Studies on the quantitation of immunoglobulin in human intestinal secretions

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SUMMARY There is increasing evidence for the importance of the secretory immune system in the gut. In studies of local antibody production it is important to have satisfactory methods for measuring immunoglobulin concentrations and to be aware of the errors which may occur. Studies on immunoglobulin measurement in intestinal secretion by the radial immunodiffusion method are reported, showing the effects of proteolytic digestion, IgA molecular size, and sampling and storage conditions. Because of the presence of monomeric IgA in addition to secretory IgA, there is no satisfactory standard for IgA in gastrointestinal secretions, and only semi-quantitative results can be given. With radial immunodiffusion, IgG and IgM when subjected to tryptic digestion, and IgA when subjected to peptic digestion, may be overestimated because of the presence of fragments of immunoglobulins. In addition, pepsin rapidly destroys IgM and IgG. Both IgM and IgG are unstable in storage. The findings suggest that immunoglobulin concentration measurements in small intestinal aspirates should be interpreted with caution. These problems are also relevant to the detection of specific antibodies in gastrointestinal secretions.

Since the initial description of the secretory IgA system (Tomasi and Zigelbaum, 1963) there has been renewed interest in the local production of antibody by the gastrointestinal tract. In studies of the local antibody system it is important to have accurate and reproducible methods for the measurement of immunoglobulin concentration in the secretions of the intestine. There have been a number of reports of measurement of intestinal immunoglobulin concentrations (Plaut and Keonil, 1969; Asquith, Thompson, and Cooke, 1970; Douglas, Crabbé, and Hobbs, 1970; Brown, Savage, Dubois, Alp, Mallory, and Kern, 1972), and a variety of technical difficulties have been described. We report here studies on some of the factors which can cause problems in these measurements, in particular the effect of proteolytic digestion and of molecular size on immunoglobulin quantitation. We have also investigated the effects of various sampling conditions and methods of storage of intestinal samples.

Subjects, Materials, Methods

SUBJECTS

Samples were obtained from normal volunteers, and from individuals undergoing intubation for pancreatic exocrine function testing and for jejunal biopsy (before the biopsy was taken).

SAMPLING

Small intestinal intubations were carried out under radiological control and tubes were positioned with the tip 5-10 cm beyond the duodenojejunal flexure unless specified otherwise. Pancreatic function tests were performed as described previously (Nimmo, Finlayson, Smith, and Shearman, 1970) using sequential stimulation with secretin (Karolinska Institute) 1·0 clinical units/kg and pancreozymin (Boots) 1·7 units/kg. Small intestinal aspirates obtained before pancreatic stimulation were collected by gentle aspiration. Aprotinin (Trasylol) was added immediately to some samples to give a concentration of 500 units/ml of sample. This concentration was chosen after performing initial experiments which

showed that this was sufficient to inhibit all tryptic activity in control samples, as measured using the substrate benzoyl-arginyl-ethyl ester. Samples were then frozen immediately and stored at $-26°C$.

IMMUNOLOGICAL METHODS

Immunoelectrophoresis (IEP)
This was performed according to the micro-method of Scheidegger (1955).

Radial immunodiffusion (RID)
A modification of the method described by Fahey and McKelvey (1965) was used. Microscope slides (10 × 7·5 cm) were covered with 8 ml of 1% agar in 0·1 M barbitone buffer pH 8·6 containing specific anti-immunoglobulin sera. Antisera against IgG, IgA, and IgM, raised in rabbits, were provided by Hoechst Pharmaceuticals. The stated antigen-binding capacity of these antisera was as follows: IgA 0·84 mg/ml, IgG 1·23 mg/ml, IgM 3·50 mg/ml. The dilution of antiserum in agar was 1:40 for each immunoglobulin class. Each batch of antiserum was tested by IEP against normal serum and showed only a single precipitin arc. The lower limit of accurate measurement of each immunoglobulin class by RID was 5 mg/100 ml.

To assess the reproducibility of assay, the IgA concentration of 28 jejunal aspirates was measured on four to six occasions. The overall mean coefficient of variation of repeat assays was 6·3%. The coefficient of variation of the results was constant over the range of concentrations tested (table I).

Comparison of IgA Standards

Secretory IgA was prepared by the method of Newcomb, Normansell, and Stanworth (1968) and standardized by measurement of extinction coefficient (Tomasi, 1970) and by weighing lyophilized material (Schultze and Heremans, 1966). Using RID and an anti-IgA serum, serial dilutions of this standard were compared with dilutions of Hoechst stabilized human serum standard. The two standards were compared on the same plate. The experiment was performed in triplicate.

GEL FILTRATION
A column of 6% Agarose (Biogel A-5M, 100-200 mesh 2·5 × 85 cm) was used. This was equilibrated with 0·05 M phosphate-buffered saline, pH 7·2, containing 0·05 M NaCl with 0·02% sodium azide as preservative. The elution position of secretory IgA was determined by passing secretory IgA labelled with ^{125}I (McConahey and Dixon, 1966) through the column.

Before chromatography, samples of jejunal juice were thawed and centrifuged for 20 min at 3000 × g and then dialysed at 4°C for 48 hr against two changes of the phosphate-buffered saline containing sodium azide (0·02%). Of ^{125}I labelled secretory IgA, 0·0025 mg was added to each sample as a marker, and 5-7 ml of sample, containing 2·5-7 mg of IgA, was loaded. Using a flow rate of 22 ml/hr, fractions were collected at 20-min intervals. The radioactivity in fractions was measured in an IDL well type scintillation counter. Fractions were screened for absorption at 280 nm using a Unicam SP500 spectrophotometer. Selected fractions were concentrated 25-fold by freeze drying and reconstituted in distilled water before measurement of immunoglobulin concentration.

A column of Sephadex G 200, 2·5 × 90 cm, was used to fractionate immunoglobulin preparations following enzymatic digestion *in vitro*. This was equilibrated with 0·1 M phosphate-buffered saline, pH 7·2, containing 0·1 M NaCl. A flow rate of 22 ml/hr was used and the fractions collected at 20-min intervals were screened for absorption at 280 nm.

Enzymatic Digestion

Tryptic and peptic digestion of IgG, IgM, and secretory IgA was carried out to assess the possible effect of digestion on quantitation of these immuno-globulins by radial immunodiffusion.

Secretory IgA was isolated as previously indicated. Human IgG at a concentration of 1000 mg/100 ml (Lister Institute) was tested by immunoelectro-phoresis against anti-whole human serum. The only arcs visible were those of IgG and a trace of albumin. Human IgM was isolated by gel filtration of IgM myeloma serum on 6% agarose and was shown to contain no other immune arcs when tested by IEP

	IgA Concentration Range (mg/100 ml)			
	0-50	*51-100*	*101-150*	*151-650*
Number of samples tested	9	4	9	6
Mean standard deviations of assays	1·6	4·3	7·2	14·4
Mean coefficient of variation of assays (%)	6·1	5·6	5·7	5·7

Table I *Means of standard deviations and coefficient of variation from 28 jejunal aspirates following four- or six-fold assay of each sample*

against antiwhole human serum.

TRYPTIC DIGESTION OF IgG, IgM, AND SECRETORY IgA

In three separate experiments, the immunoglobulins were incubated at 37°C with crystalline trypsin (Worthington) in tris-HCl buffer, 0·01 M, pH 8·0, containing 0·15 M NaCl at an enzyme : substrate ratio of 1:10. Subsamples were removed at 10, 20, and 30 min and thereafter hourly for five hours and placed in an equal volume of tris-HCl buffer containing Lima bean trypsin inhibitor (Koch Light). The concentration of the inhibitor was sufficient to neutralize twice the amount of trypsin present in the subsample. The pH was unaltered after incubation.

As controls, immunoglobulin incubated in buffer during the five-hr period of the test and unincubated immunoglobulin was used. The samples were examined by RID and by IEP at the end of the incubation period, employing antisera to IgG, IgM, and IgA. All subsamples were frozen at − 26°C until tested.

Two further experiments were carried out. In the first a further sample of secretory IgA was incubated with trypsin for four hr under the same conditions as described above. This digest was applied to a Sephadex G 200 column and the elution profile was compared to that obtained with a comparable sample of undigested IgA. In the second experiment IgG was subjected to tryptic digestion for five hr and its elution profile from Sephadex G 200 recorded.

PEPTIC DIGESTION OF IgG, IgM, AND SECRETORY IgA

In three separate experiments the immunoglobulins were incubated at 37°C in HCl, 12 m-equiv/1, containing crystalline pepsin at an enzyme : substrate ratio of 1:100. The pH of the digestion mixture was 2·2 before commencing incubation. Subsamples were removed at one-minute intervals for 10 min, at 15, 20, and 30 min, and hourly for five hr and added to an equal volume of sodium bicarbonate, 20 m-equiv/l. The pH of the subsamples was approximately 7·3. Controls were as described under tryptic digestion, and all samples were examined by RID and by IEP at the end of the incubation period.

In two further experiments, the elution profiles from Sephadex G 200 of a four-min peptic digest of secretory IgA and a two-hr digest of IgG were compared with the respective profiles of undigested samples.

Immunoabsorption of IgA in Gastrointestinal Samples

Four samples of jejunal aspirate were studied to determine whether all the material detectable as IgA by RID could be removed by absorption with specific

anti-IgA serum (Hoechst Pharmaceuticals). Two dilutions of a secretory IgA standard were included as controls. Using the supplier's value for the antigen-binding capacity of the anti-IgA serum, an estimate was made of the amount required to bind all the IgA in the samples. This volume of antiserum was added to each sample, and an equal volume of 0·15 M saline was added to the control tubes. The tubes were then incubated at room temperature for four hr and overnight at 4°C, after which they were centrifuged for one hr at 4000 × g and the supernatants tested for IgA by radial immunodiffusion.

Assessment of Effects of Storage and Trypsin Inhibitors on Jejunal Samples

Twenty-six samples were taken which, when assayed within one week of collection, contained high levels of IgA and IgM. Nineteen of these samples also contained measurable levels of IgG. Eleven of the samples were stored with added aprotinin: the remainder were stored without aprotinin. The samples were thawed and reassayed after periods of one month (two samples), two months (three samples), four months (two samples), six months (19 samples), and eight months (one sample).

Assessment of the Effect of Sampling Conditions in Individual Patients

During routine pancreatic function testing, samples were obtained from the distal duodenum with the patient fasting, and at intervals following stimulation with secretin. A sample was also taken following stimulation with pancreozymin. The immunoglobulin concentrations of these samples were measured. A study of four normal volunteers was also carried out to compare the jejunal immunoglobulin concentrations in the fasting state with those following ingestion of a standard meal consisting of glucose, albumin, corn oil, and water.

Results

COMPARISON OF IgA STANDARDS

Figure 1 illustrates the standard plots when purified secretory IgA was compared with the IgA in dilutions of Hoechst stabilized human serum which contains 87% 7S IgA and 13% 1OS IgA (manufacturer's data). These lines are seen to diverge with increasing concentration. Thus a sample of secretory IgA measured against a serum IgA standard could be underestimated by a factor of 3 at low concentration. Because of the divergence of the standard curves this factor increases sharply with increasing concentration, giving a 10-fold underestimate at the
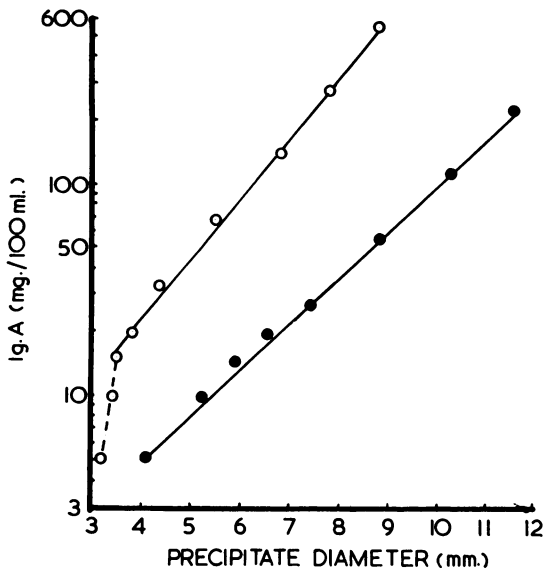
Fig 1 *Comparison of secretory and serum IgA standards by radial immunodiffusion (solid circles represent serum IgA, and open circles colostral IgA).*

highest concentration tested. Preliminary studies indicate that the relationship of the two standard curves is also dependent on the concentration of antiserum used.

IMMUNOELECTROPHORESIS OF INTESTINAL
SAMPLES
Samples were examined by IEP against antisera to IgA and antiwhole human serum. All samples showed an IgA arc with both antisera. IgM and IgG arcs were visible in some samples.

REMOVAL OF IgA FROM INTESTINAL SAMPLES
BY IMMUNOABSORPTION
Table II shows the IgA concentration of two standard solutions and four jejunal samples to which were added antiserum to IgA or the same volume of saline as a control. Following absorption with antiserum (in a quantity calculated to be just sufficient to remove all the IgA from the most concentrated standard), there was no detectable precipitate formed by three of the samples and the 50 mg/100 ml IgA standard. The 100 mg/100 ml IgA standard and the sample with the highest IgA concentration still contained trace amounts of IgA. It is concluded that the absorption procedure effectively removed from
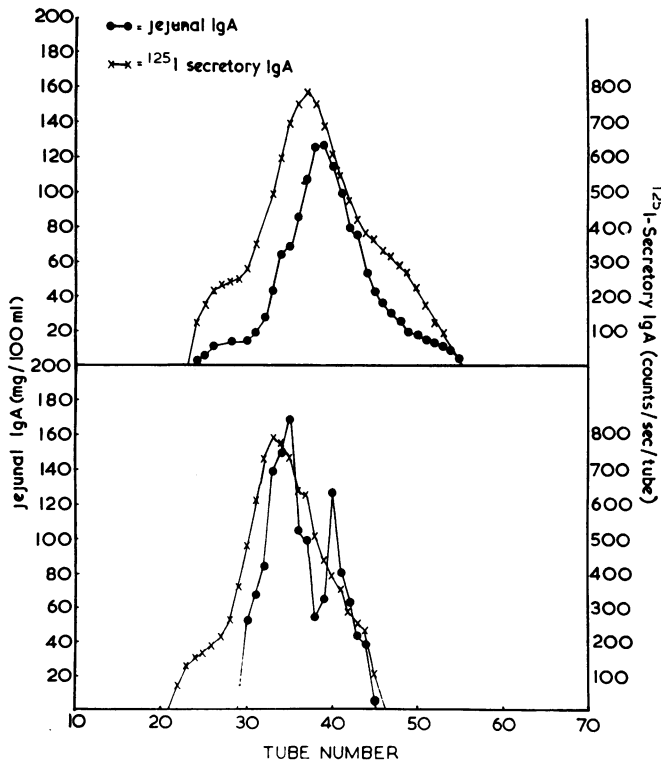

Fig 2 *IgA elution profile of two jejunal aspirates fractionated on 6% agarose: the elution profile of a* ^{125}I *labelled secretory IgA marker is also shown.*

Fig 2

	Jejunal Aspirate				IgA Standard	
A	87	62	44	50	100	50
B	<5	0	0	0	<5	0
C	54	39	20	30	60	30

Table II *IgA levels in standard solutions and samples of jejunal aspirate before and after absorption with specific anti-serum.*

A = IgA concentration (mg/100 ml) of samples
B = IgA concentration (mg/100 ml) of samples following absorption with specific antiserum at calculated equivalence
C = IgA concentration (mg/100 ml) of control samples following dilution with phosphate-buffered saline

the samples the material detected by RID using anti-IgA serum.

GEL FILTRATION OF INTESTINAL ASPIRATES

Four samples of jejunal juice were examined. Figure 2 shows the concentration profiles of IgA in two samples. The fractions were measured after 25-fold concentrations because the IgA levels in the unconcentrated fractions were too low for accurate measurement. The elution position of the ^{125}I-labelled secretory IgA marker is shown on each profile. In three of the four samples, the IgA appeared to be heterogeneous, comprising material which behaved as 11S IgA and lower molecular weight material.

EFFECT OF TRYPTIC AND PEPTIC DIGESTION ON IgG, IgM, AND SECRETORY IgA AS MEASURED BY RID
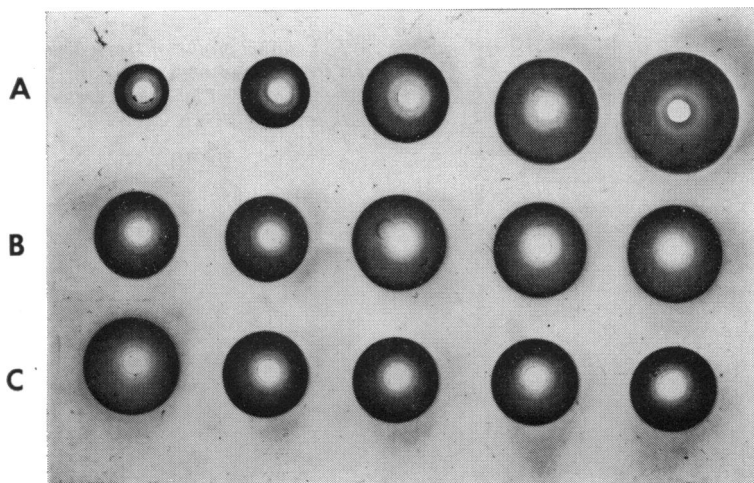
Tryptic digestion
IgA concentrations measured by RID did not alter over the five-hr incubation period and the immunoelectrophoretic arc did not change in appearance. The elution profile on Sephadex G 200 was identical before and after incubation.

The diameter of the ring formed on the RID plates was larger with incubated samples than with the control IgG and the diameter increased progressively with increasing periods of incubation (fig 3). This increase in diameter had a substantial effect on the apparent amount of IgG in the fractions, as shown in figure 4. (These results are the means of five experiments.) Immunoelectrophoretic analysis of the sample is shown in figure 5. With fractions incubated for periods up to 1·5 hr, the IgG did not appear altered. With samples incubated for longer periods an additional arc was seen in the fast gamma region. Figure 6 shows the elution profile of a two-hr tryptic digest of IgG on Sephadex G 200, compared with that of the control protein: the height of the main IgG peak is reduced and there are two subsidiary peaks.

The results of quantitation of IgM in the digests

Row A *Five standard concentrations of IgG.*

Row B *Control IgG (two wells) and IgG following tryptic digestion for two, three, and four hours.*

Row C *IgG following tryptic digestion for five hr and one, two, three, and four minutes.*

Fig 3 *The effect of tryptic digestion on IgG measured by radial immunodiffusion. The diameter of precipitate is seen to increase when samples are incubated with trypsin.*
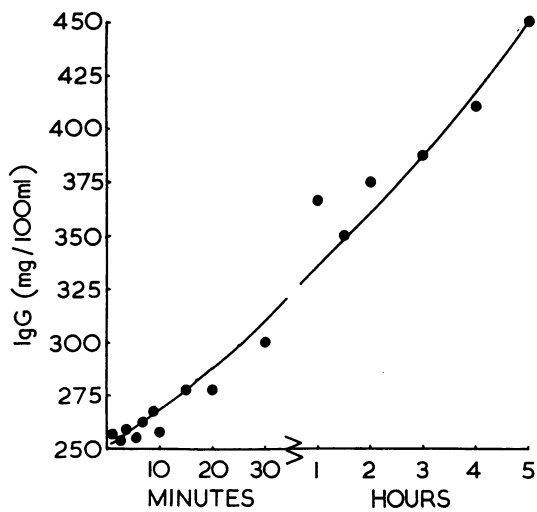*Each row should be read from left to right.*

Fig 4 *The effect of tryptic digestion on the apparent concentration of IgG measured by RID. The initial IgG concentration of the solution was 250 mg/100 ml.*
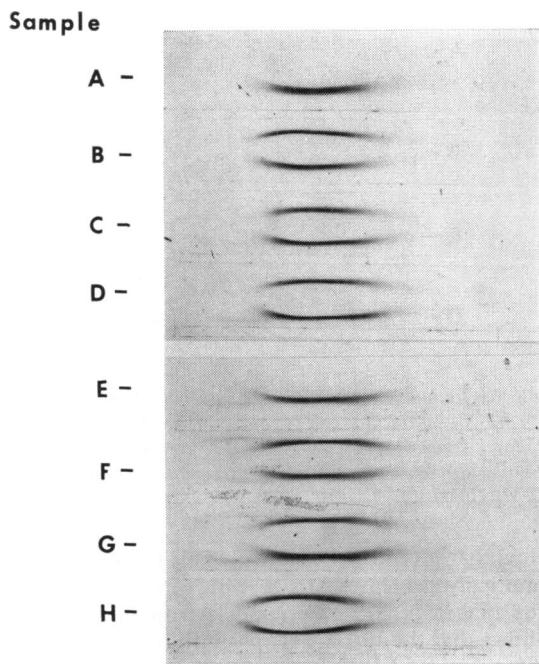

Fig 5 *Immunoelectrophoretic analysis of IgG following tryptic digestion. Samples A to H are samples following digestion of 0·5, 1, 1·5, 2, 3, 4, and 5 hr, respectively. Sample H is the untreated control. Antiserum: Anti-human IgG (Hoechst).*

by RID were similar to those found following tryptic digestion of IgG. The progressive increase in diameter as incubation with enzyme proceeded produced an apparent four-fold increase in concentration of IgM after incubation for five hours.

Immunoelectrophoretic analysis of the sample is shown in figure 7. With fractions incubated for periods up to two hours the IgM did not appear altered. With samples incubated for longer periods an additional arc was seen.

Peptic digestion
The effect of peptic digestion on the appearance of the fractions examined by RID is shown in figure 8. After one min incubation, the appearance of the ring becomes pale and diffuse, and the diameter is greater
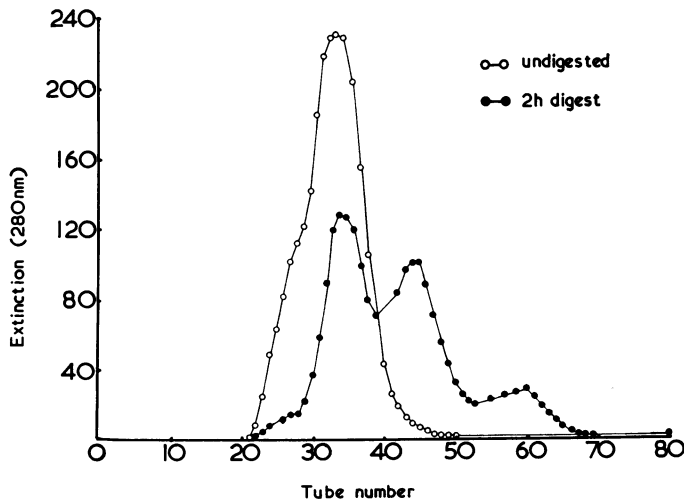

Fig 6 *Elution profile on Sephadex G 200 of undigested IgG and a two-hr tryptic digest.*
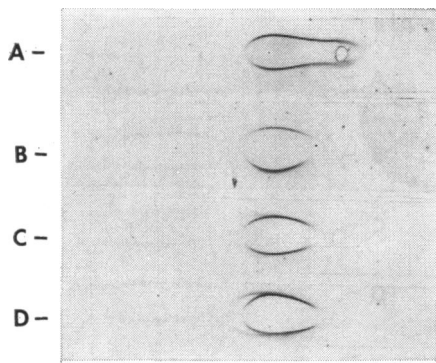
Fig 7 *Immunoelectrophoretic analysis of IgM following tryptic digestion. Sample A is control IgM. Samples B, C, and D are samples following digestion for three, four, and five hr respectively. Antiserum: Antihuman IgM (Hoechst).*

than that given by the control. This hazy precipitate gradually decreases in size with samples incubated for longer periods. Immunoelectrophoresis (fig 9) shows that the IgA arc is split into two distinct arcs after one-min incubation, and this appearance persists in the fractions up to six min. Thereafter the arc becomes hazy and is not visible with samples incubated for longer than one hour. The appearance of the four-min digest on Sephadex G 200 is shown in figure 10. There is a broad peak of lower molecular weight material in the pepsin-treated sample and the height of the main IgA peak is reduced.
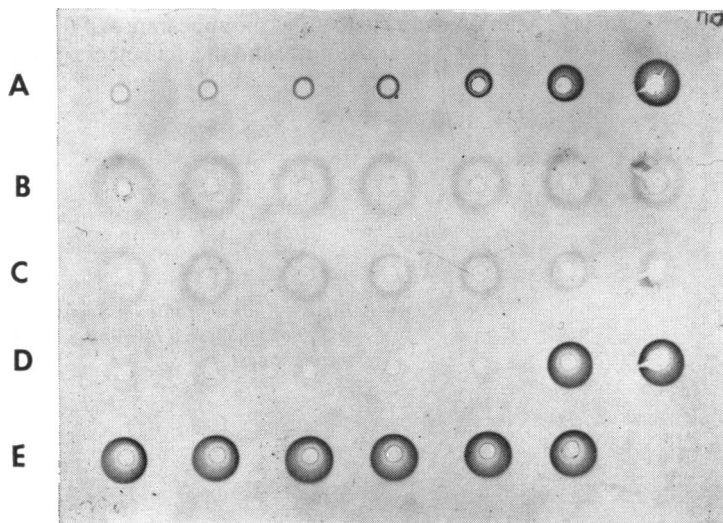
Incubation with pepsin markedly altered the appearance of the IgG rings on RID plates (fig 11). The clear ring of precipitate seen with the control IgG is greatly reduced in size, and disappears after seven-min incubation: an additional, faint outer ring is seen which progressively falls in diameter with longer periods of incubation. On IEP, there is one arc only, which is not seen with samples incubated for longer than 15 minutes. Gel filtration of a two-hr peptic digest on Sephadex G 200 showed the appearance of two peaks of lower molecular weight material.

The well defined precipitate rings produced by the control IgM samples were all that could be visualized after analysis by RID. Following one-min incubation with pepsin no IgM ring was visible. Immunoelectrophoretic analysis of all subsamples gave similar results; only control IgM arcs were visible.

EFFECT OF STORAGE + APROTININ ON
JEJUNAL SAMPLES
Over the whole range of samples, the mean fall in IgA levels on reassay was 3%, and the mean fall in IgM was 20%. In all the samples, including the two stored for one month only, the IgG fell to undetectable levels. The addition of aprotinin to the samples had little effect on the change in levels. Analysis of IgA concentrations in all samples containing aprotinin revealed a 4% drop after storage. Samples stored without aprotinin showed an overall drop of 3%. IgM concentrations showed a 23% overall drop in samples containing aprotinin and a 16% drop in those which contained no aprotinin.

Row A *Seven standard concentrations of IgA.*

Row B *Subsamples from peptic digest of IgA taken at minute intervals (one to seven min).*

Row C *As row B but taken at eight, nine, 10, 15, 20, 30 and 60 min.*

Row D *As row B but taken at 1·5, two, three, four, and five hr; control IgA (two wells).*

Row E *Control IgA (six wells), pepsin (one well).*

Fig 8 *Measurement of secretory IgA by radial immunodiffusion following digestion with pepsin. Each row should be read from left to right.*
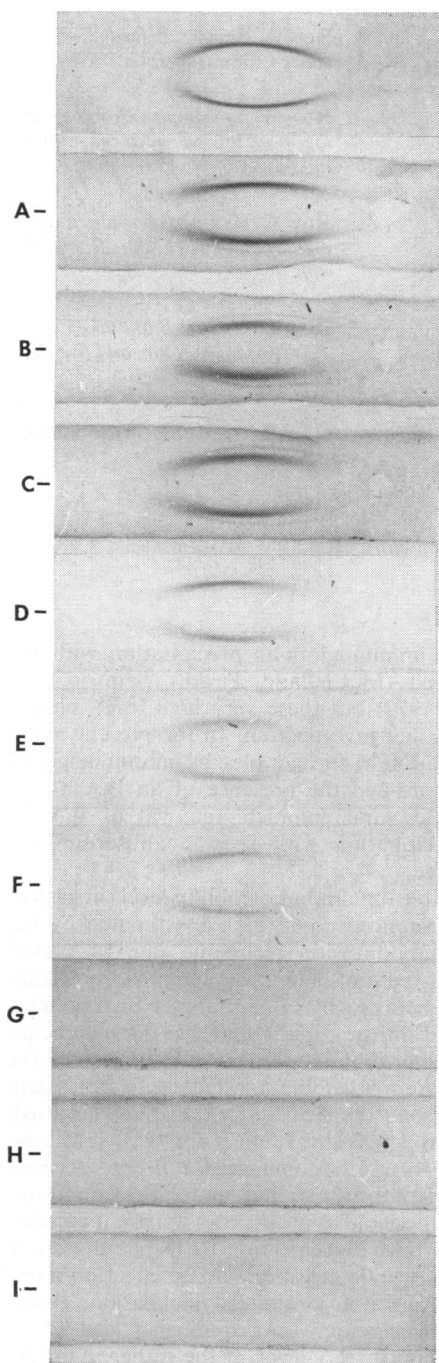
Fig 9 *Immunoelectrophoretic analysis of secretory IgA following peptic digestion. The top sample is the control and samples A to I are subsamples from a peptic digest taken at one-min intervals over nine minutes. Antiserum: Antihuman IgA (Hoechst).*
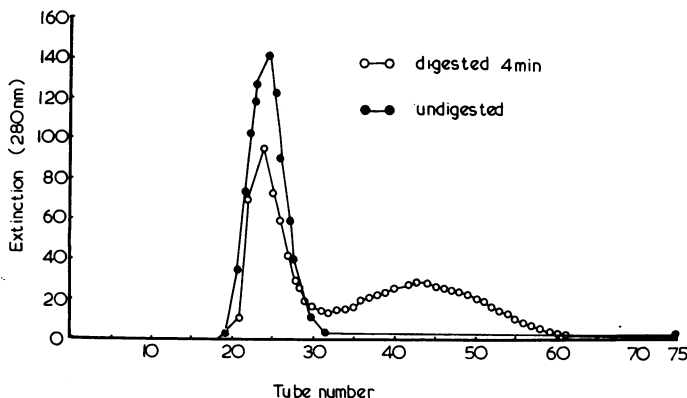

Fig 10 *Elution profile on Sephadex G 200 of undigested secretory IgA and a four-min peptic digest.*

It should be noted that the overall figure for IgA contains the results of two samples which showed a significant rise after storage (initial levels 76 and 34 mg/100 ml, rising to 91 and 50 mg/100 ml respectively after storage).

EFFECTS OF SAMPLING CONDITIONS
Table III shows the results of jejunal IgA assays on four normal volunteers in the fasting and post-prandial state. There are wide variations in the IgA concentration in the jejunum.

Figure 12 illustrates the changes in IgA concentration in the distal duodenum in patients undergoing sequential stimulation with secretin and pancreozymin. Although there is a general trend towards low levels following secretin administration, the changes in levels following administration of pancreozymin vary widely between different individuals.

Discussion

There are two main factors to consider when interpreting measurements of gastrointestinal immunoglobulin levels. These are: first, the methodological difficulties relating to enzymatic digestion, heterogeneity of molecular weight, and non-immunoglobulin precipitation; secondly, the effect of the conditions under which samples are obtained. There are further problems in determining the origin of immunoglobulin detected in gastrointestinal secretions but these are not considered in the present study.

In recent studies we have found high concentrations of IgA in human gastric juice (McClelland, Finlayson, Samson, Nairn, and Shearman, 1971) and small intestinal secretions (McClelland, Barnetson, Parkin, Warwick, Heading, and Shearman, 1972). We have demonstrated that these findings are not
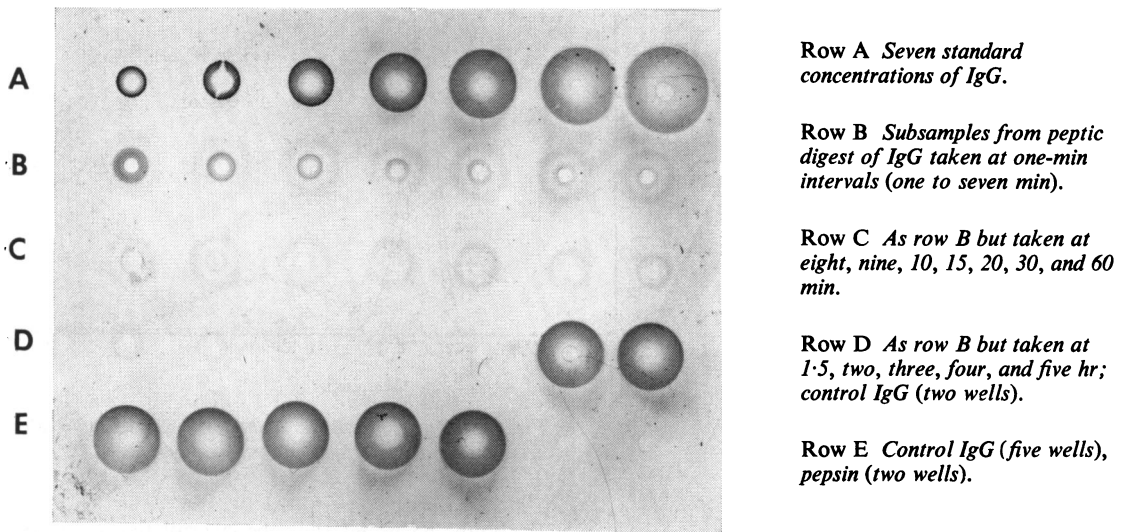
Row A *Seven standard concentrations of IgG.*

Row B *Subsamples from peptic digest of IgG taken at one-min intervals (one to seven min).*

Row C *As row B but taken at eight, nine, 10, 15, 20, 30, and 60 min.*

Row D *As row B but taken at 1·5, two, three, four, and five hr; control IgG (two wells).*

Row E *Control IgG (five wells), pepsin (two wells).*

Fig 11 *Measurement of IgG by radial immunodiffusion following digestion with pepsin. Each row should be read from left to right.*
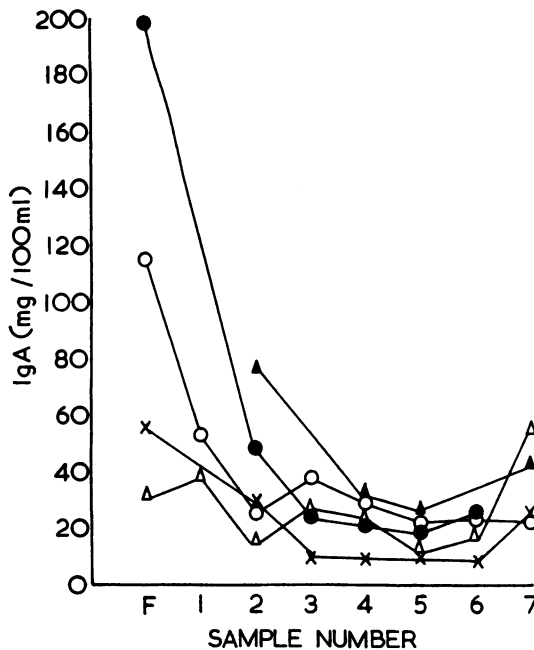

Fig 12 *IgA levels in the distal duodenum following sequential stimulation with secretin and pancreozymin. F is the fasting sample. Samples 1-7 were aspirated at 15-min intervals. Secretin was administered after taking sample F and pancreozymin after taking sample 6.*

due to non-immunoglobulin precipitation with the antisera used (McClelland, Parkin, Samson, and Shearman, 1972) but these very high levels clearly required further investigation. In the present paper the examination of the samples by immunoelectrophoresis confirmed the presence of an IgA arc in these samples, and complete removal of IgA by immunoprecipitation with specific antiserum was demonstrated.

It is possible that immunoglobulin levels in gastrointestinal secretions could be overestimated by the radial immunodiffusion technique due to partial digestion of the molecule, producing low molecular weight fragments such as Fab, Fab_2, or Fc fragments which could diffuse rapidly and give large precipitates on radial immunodiffusion. The effect of the presence of immunoglobulin fragments in any system will depend on the specificity of the antiserum used. We have investigated this effect for IgG, IgM, and secretory IgA using the commercial antisera described, and have demonstrated that under the conditions used it was possible to produce an apparent increase in IgG and IgM concentration by tryptic digestion. By contrast, tryptic digestion had no effect on secretory IgA even after prolonged incubation. Peptic digestion of secretory IgA could also lead to an overestimation of IgA levels if the rather indistinct precipitin ring (fig 8) was measured. Peptic digestion of IgG produced a rapid fall in concentration as measured by RID (fig 10). After one min peptic digestion no precipitable IgM was detected by RID or immunoelectrophoresis.

Subject	Site	State	IgA	IgG	IgM
1	Jejunum	Fasting	15	0	0
		Postprandial	34	0	0
2	Duodenum	Fasting	15	0	0
		Postprandial	0	0	0
3	Jejunum	Fasting	30	0	0
		Postprandial	83	7	9
4	Duodenuo-jejunal	Fasting	26	0	0
	flexure	Postprandial	14	0	0

Table III *Immunoglobulin levels (mg/100 ml) of four jejunal aspirates taken during the fasting state and following a standard meal*

The indistinct precipitin rings formed in RID plates by partially digested immunoglobulin (figs 7 and 10) may well account for some of the multiple precipitin rings described in previous reports. For example, a sample of jejunal fluid contaminated with gastric juice could contain both intact IgA and fragments resulting from peptic digestion in the stomach. A similar problem is likely to occur with IgG measurements. Gel filtration and immunoelectrophoretic analysis of the peptic digestion of IgA and the tryptic digest of IgG show the appearance of low molecular weight fragments which presumably account for the enlarged precipitin rings.

These results indicate that the presence of IgA, IgM, and IgG fragments could lead to overestimates of concentrations, and although secretory IgA is highly resistant, the possibility of tryptic cleavage of monomeric IgA in the small intestine is not excluded by the present experiments.

The gel filtration studies of jejunal secretions show that the IgA is heterogeneous with regard to molecular size. This raises serious difficulties in the choice of an appropriate standard. It has been shown (Tomasi and Bienenstock, 1968; Brandtzaeg, Fjellanger, and Gjeruldsen, 1970; Hanson, Holmgren, and Wadsworth, 1971) that the use of a serum IgA standard to measure secretory IgA introduces an underestimate of up to 10-fold, and the present study confirms this. A secretory IgA standard has been used in our work, but it is clear that the presence of lower molecular weight material makes this unsatisfactory. The recent report (Hanson *et al*, 1971) of a combined thin-layer gel filtration and immunodiffusion technique may partly resolve this problem although this would be a time-consuming technique for routine sample assays.

Because of variable amounts of monomeric IgA in gastrointestinal secretions the measurement of IgA levels in these specimens must be considered semi-quantitative; minor differences in IgA concentrations cannot be interpreted unless the proportion of low molecular weight IgA is known.

Little information is available on the effect of storage on jejunal immunoglobulin levels. The use of

heating to inactivate trypsin has been mentioned (Plaut and Koenil, 1969) and addition of a trypsin inhibitor has been used (Douglas *et al*, 1970). The latter authors reported little difference in immunoglobulin concentrations using epsilon-amino-caproic acid although no data were given. Our observations show that IgA is fairly stable when stored at −26°C, and that there is a slow fall in IgM levels. IgG, however, is unstable. The use of aprotinin in a concentration sufficient to abolish the tryptic activity of the samples did not appear to affect the stability of any immunoglobulin class. The apparent increase in IgA concentration of two samples may be due to depolymerization in storage, but this requires further investigation.

In the present study we have minimized the repeated freezing and thawing of samples by performing duplicate assays on the same day. However, repeated freezing and thawing may clearly accelerate immunoglobulin breakdown: this has not been systematically examined. More information is also required about the stability of immunoglobulins in secretions at room temperature, as it is likely that significant breakdown may occur during the period of incubation of the RID plate at room temperature.

Sampling conditions are clearly important in determining the immunoglobulin concentration found in a given individual, and our results illustrate the wide and rather unpredictable variation between basal samples of small intestinal secretion and samples obtained following food or administration of secretin and pancreozymin. Further studies are in progress to assess the reproducibility of repeated fasting samples from a number of subjects. The control subjects (normal young adults) for whom data are presented in table III had very low levels or no IgG and IgM in the samples obtained. This is not unusual in our experience with normal individuals and low levels have been reported in previous studies (Plaut and Keonil, 1969; Douglas *et al*, 1970).

The observations reported here indicate the need for caution in interpreting gastrointestinal immunoglobulin measurements. It is probable that a useful estimate can be obtained by careful basal sampling,

choice of suitable antisera, and the use of a secretory IgA standard, but more precise studies of gastro-intestinal mucosal synthesis of immunoglobulin will require wider application of techniques such as those described by Loeb, Strober, Falchuk, and Laster (1971) and Falchuk and Strober (1972), which allow quantitative study of immunoglobulin synthesis *in vitro*.

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