubes were covered with parafilm and incubated for 24 hours at 37°C ., after which they were treated in the same way as the unincubated tubes.

The optical densities of the clear filtrates were measured in a Hilger Uvispek spectrophotometer at $280\text{ m}\mu$ (Northrop, Kunitz, and Herriott, 1948). The difference between the mean density for the two incubated tubes and that for the two unincubated tubes was used to calculate the result, which was expressed as micrograms tyrosine-like substances produced per millilitre serum in 24 hours by comparison with the absorption of pure tyrosine at $280\text{ m}\mu$. No allowance was made for the effect on the substrate of incubation, which in nine separate determinations was shown to produce a fairly constant increase in optical density equivalent to about $90\text{ }\mu\text{g.}$ tyrosine per ml. serum in 24 hours. All the results are therefore too high by this amount. In 12 tests in which samples of serum were diluted with buffer in place of substrate and incubated for 24 hours, there was no consistent increase in the optical density of the filtrates after incubation. Incubation produced a decrease in optical density with four samples, no change in two, and a small increase in the other six. On average there was an increase of density due to incubation corresponding to only $13\text{ }\mu\text{g.}$ tyrosine per ml. serum in 24 hours.

RESULTS

EFFECT OF CHARCOAL Early in this study it was found that the filtrates from unincubated mixtures of untreated serum and untreated substrate had optical densities at 280 $m\mu$ which were between 70% and 84% of those of the corresponding incubated mixtures. Such high blank values would cause errors in the results. Treatment of the substrate with charcoal produced a tenfold reduction in the optical density of the filtrate. Treatment of 10 samples of serum with charcoal reduced the contribution of the serum to the blank by between two and six times without affecting the proteolytic activity (Table I). Larger quantities of charcoal (20-30 mg. per ml. serum) reduced the proteolytic activity by up to 25%.

TABLE I

EFFECT OF TREATMENT WITH CHARCOAL (5 mg./ml. serum) ON THE PROTEOLYTIC ACTIVITY OF SERUM

Experiment No.	Sample No.	Tyrosine (μ g.) Produced per Millilitre Serum in 24 Hours	
		Untreated Serum	Serum Treated with Charcoal
1	1	500	520
	2	370	350
2	3	1,490	1,580
	4	1,070	1,170
3	5	470	390
4	6	530	520
5	7	570	610
6	8	710	700
	9	720	740
	10	1,090	1,110

pH OF THE MIXTURES OF SERUM AND SUBSTRATE The pH of mixtures of the substrate with 38 different samples of serum was measured with a glass electrode. With one exception (pH 2.4) all the values were between 1.8 and 2.2. To see whether this variation was likely to affect the comparison of results which might be obtained at any pH value within this range, the pH of mixtures using 10 different samples of serum was adjusted to between 1.7 and 1.8 and to between 2.2 and 2.3 by adding HCl or NaOH. With three samples, mixtures at pH 1.9 were also included. The results (Table II) show that over this range the pH had no consistent effect on the result.

TABLE II

EFFECT OF CHANGE IN pH ON THE PROTEOLYTIC ACTIVITY OF SERUM

Experiment No.	Sample No.	Tyrosine (μ g.) Produced per Millilitre Serum in 24 Hours at		
		pH 1.7-1.8	pH 1.9	pH 2.2-2.3
1	1	440	—	470
	2	1,110	—	1,170
	3	690	—	730
2	4	1,060	—	1,090
	5	400	—	380
	6	860	—	860
	7	780	—	820
3	8	480	480	470
	9	560	520	570
	10	550	560	530

RESULTS FROM DIFFERENT QUANTITIES OF SERUM As one check on the validity of the method, the figures obtained by the standard technique were compared with those using 0.5 ml. of a 1:2 dilution of serum in water. The results (Table III), which in this instance do not include the small amount of tyrosine due to the effect of incubation on the substrate, show that although on average the values using 0.25 ml. of serum were slightly higher than those using 0.5 ml. serum, the difference was not significant ($t = 0.394$, $P > 0.1$).

TABLE III

SERUM PEPSINOGEN VALUES OBTAINED WITH DIFFERENT QUANTITIES OF SERUM

Experiment No.	Sample No.	Quantity of Serum Used	
		0.25 ml.	0.5 ml.
		Tyrosine (μ g.) Produced per Millilitre Serum in 24 Hours ¹	
1	1	610	600
	2	910	850
2	3	720	670
	4	1,220	1,030
3	5	790	650
4	6	250	290
	7	720	730
	8	510	480
	9	460	420
	10	420	450
	Means	661	617

¹ These results do not include the tyrosine produced during incubation of the substrate alone.

EFFECT OF DIFFERENT INCUBATION TIMES The results obtained by the standard technique from 10 samples of serum were compared with those from the same samples after six and 12 hours' incubation. The results (Table IV), which have again been corrected for the effect of incubation on the substrate, show that there is an approximately linear relationship between the amount of tyrosine produced and the time of incubation.

TABLE IV

YIELD OF TYROSINE FROM THE SAME QUANTITY OF SERUM AFTER DIFFERENT INCUBATION TIMES

Experiment No.	Serum No.	Tyrosine (μ g.) Produced per Millilitre Serum after		
		6 Hours ¹	12 Hours ¹	24 Hours ¹
1	1	340	620	1,180
	2	370	730	1,300
	3	230	520	990
	4	61	150	320
	5	250	540	1,040
2	6	370	690	1,200
	7	190	410	820
	8	240	480	900
	9	280	590	1,100
	10	280	540	1,050
	Means	261	527	990

¹ These results do not include the tyrosine produced during incubation of the substrate alone.

REPRODUCIBILITY OF RESULTS AND THE EFFECT OF STORAGE The proteolytic activity of a pooled sample of serum was measured 35 times at intervals during 10 months. No loss of activity was detected during this period, and the results varied from 650 to 920 μ g. tyrosine/ml. serum in 24 hr. (mean 789, S.D. 58). The activity of 19 samples of serum was measured twice on different days. The differences between the pairs of results, expressed as percentages of the means, varied from 0.0 to 33% (mean 9.2%).

RESULTS FROM CONTROL SUBJECTS AND PATIENTS WITH PERNICIOUS ANAEMIA The results are shown

in Figure 1. Sera were studied from 135 healthy control subjects, 45 men and 90 women aged between 17 and 85, who were either members of the hospital staff or visitors to patients on medical wards. Of the total, 106 were aged 40 or over. The sex distribution of the group was designed roughly to match that of the patients with pernicious anaemia. The results ranged from 310 to 2140 μ g. tyrosine/ml. serum in 24 hr. (mean 833, S.D. 290). In 45 men and 92 women with pernicious anaemia the values were between 130 and 910 μ g. tyrosine/ml. serum in 24 hr. (mean 373, S.D. 139). Only nine (6.6%) of the values in this group were above 600 μ g.

FIG. 1. The serum pepsinogen levels of 135 control subjects compared with those from 137 patients with pernicious anaemia.

FIG. 2. The serum pepsinogen levels of 42 patients classified according to the findings on gastric biopsy, which are shown as follows; 1 patients with normal gastric mucosa; 2 patients with superficial gastritis; 3 patients with atrophic gastritis; 4 patients with gastric atrophy. The broken lines indicate the mean values for each group of patients.

tyrosine/ml. serum in 24 hr. There is a highly significant difference between the mean value for the control subjects and that for the patients with pernicious anaemia ($d = 16.7$, $P < 0.001$).

RELATIONSHIP OF SERUM PEPSINOGEN TO GASTRIC HISTOLOGY Results were obtained from 42 subjects on whom gastric biopsies were performed. The histological appearance of the specimens was kindly examined by Dr. W. C. D. Richards. The values are classified according to the histological reports in Figure 2. The mean value for subjects with normal gastric mucosa was 710 μg . tyrosine/ml. serum in 24 hr., 786 for those with superficial gastritis, 464 for those with atrophic gastritis, and 363 for those with gastric atrophy.

DISCUSSION

Our results show that under the conditions described serum pepsinogen can be estimated with reasonable accuracy. The degree of digestion of the substrate is directly related to the amount of serum used and to the time of incubation. The buffer solution gives satisfactory control of the pH of the mixtures of substrate and serum. Preliminary treatment of substrate and serum with charcoal effects large reductions in the blank values, which otherwise would be so high as to cause errors in the results. Estimation of tyrosine by measuring the absorption of the filtrates at 280 $m\mu$ eliminates the tedious use of the Folin-Ciocalteu reagent, and satisfactory results are obtained with smaller quantities of serum than are required for most other methods. Serum can apparently be stored frozen for up to 10 months without any loss of activity.

The highly significant reduction in the values from patients with pernicious anaemia compared with those from control subjects confirms previous findings. Nevertheless there is a considerable overlap between the results in these two groups of subjects, in agreement with the results of Chinn (1953), Hoar and Browning (1956), and Mirsky *et al.* (1952). Smaller degrees of overlap were found by Bock *et al.* (1963), Nolan (1958), and Singh and Shinton (1965). We are not aware of any reports of clear separation between the results from these two groups of subjects. This overlap limits the diagnostic usefulness of the test in individual patients. A result could not be regarded as abnormal unless it were below about 300 μg . tyrosine/ml. serum in 24 hours, and such low values were found in only 46 (34%) of the patients with pernicious anaemia. However, the estimation has a place with other screening tests in studies of large numbers of subjects in whom simple indirect assessments of gastric

function are required. Thus any subject in whom the serum pepsinogen value was less than 600 would be worthy of further study for evidence of gastric dysfunction. This is supported by the progressive lowering of the mean serum pepsinogen with increasing degrees of atrophy of the stomach (Fig. 2).

The number of observations on the relationship between serum pepsinogen and the histology of the stomach is too small for definite conclusions to be drawn, but as far as they go the results support the conclusions of Bock *et al.* (1963), except that in the present series the mean value for the patients with superficial gastritis was slightly higher than that for those whose gastric biopsies were normal. However, the difference is not significant ($t = 0.47$, $P > 0.10$). One patient whose biopsy was normal had a low serum pepsinogen value, but re-examination of the sections confirmed their normal appearance. The mean value for the 17 patients with gastric atrophy was almost the same as that for the patients with pernicious anaemia.

SUMMARY

A modified method for the estimation of serum pepsinogen is described, using haemoglobin as substrate and incorporating a buffer system to control the pH of the mixtures of serum and substrate. Both serum and substrate are treated with charcoal to reduce the blank values, and the tyrosine produced is estimated by measuring absorption at 280 $m\mu$. The results are presented of control experiments carried out to evaluate the method.

In 135 control subjects the results were between 310 and 2140 μg . tyrosine/ml. serum in 24 hours (mean 833, S.D. 290), compared with a range of 130 to 910 (mean 373, S.D. 139) in 137 patients with pernicious anaemia. The difference between these mean values is highly significant.

The mean value for 10 patients whose gastric biopsy specimens showed superficial gastritis was 786 μg . tyrosine/ml. serum in 24 hours, which was slightly but not significantly higher than the mean of 710 for eight patients with normal gastric mucosa. In seven patients with atrophic gastritis the mean value was 464, compared with 363 for 17 patients with gastric atrophy.

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