Methods and techniques

Simplified technique of immuno-electrophoretic assay of human intrinsic factor on acrylamide gel

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During an investigation into various methods which have been introduced for the assay of intrinsic factor, a technique for immuno-electrophoretic assay using polyacrylamide gel was devised. This proved to give reproducible results and was considerably quicker and simpler than the method described by Jeffries and Sleisenger (1963) using starch gel. This paper describes the technique and preliminary results.

The principle remains the same as that used by Jeffries and Sleisenger (1963). Electrophoresis of a mixture of gastric juice, radioactive vitamin B_{12}, and normal serum separates free and bound vitamin B_{12} which move cathodally and anodally respectively. The anodal peak has three components: vitamin B_{12} bound to intrinsic factor, vitamin B_{12} bound to non-specific binders, and hydroxycobalamin which may be present in ^{57}Co-B_{12} (Kennedy and Adams, 1965). Hydroxycobalamin is also responsible for the radioactivity detected in the anodal zone away from the peak (Williams and Bardhan, 1966). If serum containing antibody to intrinsic factor is substituted for normal serum then there is a marked reduction of the anodal peak, due to electrophoretic immobilization of the intrinsic factor-vitamin B_{12} complex at the point of application. The reduction in anodal radioactivity is a measure of the intrinsic factor content of the gastric juice.

MATERIALS

PREPARATION OF GELS The apparatus was set up as described by Davis (1964). The gel mixture was prepared as follows:— One part water was mixed with 1 part solution I (1 N hydrochloric acid 48 ml., 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris) 36·6 g., N.N.N′,N′-tetramethyl-ethylenediamine 0·23 ml., water to 100 ml.), 2 parts solution II (acrylamide 28 g., N, N′-methylenebisacrylamide 0·74 g., water to 100 ml.) and 4 parts ammonium persulphate (0·14 g., water up to 100 ml.). This mixture was pipetted to an 11 cm. mark in each of 24 glass tubes measuring 16 cm. long, with internal diameter 0·9 cm.

Gastric juice This was collected during an augmented histamine test. For preliminary work pools of neutralized gastric juice collected from several subjects were used. For individual assays all samples were collected in bottles cooled in ice; overnight, basal, and histamine-stimulated gastric secretions were collected separately. The latter was divided into four 15-minute samples. The volume of the samples was measured and a fixed fraction of each was neutralized to *p*H 7 with 1·0 N sodium hydroxide solution and then pooled. Excessive mucus was removed by filtration through gauze and the remainder was centrifuged at approximately 1,300 *g* for 10 minutes. The supernatant solutions were assayed for their intrinsic factor content.

^{57}Co-cyanocobalamin This was obtained from the Radiochemical Centre, Amersham. The contents of one ampoule containing 1·1 μg. (1·1 μc.) were dissolved in 11 ml. water, and the solution was stored at 4°C. in the dark.

Normal serum Normal serum was obtained from healthy young donors who had no gastric parietal-cell antibodies or intrinsic factor antibodies.

Serum containing antibody to intrinsic factor This was pooled from several patients with pernicious anaemia who had a moderate to high titre of antibody to intrinsic factor.

METHOD

Three samples of gastric juice were assayed simultaneously. The procedure for one sample was as follows. The gastric juice was diluted with an equal volume of water. One volume of diluted gastric juice was pipetted into each of two test tubes and mixed with twice the volume of ^{57}Co-vitamin B_{12} solution. The vitamin B_{12} solution was such that the 'exposure dose' of vitamin B_{12} was 400 m μg. per ml. undiluted gastric juice. The mixture was shaken and kept at room temperature for 15 minutes. To one tube, a volume of normal serum equal to that of the diluted gastric juice was added and to the other a similar volume of serum containing antibody to intrinsic factor was added. The mixtures were shaken and kept at room temperature for a further 15 minutes. Water and 2 M sucrose solution were added to make the total volume 7 ml. In practice 0·37 ml. each of diluted gastric juice and serum, 0·74 ml. B_{12} solution, 1·02 ml. water, and 4·5 ml. of 2 M sucrose solution were used; these volumes were determined by the need to compromise between the cost of ^{57}Co-vitamin B_{12} and getting measurable amounts of radioactivity in the gels.

For electrophoresis, 1 ml. aliquots of each mixture were used and the estimations were carried out in quadruplicate. Thus each sample of gastric juice required eight gels, four for the mixture containing normal serum and four for that containing antibody serum. It was most essential that the mixtures were well shaken before each aliquot was pipetted on to the gel. The mixtures were covered with Tris-glycine stock buffer (Tris 6 g., glycine 28·8 g., water to 1 l.) which had been diluted 10 times and the *p*H adjusted to 8·6 by adding more Tris. Electrophoresis was carried out using 3·75-4 mA per tube for 3½ to 4½ hours, depending on the migration of the clearly visible albumin band; a migration of 8 cm. for this band was sufficient.

Following electrophoresis the gels were removed and the first 2 cm. cut off (vide infra). The remaining 9 cm.

was placed in a plastic container and the radioactivity measured in a small sodium iodide well counter. The vials were then inverted and recounted and the average of the two counts was noted. This was done to minimize the errors involved in counting radioactivity of solids of an irregular geometry.

The difference between the mean of the net counts for the tubes with serum containing antibody to intrinsic factor and the mean for the four tubes containing normal serum was used to calculate the result, which was expressed by reference to the standard as units of intrinsic factor per millilitre gastric juice. One unit of intrinsic factor was arbitrarily defined as that amount which binds 1 mμg vitamin B_{12} (Ardeman, Chanarin, and Berry, 1965).

RESULTS

DISTRIBUTION OF THE RADIOACTIVITY IN THE GEL Gels were cut at 1 cm. intervals and the radioactivity of each segment was measured. Figure 1 shows that the main anodal peak of radioactivity due to bound vitamin B_{12} is considerably decreased in the presence of antibody to intrinsic factor. In contrast the other areas from the second centimetre onwards contain similar amounts of radioactivity. Free ^{57}Co-vitamin B_{12} is adsorbed on the first centimetre of the gel in the presence of sucrose; this is non-specific, very variable and unpredictable, and hence the first centimetre is excluded. The second and third centimetres are low in radioactivity; therefore inaccuracies in cutting between the two are unlikely to affect the results. Since the only difference in the distribution of radioactive vitamin B_{12} in the gels beyond the second centimetre is in the anodal peak, the whole gel beyond the second centimetre can be measured and the difference in the counts is proportional to the intrinsic factor content of the gastric juice.

SENSITIVITY Serial dilutions of a sample of gastric juice were measured for their contents of intrinsic factor and the results are shown in Table I. The method is sensitive and can detect as little as one unit of intrinsic factor.

TABLE I

INTRINSIC FACTOR CONTENT IN A SAMPLE OF GASTRIC JUICE DILUTED SERIALLY

Dilution	*None*	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$
Units of intrinsic factor per ml. undiluted gastric juice	41	36	45	39	36

REPRODUCIBILITY Two pools of gastric juice were assayed on consecutive days. The first pool, with a low content of intrinsic factor, was assayed 11 times and gave a mean of 22·8 units intrinsic factor per millilitre gastric juice with an S.D. of 5·2 and a coefficient of variation of 23%.

The second pool, with a high content of intrinsic factor, was assayed 13 times and gave a mean of 78·3 units intrinsic factor per millilitre gastric juice with an S.D. of 7·62 and a coefficient of variation of 9·7%.

INTRINSIC FACTOR SECRETION IN TWO GROUPS OF SUBJECTS Two groups, one with proven pernicious anaemia and the other a miscellaneous group of subjects who had an augmented histamine test and in whom the total acid output after histamine stimulation was greater than 10 mEq./hour, were studied (Fig. 2). The differences in the intrinsic factor output following histamine stimulation are very clear. No patient with pernicious anaemia secreted more than 100 units of intrinsic factor in one hour and no subject with acid gastric juice secreted less than 2,000 units in one hour.

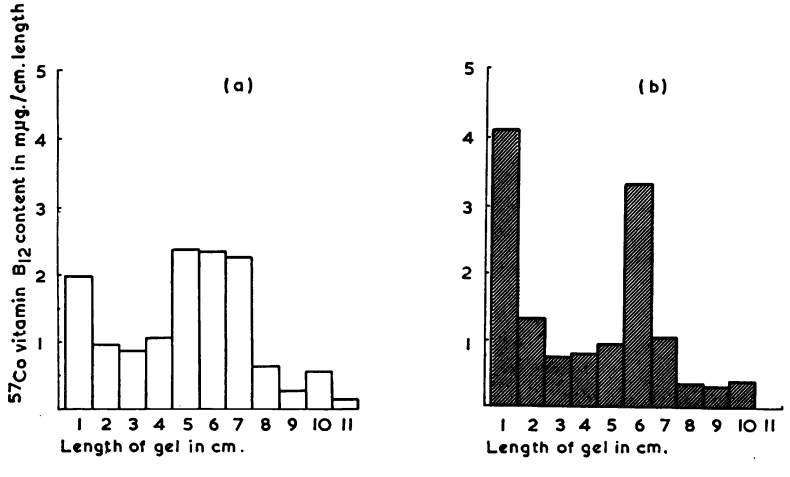

FIG. 1. *Distribution of radioactivity in gels after electrophoresis. (a) Mixture of gastric juice, ^{57}Co B_{12}, and normal serum. (b) Mixture of gastric juice, ^{57}Co B_{12}, and serum containing antibody to intrinsic factor.*

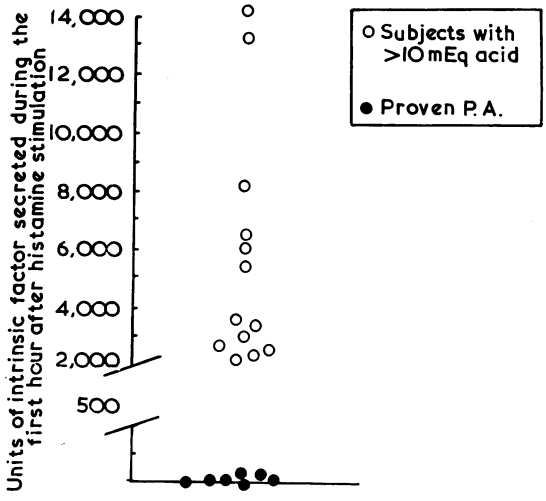

FIG. 2. *Intrinsic factor secretion following histamine stimulation.*

DISCUSSION

The main advantage of this method is speed and simplicity. Three samples of gastric juice are analysed in quadruplicate simultaneously. The method compares favourably with that of Jeffries and Sleisenger (1963). Few steps are involved; there is no need to divide the gels into many segments, and hence it takes less time. Preliminary work using starch gel electrophoresis showed that the results obtained were qualitatively similar to those obtained by the present method, but in our hands the method using starch gel gave less reproducible results.

The results with the present method show a clear distinction between the secretion of intrinsic factor in gastric juice of patients with pernicious anaemia and those with normal acid secretion following histamine stimulation.

SUMMARY

A rapid immuno-electrophoretic technique for the assay of human intrinsic factor is described; the method is simple, sensitive, and reproducible. It distinguishes clearly the secretion of intrinsic factor following histamine stimulation in subjects with pernicious anaemia and those with normal acid output.

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