

DEVELOPMENT OF A RADIOIMMUNOASSAY FOR GASTRIC INHIBITORY POLYPEPTIDE

by

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ABSTRACT

With the isolation, purification and sequencing of Gastric Inhibitory Polypeptide (GIP), interest turned towards its physiological significance. In order for this to be properly evaluated a means had to be developed for measuring its concentration in serum and tissues. A radioimmunoassay was the method of choice due to the sensitivity, specificity, accuracy and precision.

This thesis describes the steps involved in the development of a radioimmunoassay. Specific and sensitive antiserum had to be produced. A method had to be standardized for obtaining a high purity 125I labelled GIP. With the attainment of these two goals the assay environment had to be evaluated to yield an optimum incubation volume, Trasylol concentration, plasma concentration, pH and incubation timing. Since the antibody/antigen complexes do not spontaneously precipitate, a method of separation was decided upon which yielded efficient separation coupled with technical practicality. Analysis of data was evaluated and the most appropriate means of correcting for non specific binding was determined.

Once the assay was established it had to be investigated qualitatively and quantitatively. First the specificity of the antiserum was tested against other gastrointestinal polypeptides. A serum at various dilutions was measured to compare unknown and standard GIP reactivity with antibody. A quality control chart utilizing 10% CCK-PZN was set up. The fasting normal range for GIP was determined.

The assay was then utilized in a series of preliminary studies

to evaluate the GIP response to feeding and oral glucose tolerance tests. Initial evidence for the insulinotropic action of GIP was presented. Indicated also was an implication of GIP involvement in various pathological conditions.

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INTRODUCTION

Investigation by Kosaka and Lim (1930a) established that a crude preparation of cholecystokinin (CCK-PZN) inhibited gastric secretion stimulated by a meat meal and histamine in dogs. They interpreted the results to indicate that the crude preparation was contaminated with an inhibitory material. They also obtained extracts of duodenal mucosa following stimulation with olive oil which exhibited the same type of inhibition (1930b). Enterogastrone was the name given by them to the inhibitory material liberated by fat in the duodenum. This definition was extended by Gregory (1967) to "liberation by fat digestion products, hypertonic solution and acid of a hormone which inhibits gastric secretion and motility."

Subsequently it was found that crude CCK-PZN preparations inhibited exogenous (Gillespie and Grossman, 1964) and endogenous (Brown and Magee, 1967) gastrin stimulated acid secretion. Pure CCK-PZN when injected without gastrin, however, stimulated acid secretion. Brown and Pederson (1970) compared the response to 10% and 40% CCK-PZN preparations in sympathetically and vagally denervated fundic pouches to attempt to resolve the conflicting responses. They found that 40% CCK-PZN stimulated acid and pepsin secretion to a greater extent than the 10% preparation. During pentagastrin stimulation, 40% CCK-PZN resulted in significantly lower inhibition of acid secretion. The effects on gall bladder and antral pouch motility were the same.

There were two possible explanations for these results. Either an inhibitory material was being eliminated during the purification procedures or a stimulatory substance was being concentrated. Since CCK-

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PZN and gastrin share many structural similarities it appeared likely that acid stimulation would be an inherent property of the CCK-PZN molecule, the former hypothesis was pursued. Brown, Pederson, Jorpes, Mutt (1969) began purification of the possible inhibitor utilizing crude hog 10% CCK-PZN as the starting material.

Assay of the starting material, designated EGI, in the guinea pig and cat indicated no secretin activity and less than 10 IDU CCK-PZN/mg. They obtained a significant inhibition of exogenous pentagastrin stimulated acid and pepsin secretion. Whereas 10% CCK-PZN stimulated antral motor activity, EGI was found to inhibit it.

Brown, Mutt and Pederson (1970) continued the purification of the polypeptide from EGI through to EGIII and named the material Gastric Inhibitory Polypeptide (GIP). Chemical evaluation was then initiated to determine the amino acid composition and sequence. The polypeptide was found to have a molecular weight of 5105. It consisted of forty three amino acids and the sequence was determined to be TYR-ALA-GLU-GLY-THR-PHE-ILE-SER-ASP-TYR-SER-ILE-ALA-MET-ASP-LYS-ILE-ARG-GLN-GLN-ASP-PHE-VAL-ASN-TRP-LEU-LEU-ALA-GLN-GLN-LYS-GLY-LYS-SER-ASP-TRP-LYS-HIS-ASN-ILE-GLN (Brown, 1971; Brown and Dryburgh, 1971).

GIP was chemically distinct from CCK-PZN by absence of proline, the presence of a high content of glutamic acid or glutamine and a preponderance of lysine over arginine. Comparisons were also made with other gastrointestinal polypeptides. Structural similarities were found with both procine glucagon and secretin. GIP shares fifteen of the first 26 amino acids of glucagon and nine of secretin.

Once GIP purification had proceeded to the EGIII stage a series of

studies were undertaken to determine its range of physiological activities. Pederson (1971) compared the actions of GIP on acid stimulation by pentagstrin and the whole gastrin molecule (SHG-1). Inhibition of acid and pepsin secretion and antral pouch motor activity were all greater following SHG-1 stimulation.

Kosaka and Lim (1930a) found that fat in the duodenum inhibited histamine stimulated acid secretion. Studies with both CCK-PZN and secretin (Johnson and Grossman, 1969) yielded no inhibition of histamine stimulated acid secretion and therefore neither of them fulfilled the requirements for enterogastrone. It therefore became important to look at GIP with respect to histamine. Pederson and Brown (1972) determined the extent of inhibition of acid and pepsin secretion following histamine stimulation. They obtained results similar in degree to those obtained with fat in the duodenum (Johnson and Grossman, 1969, and Alley <u>et al</u>, 1934). The acid and pepsin secretion stimulated by insulin hypoglycemia was also inhibited by GIP.

Barbezat and Grossman (1971a) compared the ability of a series of gastro-intestinal polypeptides to stimulate secretion from isolated loops of jejunum and ileum in conscious dogs. GIP was a potent stimulator of both jejunal and ileal secretion. Although glucagon and pentagstrin were also stimulatory they were much less effective than GIP, particularly when comparing doses used on a molar basis. CCK-PZN and secretin were essentially without effect.

In terms of the enterogastrone definition, GIP's actions are much closer than any of the previously investigated gastro-interstinal polypeptides. Secretin inhibited acid secretion but stimulated pepsin

secretion (Nakajima and Magee, 1970). CCK-PZN effects vary with the presence or absence of exogenous gastrin. More recent work by Grossman on the kinetics of CCK-PZN-gastrin interaction indicates that CCK-PZN is a competitive inhibitor of gastrin, both interacting with a single receptor. When CCK-PZN was given alone, therefore, it acted at the receptor without competition yielding stimulation. With exogenous gastrin infusion, however, the resulting inhibition or stimulation with CCK-PZN depended on whether CCK-PZN was as effective as gastrin in stimulating acid secretion. This was apparently species dependent. Neither CCK-PZN nor secretin inhibited histamine stimulated secretion. GIP, however, inhibited acid, pepsin and antral motor activity stimulated by gastrin pentapeptide, gastrin, insulin hypoglycemia and histamine. It appeared to counteract all effects of gastrin action and mimicked the effects of fat in the duodenum. Since GIP inhibited the action of stimulators for acid secretion other than gastrin its action was distinct from CCK-PZN.

Itoh, Lucien and Schally (1972) have proposed that their "enterogastrone" inhibits acid secretion at the same site as histamine, distinct from the gastrin site. This explanation was untenable for their "enterogastrone" as well as GIP because both inhibit gastrin stimulated acid secretion. Thus a proposal must be made to account for interaction at more than one receptor. Although the "enterogastrone" of Itoh yields similar degrees of inhibition of gastrin and histamine stimulated acid secretion it is impossible, at present, to say if the substances are identical as no chemical data is available on "enterogastrone". The use of 0.5 mg doses of the Itoh material to obtain inhibition suggests a fairly low degree of purity or a very low activity level.

It is not difficult to evaluate the physiological importance of such an inhibitor. If an enterogastrone was released by the presence of fat, acid and hypotonic or hypertonic solutions in the duodenum it would protect the small intestine and stomach mucosa from excess H⁺ secretion. Inhibition of acid would prevent further pH decreases and inhibition of antral motor activity would delay gastric emptying. The antral effect coupled with stimulation of intestinal secretion would improve absorptive functions in the small bowel.

Once the inhibitory action of GIP had been evaluated, a means had to be developed for measuring GIP levels in blood and tissues. The availability of such an assay would enable the determination of the physiological secretagogues for GIP release. This would allow an evaluation of the physiological role of GIP and also determine if it can be assigned hormonal status.

Peptide hormones have characteristics invivo which differentiate them from the thyroidal and steriod hormones. They have a lower circulating basal level, which sporadically increase 1000% or greater over a short period of time. Their length of action is limited and thus the plasma one half life is usually less than thirty minutes. Chemically they cannot at present be separated from each other for routine analysis. Attempts at bioassay to date have not yielded sufficient sensitivity to measure plasma levels. Even if a bioassay was developed, they are usually too tedious for routine clinical application.

Similarity in the action and structure of the gastro-intestinal polypeptides was mentioned previously. There is also a considerable amount of interaction between peptides as indicated by CCK-PZN and

gastrin receptor activity. Thus with any assay utilized, an evaluation of cross reactivity must be considered.

Radioimmunoassays are well known for their sensitivity and specificity. Since the chemical reaction between antibody and antigen is extremely specific, peptide antigens can be detected in the presence of a large number of other proteins without any prior extraction procedures. Specificity can be evaluated for each antiserum utilized in the assay. Antibody and antigen concentrations can be reduced proportionately to obtain sensitivities in the low picogram range. The assay procedure is well suited for testing large numbers of samples at one time, thus making it clinically practical.

The present research was undertaken to develop a radioimmunoassay. for GIP. The assay could then be utilized to evaluate GIP's physiological role and possible pathological involvements. Clinically the only presently available GI peptide radioimmunoassay is for gastrin. A considerable amount of data on gastrin levels in pathological conditions has been published (Yalow and Berson, 1972) and the test is used diagnostically in suspected cases of Zollinger-Ellison Syndrome.

METHODS

PRODUCTION OF ANTISERA

I Immunization Schedule

Initially the procedure of GO, Ryan and Summerskill (1971) for CCK-PZN was used for immunization. Their schedule consisted of six subcutaneous injections of 10% pure CCK-PZN at 10 day intervals (2.5 mg/ guinea pig) emulsified with Freunds Complete Adjuvant (FCA). Ten days following the last injection blood was taken by cardiac puncture. The antibody titers were boosted by subsequent immunizations with 50% purified CCK-PZN (1 mg) in 1 ml of FCA on three occasions at teneday intervals. GIP immunization schedules for six rabbits are found in Table 1 and ten guinea pigs in Table 2.

The GIP side fraction (Brown, Mutt and Pederson, 1970) used for initial immunization was approximately 70% pure. Parker (1971) indicates that pure material is not required but one means of improving antibody affinity is to increase the purity of the antigen with subsequent immunization. GIP III was thus substituted as immunization proceeded.

Since the amount of antigen injected is an important variable in the production of antisera, this was also varied as the schedule progressed. Exceeding the optimum amount can result in decreased antibody titer due to complex formation invivo and may even induce tolerance. Insufficient antigen results in a deficient response (Odell, 1969). Throughout the assay development GIP was extremely scarce and as a result a controlled study of antibody response versus antigen quantity could not be undertaken. The amount injected was decreased throughout

TABLE ONE

IMMUNIZATION SCHEDULE FOR THE INDUCTION OF ANTIBODIES TO GIP IN RABBITS

DAY	INJECTION	BLEEDING
0	0.5 mg GIP side A with FCA	
22	0.5 mg GIP side A with FCA	12 days post marg, ear
56	0.5 mg GIP side A with FCA	13 days post card, punc,
86	0.5 mg GIP side A with FCA	20 days post C.P.
128	1.0 mg GIP side A with FCA	5 days post C.P.
166	1.0 mg GIP II with FCA	10 days post C.P.
183	1.0 mg GIP II with FCA	ll days post C.P.
226	0.5 mg GIP III with FCA	6 days post C.P.
281	0.5 mg GIP III with FCA	ll days post C.P.
327	0.5 mg GIP III with FCA	12 days post marg, ear
420	0.5 mg GIP III with FCA	10 days post marg. ear
431	0.2 mg GIP III + BSA with FCA	10 days post marg, ear
482	0.2 mg GIP III + BSA with FCA	10 days post marg. ear

TABLE TWO

IMMUNIZATION SCHEDULE FOR THE INDUCTION OF ANTIBODIES TO GIP IN GUINEA

PIGS

DAY	INJÈCTION	BLEEDING
0	2.5 mg GIP side A with FCA	
9	2.5 ng GIP side A with FCA	
20	2.5 mg GIP side A with FCA	
27	2.5 mg GIP side A with FCA	6 days post C.P.
37	1.0 mg GIP II with FCA	7 days post C.P.
44	1.0 mg GIP II with FCA	10 days post C.P.
62	1.0 mg GIP II with FCA	16 days post C.P.
82	1.0 mg GIP II with FCA	3 days post C.P.
114	0.5 mg GIP III with FCA	6 days post C.P.
136	0.5 mg GIP III with FCA	12 days post C.P.
238	0.5 mg GIP III with FCA	ll days post C.P.
269	0.2 mg GIP III + BSA with FCA	11 days post C.P.
304	0.2 mg GIP III + BSA with FCA	17-days post C.P.
305	0.2 mg GIP IIII + BSA with FCA	10 days post C.P.

the schedule. At no time could an insufficient response be attributable to decreased antigen. Thus at the end of the schedule, 0,2 mg of GIP III was found to be effective and was the optimum quantity chosen for GIP immunizations.

The frequency of immunization yielding the largest titer increase varies for each antigen (Odell <u>et al</u>, 1971). Unlike CCK-PZN (Go <u>et al</u>, 1971) frequent boosters were not found to be effective with GIP. Determinations of the frequency of booster injections will have to wait until a larger series of animals have been employed. At present animals are maintaining their level of antiserum three months post booster. Incorporating the above information, two new animals series were begun (Table 3),

II Vehicle of Injection

The GIP was dissolved in one volume of saline which was emulsified with an equal volume of Freunds Complete Adjuvant (EAC). The EAC was employed to reduce the rate of release of antigen thus sustaining the stimulus to antibody production. To obtain and even slower rate of release the saline to FCA ratio was changed to 2/3 (Hurn and Landon, 1971).

III Injection Route and Volume

Originally a single subcutaneous injection of 1.0 ml was given to the rabbits and 0.5 ml to the guinea pigs. The volume was later reduced to two 200 μ l subcutaneous injections at different sites. This reduced the local reaction sores. Although sores are the result of a local inflammation and therefore, indicative of a good response, the sores were severe and threatened the preservation of the animals. With a 200

TABLE THREE

IMMUNIZATION SCHEDULE FOR THE INDUCTION OF ANTIBODIES TO A NEW SERIES OF ANIMALS (7 GUINEA PIGS)

DAY	INJECTION	BLEEDING
0	0.5 mg GIP III in FCA	
29	0.2 mg GIP III + BSA in FCA	
70	0.2 mg GIP III + BSA in FCA	13 days post

IMMUNIZATION SCHEDULE FOR THE INDUCTION OF ANTIBODIES TO GIP IN GUINEA PIGS

DAY	INJECTION	BLEEDING
0	1.0 mg GIP II with FCA	
135	0.5 mg GIP III with FCA	
258	0.2 mg GIP III + BSA with FCA	ll days post C.P.
290	0.2 mg GIP III + BSA with FCA	10 days post C.P.
341	0.2 mg GIP III + BSA with FCA	10 days post C.P.

 μ l volume sores were still observable but did not require treatment,

The order of increasing effectiveness of injection sites is: intravenous, subcutaneous, intraperitoneal, intramuscular, intradermal, intra articular (knee joint) and lymphatic nodes (Hurn and Landon, 1971) Due to the relative ease of subcutaneous injections this route was retained, Utilizing two injection sites in place of a single one, however, better distribution to the lymph nodes of both back and abdomen was obtained.

Another factor favoring the continued use of back and abdominal injection sites is that the sensitized cells formed during the initial immunization tend to localize in lymph nodes near the site of injection. Thus subsequent injections are most effective when given to the same area (Parker, 1971).

IV Conjugation

Following 11 injections even the best animals were producing antiserum of only low titer. Although Yalow and Berson (1970) indicate that a 5,000 molecular weight protein should be antigenic on its own, conjugation to bovine serum albumin (BSA) was tested. Conjugation was obtained by activation with CDI (I-ethyl-3-[3-dimethyl-aminipropyl]-carbodimide), which form CO-NH bonds. CDI is water soluble and has an optimum pH of 5.5 (Parker, 1971).

The method used for conjugation was that of Cataland (personal communication). Forty mg of bovine serum albumin, 1.6 mg of GIP IV and 400 mg of CDI were dissolved separately in, respectively 1.0 ml, 500 μ l and 500 μ l of deionized water. The reagents were then added to the GIP solution and mixed thoroughly. Following incubation for one hour at room temperature, the mixture was dialysed against deionized water

at 4°C. At the end of 24 hours, 10 μ l of the dialysate was removed for a high voltage run at pH 2,1, and the remainder was lyophilized.

Although the optimum pH for CDI activation is 5,5, the procedure indicated utilizes a pH of 7.0. Sufficient time was not available for evaluating the best pH for GIP conjugation. One factor in favor of pH 7.0 is that it would reduce the degree of protein dimerization, which can be a problem with carboxyl group activation (Parker, 1971).

The initial response to conjugation was striking as evaluated by titer increase. Subsequent booster shots yielded one further increase followed by a plateau after the second injection (Figure 1), Since BSA will also be inducing antibody formation, further boosters consisted of unconjugated GIP.

One important finding from the motilin radioimmunoassay was that conjugate was most effective when preceded by unconjugated antigen. Thus new GIP immunization schedules were begun with two GIP III injections prior to the BSA conjugation (Table 3).

V Bleeding and Treatment of Antisera

Initially all bleeding was by cardiac puncture but due to fatalities the rabbits were bled by marginal ear vein. Holding the guinea pigs securely and bleeding them only if the heart was punctured on the first attempt was found to reduce mortality. Serum was obtained by centrifuging the samples for 15 minutes at 4.000 rpm.

Initially the serum was aliquoted into approximately 100 μ l samples which were frozen in dry ice and methyl cellosolve and stored at -20°C, No preservative agents were required. Serum has been used without loss





of affinity for periods as long as six months.

Once the 100 μ l aliquots had been thawed they were realiquoted into 10 μ l quantities, and refrozen. A 10 μ l aliquot was diluted up to 1.0 ml for preparing an individual assay's antibody. The l ml dilution reduced the amount of gamma globulin adsorbed to the tube yielding greater consistancy of final dilutions.

Lyophilization of serum was considered but the literature reports conflicting results as to the stability (Hurn and Landon, 1971). Since there was no problem in stability with freezing, it was considered the method of choice.

LABELLING OF GIP

The original labelling procedure involved the addition of the following reagents in rapid succession to 5 μ g of GIP IV in 100 μ l of 014M PO₄ buffer pH 7.5 (Deftos, personal communication to D.H. Copp):

	REAGENT	VOLUME	AMOUNT
1.	Na I ¹²⁵	5.µÎ	1 mCi
2.	Chloramine-T	20 µl in D.I. water	176 µg
3.	Sodium metabisulphite	20 µl in D.I. water	252 µg
Steps	1-3 are performed with bu	Ibbling to insure proper	mixing.
4.	Quso	suspended in	10 mg
Mix or	n vortex mixer and spin do	own.	
5.	40% acetone/acetic acid 1	.0 m1	

Mix on vortex

6, Dowex

Mix and spin down.

The supernatant was decanted and divided into aliquots for further purification on a column. Aliquots were stored at -20°C until used at which time they were run on a 0.6 x 22 cm G25 Sephadex column.

The Deftos! method yielded adequate labelled GIP for antibody evaluation assays. Modification had to be introduced, however; to yield a 125I-GIP product suitable for standard curves.

A basic modification was the purification of the labelled product on a Sephadex G25 column (0.6 x 22 cm) immediately following iodination. Although primary internal radiation damage (decay of ^{125}I) cannot be avoided, primary external (decaying ^{125}I interacting with other labelled protein molecules) and secondary (secondary ions produced in the liquid medium by gamma rays) can be reduced by eliminating as quickly as possible all unreacted ^{125}I and labelled peptide fragments. Thus the Quso and Dowex were substituted by immediate gel filtration on the Sephadex column.

The fractions were counted on a gamma counter (Model 4227, Nuclear Chicago). Counts/minute were plotted against tube fractions to yield a column profile. A typical profile at this stage is shown in Figure 2. The first peak was the labelled GIP molecules and fragments while the second peak is unreacted 125 I. The active peaks were aliquoted in 100 μ l quantities and frozen. Both procedures reduce secondary radiation damage.

A method had to be developed for testing the column fractions to



Column Fractions 400 Jul. Aliquots



determine which contained the immunoreactive ¹²⁵I-GIP. The laboratory facilities precluded the use of chromatoelectrophoresis (Yelow and Berson, 1960) so a simple incubation in excess antibody was chosen and referred to as a damage assay. Essentially the assay evaluated the fractions on their ability to react with antibody compared with their non specific interaction with the diluting buffer.

To improve peak separation a 33 cm Sephadex G-15 column was substituted for the 22 cm G-25. Figure 3 is the resulting column profile indicating a marked increase in peak separation.

At this time, the eluting agent was the same 0.04 PO_4 buffer used for the assay. ¹²⁵I-GIP storage life was approximately ten days. To increase this time 0.2M acetic acid was substituted for the phosphate buffer (Hunter, 1971). Stored in the acetic acid the shelf life of labelled GIP was increased to three weeks.

Another area of concern was with the use of chloramine-T, a powerful oxidizing agent used to convert iodide to iodine during labelling. Preliminary evaluation with polyacrylamide gel electrophoresis indicated GIP molecules undergo fragmentation with exposure to chloramine-T. Subsequent evaluation indicated that the chloramine-T acted on the trytophan in GIP (Brown, personal communication) and thus the reaction was specific to some extent to peptides containing this amino acid.

This sensitivity of GIP to chloramine-T eliminated the possibility of improving iodination by reducing the iodination volume (Hunter, 1971), since this increased considerably the chloramine-T concentration during iodination. The alternative available for improving ^{125}I -GIP specific activity was to alter the GIP/chloramine-T/Na ^{125}I ratios. Labellings



FIGURE THREE: Column profile following gel filtration separation of iodination products on a 0.6 x 33 cm Sephadex G-15 column.

were evaluated with 5/176/1, 5/50/1, 5/50/2, 5/35/2. 5/25/2, 5/40/2 and 6/40/2 (μ g/ μ g/mCi) ratios. The final ratio of 6/40/2 was found to result in the highest precentage yield of immunoreactive GIP plus the lowest non specific binding of the active peaks (Figure 4). The immunoreactivity and non specific binding were determined by a damage assay and the percentage yield evaluated from the counts per minute of 10 μ l aliquots of column fractions.

Another indication of improved iodination was the increase in usable fractions. Initially two fractions near the peak were immunoreactive. With the new ratio all fractions from the peak and on the descending limb could be utilized in assays. The 6/40/2 ratio was therefore optimum for GIP labelling.

The labelling procedure for GIP IV was then modified to: GIP IV that had been previously divided into 6 μ g aliquots and lyophilized, was used for labelling. A siliconized tube (12 x 75 mm) comtaining 6 μ g of GIP IV was reconstituted by the addition of 100 μ l of 0.4 PO₄ buffer at pH 7.5. The whole labelling procedure was carried out in one tube to prevent adsorption losses. Added in the order listed were:

1) 2 mCi of ${}^{125}I - - - - - - - - 20 \mu l$ 2) 40 µg of chloramine-T - - - - - - 10 µl 15 second time delay.

3) 252 μ g of sodium metabisulphite - - 20 μ l Steps were performed with bubbling to insure proper mixing and reagents added as quickly as possible except where indicated.

After completion of the labelling the 150 μ l reaction volume was added immediately to a Sephadex column (0.6 x 33 cm). The column was



FIGURE FOUR: Column profile following iodination of 6 mcg of GIP with 2 mCi of Na 125 I, oxidized with 40 mcg of chloramine-T.

2]

eluted with 0.2M acetic acid buffer to which had been added 1% human plasma (to reduce peptide adsorption to the column) and 2% Trasylol (25,000 KIU/5 ml, a protease inhibitor). Approximately 40 tubes of 8 drops/tube (approximately 300 ul) were collected and 10 ul aliquots were counted. Once the fractions were counted the active tubes were divided into 50 ul aliquots, frozen in dry ice and methyl cellosolve, and stored at -20^oC. The 10 ul aliquots were used to set up a damage assay to evaluate the labelling.

STANDARDS

Two mg of GIP IV were weighed accurately on a micro balance (Cahn) and dissolved in 33.3 ml of deionized water. Two hundred quantities (6 ug) were aliquoted into vials; which were lyophilized, sealed and stored at -20⁰C until required.

CONDITIONS OF ASSAY

I. Diluent Buffer

The standard diluent buffer used in the assay is 0.04M PO₄ pH 6.5 with 5% Trasylol (S.B.A. Pharmaceutical Ltd.) and 10% outdated human plasma. All buffer variables were tested separately to determine optimum assay conditions. Phosphate buffers at pH's 6.5, 7.5 and 8.5 were tested and pH 6.5 yielded the best standard curve (Figure 5).

Trasylol prevents protease activity thus protecting peptides. Originally 1% (5,000 KIU/ml) was added to the diluent buffer. It was subsequently found that 5% increased the sensitivity of the standard curve.



FIGURE FIVE: Comparison of variations in the pH of the phosphate buffer.

Figure 6 shows standard curves with 0, 2.5, 5 and 10% Trasylol in the diluent buffer. Sensitivity was increased when 5% Trasylol was used and depressed with 10%. The 10% depression must be due to an action of Trasylol on the GIP molecule. The results of this study and others indicated that 5% Trasylol produced the maximum protection without a suppresive action. This was supported also by an assay comparing 0, 2.5 and 5% Trasylol in diluent buffer used for standard curves evaluating known serum samples. Again 5% yielded the best results.

Plasma addition also had to be evaluated. Standard curves were set up to compare the effect of varying concentrations of plasma in the diluent buffer. The 2% and 10% curves are shown in Figure 7 indicating the increased sensitivity of the curve (evaluated by the slope) with 10% human plasma.

The plasma used in the assay is outdated blood bank plasma, Originally it was added without treatment as polypeptides and protease activity should be short lived. Routine screening of the new plasma in the assay indicated readable GIP levels, however, so an overnight charcoal extraction at 4°C (1.0 mg charcoal/300 ml plasma), is now routine procedure.

II Reaction Volume

The standard reaction volume for the assay was 0.5 ml. Larger volumes were tested but no gain in sensitivity was found. This volume was used as it conserved antibody and provided for best counting characteristics. ¹²⁵I decays by very weak gamma radiation. This low energy level can be absorbed by the solution (self-absorption) and thus the larger the counting volume the lower the number of counts obtained. Since both the



FIGURE SIX: A comparison of the effect on standard curve sensitivity of various concentrations of Trasylol in the diluent buffer,



FIGURE SEVEN: A comparison of different plasma concentrations in the diluent buffer on standard curve sensitivity.
precipitate and supernatant are counted, a difference in volume could prejudice the count rate in favor of the smaller volume. An analysis of volume effects on count rate (Figure 8) indicated that within the range of 0.1 to 1.5 ml the counts were within one S.D. Thus to reduce error due to volume differences the precipitate and supernatant should be within the 0.1 to 1.5 ml range.

III Assay Tubes

The reaction volume was incubated in glass culture tubes (Kimble, 10 x 75 mm). Initially the tubes were used without siliconizing. When 100 μ l of antigen was added alone to nonsiliconized tubes, and left to stand over the incubation period only 1/3 of the radiation could be separated out by dioxane. This indicated a very high degree of tube absorption. It was eliminated to a great extent by siliconization. It also allowed for greater ease in decanting the supernatant following centrifugation, thus reducing the amount of supernatant left in the precipitate.

IV Incubation Time

A comparison of different incubation times was undertaken. Equilibrium assays for 24, 48, and 72 hours were compared to disequilibrium assays with a 24 and 48 preincubation without antigen followed by 24, 48 and 72 hour post antigen addition incubation. Figure 9 illustrates the affect of incubation time on the standard curve. The 24 hour pre and 48 hour post disequilibrium and 48 hour equilibrium yielded similar results. Prior to the use of this antibody at the present 1/40,000 dilution, the disequilibrium assay was far superior. Since the 48 hour



FIGURE EIGHT: An analysis of the extent of ¹²⁵I self-absorption in different volumes of diluent buffer.



FIGURE NINE: Evaluation of incubation times to determine which yielded optimum sensitivity of the standard curve.

eliminates one day it was preferred over the disequilibrium assay, and was used in subsequent assays.

V) Assay Procedure

The optimum conditions of the assay for GIP were found to be:

1) The total incubation volume was 0.5 ml. Antibody, ^{125}I -GIP, standards and unknown were diluted with diluent buffer (0.04M PO₄ pH 6.5 with 10% human plasma and 5% Trasylol) to yield the desired concentration in a 100 µl volume. The various components of the assay were added with 100 µl sampler pipets (Oxford Laboratories) and the volume was adjusted to 0.5 ml by addition of diluent buffer via an Oxford Pipetor (Model NO. 13-687-90). The tubes (siliconized, glass culture tubes, Kimble 10:x 75 mm) were then mixed briefly on a Vortex-Genie (Scientific Industries Inc., Model K 550-6) and incubated at 4°C.

2) All standard curves were set up as either disequilibrium assays with a 24 hour preincubation of the antibody and standard or unknown and a 48 hour post incubation following antigen addition, or a 48 hour equilibrium assay.

3) Control mixtures containing no antibody were set up for all samples to permit calculation of incubation damage. All samples were assayed in triplicate.

SEPARATION PROCEDURES

Three basic separation procedures have been employed to separate the GIP-antibody complex from the unreacted GIP; dioxane precipitation, double antibody and peptide adsorption.

Initially dioxane (a diethylene dioxide which denatures and precipitates the antibody-antigen complex) was utilized but it became increasingly apparent that it was not adequate for separation. As the amount of plasma in the assay was increased, the amount of nonspecific precipitation increased to a level incompatible with assay sensitivity. This was also reported by Thomas <u>et al</u> (1969).

A series of assays were then set up to find a better means of separation. Gastrin (a negatively charged polypeptide) had been separated with amberlite, an anion exchange resin (Yalow and Berson, 1970). At a pH of 6.5 GIP is positively charged and so cationic exchange resins were evaluated first. As was feared the antibody was also positive at pH 6.5 and thus CM11 and SE Sephadex both provided inadequate separation. Separation on the basis of size with Sephadex G-50, quso and talc were also inefficient.

An assay was performed to compare various quantities of charcoal and dextran coated charcoal with dioxane separation (Palnieri, Yalow and Berson, 1971). Both separation procedures were superior to dioxane (Figure 10). Charcoal-dextran was superior to charcoal alone due to a lower damage and greater consistancy of results with increasing plasma concentrations. As a result of a series of experiments (Figure 11), 2.5 mg of charcoal with 0.5 mg of dextran per tube was found to be optimum.



FIGURE TEN: A comparison of charcoal-dextran, methods for separating antibody/ antigen complexes from free antigen.



FIGURE ELEVEN: A determination of the charcoal concentration required to yield optimum GIP/GIP antibody separation.

The charcoal-dextran was added to the assay in 200 μ l of diluent buffer (pH 6.5 0.04M PO₄ buffer with 2% human plasma and 1% Trasylol) This was higher than the maximum 20% of assay volume suggested by Painieri <u>et al</u> (1971), but it was found to be the minimum volume required for adequate separation of precipitate and supernatant. The suggestion of various workers (Hunter and Ganguli, 1971) to add 1 ml of diluent buffer just prior to centrifuging, reduced rather than improved the results.

One further modification of the separation procedure was introduced. The charcoal-dextran was made up just prior to use. Arnaud (1971) prepares the mixture the night before and leaves it mixing overnight. This procedure was found to yield improved assay sensitivity.

A comparison was also made with the double antibody method, Utilizing the method and materials of Cataland (personal communication) for double antibody separation, charcoal-dextran separation was found to yield superior separation.

The separation procedure adopted was: the day prior to separation of the assay the charcoal-dextran was prepared. The buffer (0.04M PO₄ buffer pH 6.5 with 2% human plasma and 1% Trasylol) was cooled prior to the addition of the dextran (T 70, Pharmacia Fine Chemicals). Once a dextran solution had been obtained, the charcoal (carbon decolorizing neutral C-170, Fisher) was added. The amounts added yielded a final concentration of 2.5 mg of charcoal and 0.5 mg of dextran for each 200 μ l of buffer. The mixture was left to stir on a mag mixer overnight at 4°C.

In the morning a half hour timing was started with the first 200 μ l

addition of the charcoal-dextran to a tube. After completing the addition of the mixture to the assay, the tubes were vortexed and left to stand at 4°C until the half hour was completed. The tubes were then centrifuged at 2800 rpm for 15 minutes and the supernatant was decanted. The tubes were corked and placed into plastic carrier tubes for counting.

TYPES OF ASSAYS

There were basically four types of assays set up which tested, antigen labelling (damage assay), antibody bleeding (dilution assay), antibody-antigen reaction (sensitivity assay), and GIP levels (standard curves).

I Damage Assay

This assay consisted of two types of tubes. One contained only diluent buffer and antigen. This was a measure of the nonspecific binding of the antigen (adsorption and nonspecific protein interactions). The second tube contained a certain constant dilution of antibody and measured the maximum antibody-antigen binding. This assay was set up after each new labelling to evaluate the quality of the new labelled material.

II Dilution Assay

Damage tubes were set up as correction factors. Instead of varying the antigen, however, different antibody dilutions were included to find the dilution at which 30 to 50% of the antigen was bound. These assays were usually set up for a quick evaluation of a new bleeding (Figure 12).



FIGURE TWELVE: Dilution assay for antibody evaluation.

III Sensitivity Assay

In this assay both antibody and antigen concentration were variable. This tested the affinity of the antibody for antigen and also yielded exact information on optimum antibody and antigen concentration. Figure 13 indicated that the antibody can distinguish between the addition of antigen yielding 40,000, 20,000 and 10,000 cpm. This type of assay was usually used during the development stages of the assay. Once standard curves were available evaluations comparing curve sensitivity were more profitable.

IV Standard Curves

Sensitivity (considered to be either the slope of the standard curve or the lowest standard which can significantly be distinguished from zero competition) was the key to the standard curve. Once all the assay conditions had been standardized this depended primarily on the energy of the antibody/antigen reaction. Once a reasonable titer (final dilution usable in the assay) was reached, increasing the sensitivity required an increase in the affinity of the antibody for the antigen. Changes in antigen labelling as well as antibody avidity affected this reaction.

Once the sensitivity of the assay was established in the low picogram range one inconsistent problem had to be evaluated. In many of the standard curves the low picogram standards yielded higher B/F's than the zero competition (hook effect). Immunologically the hook effect could be explained on the basis of excess antibody. If this were the case, in the upper part of the curve where antigen quantities were lowest; less complex antibody/antigen formations would occur. Charcoal-



FIGURE THIRTEEN: Sensitivity assay indicating antibody affinity for GIP

dextran separation would be less efficient with the smaller complex thus prejudicing the free component and yielding a decreased B/F. To test this hypothesis, standard curves were set up with final dilutions of 1/20,000. 1/30,000 and 1/40,000 (Figure 14). The hook effect disappeared with the 1/40,000 dilution thus supporting the theory.

The elimination of the hook effect at a 1/40,000 final dilution results in a zero competition B/F in the range of 0.7 to 0.5. This is in opposition to the generally held concept that assays should aim for 50% binding or a B/F of 1.0. For GIP a B/F of 0.5 yields a superior standard curve and was used routinely.

Another standard practice is the addition of 10,000 cpm of labelled antigen to assays. This practice does not take into consideration differences in specific activity of the labelled material. 10,000 cpm at could mean the addition of 100 pg or 10 pg depending on the extent of iodine substitution of the peptide. Yalow and Benson (1971a) indicate that one means of improving the sensitivity is to decrease the amount of antigen added. This decrease is limited, however, by the increasing counting errors introduced if adequate total counts are not collected. Also if the antibody is not of sufficient sensitivity to distinguish between 100 pg and 10 pg of antigen, a reduction of labelled antigen to the 10 pg range will not yield an increase in sensitivity. With GIP the counts added to the assay depends on the efficiency of the labelling and the shelf life of the material. With a new labelling a reduction to 4,000 cpm (approximately 10 pg) will improve sensitivity. As the label ages, the labelled fragments increase and 8,000 cpm was the lowest practical level. This occurs at about two and one half weeks.





FIGURE FOURTEEN: Hook effect evaluated at increasing antibody dilutions.

SOURCE OF HORMONE PREPARATIONS

- Synthetic Human Gastrin (SHG-15-Leu): Max-Planck Institute für Eiweiss und Lederforschung (Munich).
- CCK-PZN (250 I.D.U./mg and 1500 I.D.U./mg): G.I.H. Research Laboratories (Stockholm, Sweden).
- Synthetic Secretin: Max-Planck Institute für Eiweiss und Lederforschung (Munich).
- Natural Porcine Secretin: G.I.H. Research Laboratories (Stockholm, Sweden).
- Synthetic Glucagon: Max-Planck Institute für Eiweiss und Lederforschung (Munich).

ANALYSIS OF DATA

There are numerous methods available for calculating and expressing assay results. Methods tested for GIP include: CPM bound, percent bound (using zero competition as 100%), Y (where Y equals the bound of the standard/bound of zero competition) and B/F. Each result was plotted against the log of the standard concentration.

Only the B/F calculation involves counts of both precipitate and supernatant. This double count corrects and/or controls for unequal antigen addition, incomplete separation of precipitate and supernatant and counting errors. The B/F calculation also involves a correction factor D (damage) for non specific binding to glass or plasma proteins. The formula used for B/F calculations was:

 $B/F = \frac{B - (B + F) \times 0.D}{F} \text{ where } D = \frac{B_d}{B_d + F_d} \text{ in damage tubes (Deftos,}$ personal communication to D.H. Copp). All serum samples were corrected

with their separate damage tubes containing the individual serum tested.

To determine the sensitivity of the standard curve; the Student t test and Fisher's modification of the Student t test were used to determine the significance of the difference between the means of two small non correlated samples. The sensitivities indicated during the evaluation of standard curve improvement were determined as 10% of the lowest readable standard concentration. This was used in place of the Fisher t test (Smith, 1964) due to lack of adequate statistics on earlier curves. This approach can be justified, in that case, as the values were used as qualitative comparison of standard curve improvement and not for evaluating test samples.

Inter and intra assay precision determinations were based on the coefficient of variation which, expressed as a percent, is equal to the standard deviation divided by the mean, times 100. A value of 10% or less is considered to be necessary for radioimmunoassays (Loraine and Bell, 1971).

The normal ranges and quality control charts were set up according to the procedures of Hoffman (1971). The limits of the quality control chart were based on +2 S.D. of 20 repeat values of the 10% CCK-PZN standards. Normal ranges were based on the averages-of-normals method. A cummulative distribution was determined followed by a calculation of the cummulative percent. The data was then plotted on normal probabilits graph paper from which the normal range could be determined.

Utilizing the Nadeau and Zahnd (1971) correction for damage and non specific protein interaction, Ba/F ratios were obtained:

Where: Bo = Bound cpm for excess antibody tubes, Bc = Bound cpm for unknown or standard, and Be = Bound cpm for damage tubes.

The specific activity of the labelled GIP was determined. The evaluation was based on the percent of activity corresponding to immunoreactive ¹²⁵I-GIP fractions in a column profile (Figure 4). This is a deviation of the method reported (Hunter, 1971) which estimates the number of ¹²⁵I molecules substituted/polypeptide molecule. Both methods are rough approximations.

Specific Activity of ¹²⁵I used: 14 mCi/mcg

Therefore in 1 mCi there are 72 ng 143 ng of ^{125}I were reacted with 6 mcg of GIP On a molar basis 1.144 nM of ^{125}I (MW ^{125}I is 125) were

reacted with 1.175 nM of GIP (MW is 5105) Determined from the column profile, 78.9% of the ¹²⁵I was reacted with the GIP or 0.902 nM/1.175 nM of GIP

The specific activity of the GIP was therefore:

0.902 nM x 125 (MW) = 112.8 ng of 125I

112.8 ng/6 mcg GIP

or 112.8/72 = 1.57 mCi/6 mcg GIP or 262 µCi/mcg GIP

This calculation was representative of one column profile and had to be estimated for each new labelling.

To determine the quantity of ^{125}I -GIP actually added to the assay the cpm (counts per minute) are converted to dpm (disintegration per minute).

 $dpm = \frac{cpm}{counter counting efficiency \times 100\%}$ $dpm = \frac{10,000 cpm}{68.1\%} \times 100$ = 14,700

 $1 \ \mu \text{Ci} = 2.22 \ \times \ 10^6 \ \text{dps} \ (1 \ \text{Curie} = 3.7 \ \times \ 10^{10} \ \text{dps})$ Therefore 14,700 dpm = $\frac{1.47 \ \times \ 10^4}{2.22 \ \times \ 10^6}$ = 6.62 $\times \ 10^{-3} \ \mu \text{Ci}$ Since there are 262 $\mu \text{Ci/mcg GIP}$ 6.62 $\times \ 10^{-3} \ \mu \text{Ci}$ represents $\frac{6.62 \ \times \ 10^{-3}}{262}$ = 0.025 $\times \ 10^{-3} \ \text{mcg of GIP}$ or 25 pg of $\frac{125}{1-\text{GIP}}$

With the addition of 4,000 cpm to the assay, 4/10 of 25 or 10 pg of 125_{I-GIP} was added.

RESULTS

I Standard Curve Improvements

The series of standard curves in Figures 15 through to 19 depict the increase in sensitivity that occurred as a result of alterations in assay conditions, antibody production and antigen labelling. The immprovements can be accounted for by alterations in all phases of the assay. The two most prominent changes are concerned with antibody and antigen. The use of conjugate for immunizing increased the antibody final dilution from 1/500 to 1/10,000. Changes in assay conditions and labelling resulted in a further increase in usable dilution to 1/40.000.

One interesting observation was the difference in sensitivity between standard curves in Figures 18 and 19. These assays utilized antigen with the same labelling procedure and antibody. They differed only in respect to the new standard aliquots and antibody dilution, which yielded an increase in sensitivity from 40 to 10 pg. This supports the importance of evaluating each new antibody in a sensitivity assay involving standard curves.

II Specificity of Antiserum

Synthetic human gastrin (SHG-15 Leu), CCK-PZN, synthetic and natural porcine secretin, synthetic glucagon and motilin were tested for crossreactivity with the antibody routinely used in the assay (Figure 20). All yielded B/F's within the standard deviation for zero GIP addition, thus indicating no cross-reactivity with the antibody.

III Sensitivity, Accuracy and Precision

Each point and standard deviation of the curve in Figure 21 is









Antibody: 13/6/72 bleeding at a 1/6,000 final dilution Labelling: 50 mcg chloramine-T, 2 mCi ¹²⁵I, 5 mcg GIP Separation: charcoal-dextran (1.25 mg% charcoal) with overnight mixing



FIGURE SEVENTEEN: Diluent Buffer: same

Antibody: 13/6/72 bleeding at a 1/10,000 final dilution Labelling: 40 mcg chloramine-T, 2 mCi ¹²⁵I, 5 mcg GIP Separation: same Sensitivity: 60 pg



Labelling: 40 mcg_chloramine-T, 2 mCi ¹²⁵I, 6 mcg GIP Separation: same

Sensitivity: 40 pg









of each standard.

based on seven determinations. The calculated sensitivity with a P < or > 0.01 is 25 pg by both Fisher modification and the Student t test. This indicated that this GIP assay was capable of determining unknown values down to the 25 pg level. The same curve replotted to include the protein interference correction of Nadeau and Zahnd (1971) is shown in Figure 22.

GIP was added to charcoal treated plasma to yield a concentration of 50 pg/100 μ l. Ten determinations of 100 μ l and 200 μ l samples yielded values of 54 <u>+</u> 4 (S.D.) and 100 <u>+</u> 10 (S.D.) respectively. This was an accuracy of 100%.

The intra assay precision determined from nine repeats of normal pooled serum samples was 3.1%. An inter assay evaluation of precision based on ten repeat determinations of a 10% CCK-PZN standard was 11.9%.

IV Plasma Dilution Assay

A serum with high GIP content was assayed at various dilutions to determine if the GIP in serum reacted the same as the GIP IV utilized for standard curves. Figure 23 indicates that the concentration of GIP in the plasma is dependent on the dilution at which the plasma is assayed. The values fitted to a standard curve, support the conclusion that the GIP in plasma is not identical in antibody reactivity to standard GIP (Figure 24). This data plotted as pg GIP for both standard and plasma on semi log paper indicates independence of plasma analysis to dilution. This supports Yalow and Berson (1971b) contention that parallelism of the log dose response curve is not as sensitive a measure of identical reactivity as the linear plot (Figure 23).



FIGURE TWENTY TWO: A replot of the data of Figure twenty one according to the method of Nadeau and Zahnd which corrects for protein interference.



FIGURE TWENTY THREE: Linear plot of the effect of plasma dilution on measured GIP content of a single serum sample dilution.



FIGURE TWENTY FOUR: . B/F of serum dilution fitted to a GIP standard curve to

test for dilution effect

V Ten Percent CCK-PZN Assay

Due to the origin of GIP from the side fraction of CCK-PZN, 10% CCK-PZN was evaluated for GIP content. It was also established as a quality control for individual assays. The parallel nature of the GIP IV and 10% CCK-PZN curves was indicated in Figure 25.

Ten separate assay evaluations for GIP content of a 10% CCK-PZN preparation (batch number 26931) showed it to contain 107 ± 5.9 pg of GIP/1,000 pg. Lyophilized samples of this batch were prepared for use as a quality control for individual assays. The importance of retesting new batches was indicated by the finding that batch number 297271 contained only 25 pg/1,000 pg of 10% CCK-PZN.

On the basis of the ten assay values for 10% CCK-PZN (batch number 26931) a quality control chart was set up (Figure 26). The confidence limits are \pm 2 S.D. of the mean value for 10% CCK-PZN reported as pg of GIP/ng of 10% CCK-PZN. The 10% CCK-PZN values obtained from subsequent assays are indicated in Figure 26. These values would be placed on the chart immediately prior to reporting assay unknown results. Based on this control, assay results from assay 4,5,10,13,14 could not be reported as the 10% CCK-PZN values do not fall within the confidence limits set.

Another value of the chart is in indicating consistent assay changes. Assays 2, 3, 4 and 5 show a consistent increase in control value. A reevaluation of assay constituents at this point would yield a decreasing potency of antibody or a decrease in quality of ¹²⁵I-GIP.

VI Normal Fasting GIP Serum Values and Ranges

The normal fasting GIP level based on 45 determinations was



FIGURE THWENTY FIVE: A comparison of the reactivity of GIP standards and 10% CCK-PZN with GIP antibody.



FIGURE TWENTY SIX: A quality control chart based on ten determinations of GIP content of a 10% CCK-PZN standard with 10% CCK-PZN standard V

values of subsequent assay plotted.

 163 ± 17 (mean + S.E.). The normal level had been decreasing since November 1972 at which time it was 636 ± 31 (S.E.). This is consistent with other radioimmunoassays (Yalow and Berson, 1972) and accompanies improved assay sensitivity.

The normal fasting range for human GIP was established by the method of Hoffman (1971) as indicated in Table 4. As determined from the normal probability graph (Figure 27) the normal range was 0-300 pg/ml.

VII Post Prandial Release of GIP

Fasting serum levels were obtained from six subjects. Following a meal consisting of 4 oz. of orange juice, 9 oz. of whole milk, bacon, two eggs with hash-brown potatoes, toast with conserves, and coffee or tea; serum samples were taken every one half hour for a four hour period. Figure 28 shows the average serum GIP levels of the six sibjects \pm the S.E. Table 5 indicates the responses of the individual subjects.

VIII GIP Serum Levels With Oral Glucose Tolerance Tests

Serum samples were obtained following the oral administration of 75 mg of glucose to fasting normals. The GIP responses of five subjects are depicted in Table 6 and Figure 29.

GIP levels following an oral glucose tolerance test in one individual with insulin dependent diabetes is also indicated in Figure 29. TABLE FOUR

SCALE	NO. OF TESTS	CUM SUM	CUM %
25	2	2	4.4
50	2	4	8.9
75	3	7	15.6
100	7	14	31.1
125	4	18	40.0
150	5	23	51.1
175	1	24	53.3
200	4	28	62.2
225	4	32	71.1
250	5	37	82.2
275	4	41	91.1
300	4	45	100.0

.



FIGURE TWENTY SEVEN: Probability graph plot of the frequency distribution of GIP normal fasting values used to determine the GIP normal range.


FIGURE TWENTY EIGHT: Post prandial release of GIP.

TABLE FIVE

GIP SERUM LEVELS IN SIX INDIVIDUALS FOLLOWING BREAKFAST

TIME IN MINUTES	FASTING	45	75	105	135	165	195	225	255	
SUBJECTS										
01	250	1050	1500	1250	1650	1850	1250	1600	775	
02	150	1550	1515	1350	1400	1100	900	1150	1100	
03	90	1375	1325	1600	1600	1550	1075	875 [·]	875	
04	215	1325	1500	950	1260	1350	1350	500	525	
05	180	575	650	600	950	850	650	625	600	
06	73	1250	1325	1250	875 [·]	825	1125	550	725	
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MEAN	142	1215	1263	1150	1217	1135	1020	740	765	
S.D.	59	374	354	385	304	315	261	270	229	
S.E.	26	167	158	172	136	140	117	121	102	
	168	1382	1421	978	1353	1275	1137	861	867	
	116	1048	1105	1322	1081	995	903	619	663	

GIP SERUM LEVELS DURING ORAL GLUCOSE TOLERANCE TESTS												
SUBJECT	FASTING LEVEL	5 MIN.	15 MIN.	30 MIN.	60 MIN.	120 MIN.	130 MIN.					
0.1	230	350	1500	1250	500	400	380					
0.2	260	640	1340	1650	1230	1000	620					
0.3	210	580	1760	1260	1160	660	520					
0.4	280	375	700	750	750	350	300					
0.5	350	450	750	1200	650	720	420					
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MEAN	266	478	1210	1222	858	626	448					
S.D.	54	128	467	319	321	263	124					
S.E.	24	57	209	142	143	117	55					

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TABLE SIX

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FIGURE TWENTY NINE: Serum GIP levels during oral glucose tolerance tests.

DISCUSSION

Gastric inhibitory polypeptide (GIP) was isolated from a side fraction of CCK-PZN (Brown, Pederson, Jorpes and Mutt, 1969), purified (Brown, Mutt and Pederson, 1970), and sequenced (Brown, 1971; Brown and Dryburgh, 1971). The pure material was found to inhibit gastrin, pentagastrin, histamine and insulin hypoglycemia-induced acid and pepsin secretion (Pederson and Brwon, 1972). The inhibition of histamine stimulated acid secretion is particularly relevant. GIP is the only purified polypeptide shown to have this effect. The inhibition is similar in magnitude to that obtained with fat in the duodenum, This is an important requirement for the enterogastrone originally defined by Kosaka and Lim (1930a). Purified GIP was also found by Barbezat and Grossman (1971) to be a potent stimulant of jejunal and ileal secretion. This property could be relevant to the eteology of increased small bowel secretion found with vibrio cholera and pancreatic cholera.

In order to further evaluate GIP's physiological and pathological roles, a means had to be developed for measuring circulating GIP serum levels. Assay demands were based on known properties of other polypeptide hormones. Low fasting levels required a very sensitive assay. Large increases in concentration of relatively short duration must be followed accurately demanding a high degree of precision. The structural similarities between GIP and other gastrointestinal polypeptides necessitates evaluation of the degree of cross reactivity between these peptides and the GIP antiserum. Gastrin radioimmunoassays have satisfied all the above requirements and thus radioimmunoassay was the logical choice for GIP.

Radioimmunoassays are based upon the competitive inhibition of an antibody/labelled antigen reaction by unlabelled antigen. A dose response curve is obtained by challenging the labelled antigen-antibody complex with known amounts of unlabelled antigen. This standard curve is used as a reference for converting the displacement caused by unknown quantities of peptide in the serum, to absolute quantities.

The assay requirements include an antibody that is specific for the peptide of interest, a pure labelled peptide, and a standard. It is not necessary that standard and labelled peptide exhibit identical reactivity with the antibody, but it is required that standard peptide and unknown peptide show identical immunoreactivity (Yalow and Berson, 1972). Since the immune reaction will be affected by changes in the incubation environment, eg. pH, protein concentration and salt concentration, these must be constant for both standards and unknown (Yalow and Berson, 1971b).

The theoretical aspects of radioimmunoassays have been presented at numerous symposia (Yalow and Berson, 1968c; Yalow and Berson, 1971a), Basically $B/F = K(Ab^{\circ} - b[H])$ where K is the equilibrium constant for antibody/antigen complex formation, Ab° is the molar concentration of combining sites on the antibody molecules at the dilution employed in the assay and b[H] is the fraction of total hormone concentration bound.

From this relationship certain basic radioimmunoassay properties can be elucidated:

- 1) As b[H] increases the B/F decreases,
- Since b[H] cannot exceed 1 or 100% binding, the detection of small changes in b[H] requires that Ab° is not too large.
- 3) b[H] consists of both that labelled and unlabelled [H]. If a large amount of labelled [H] is added, it will limit the amount of unlabel-

led [H] detectable.

4) K is a measure of the energy of interaction of the antibody/antigen reaction and is a property of the antibody. As K increases, Ab° and b[H] can be decreased thus increasing the sensitivity of the assay.

An assay can be utilized for physiological and pathophysiological investigations following:

1) production of adequate antibody, labelled peptide and standard

2) optimizing Ab° and labelled [H]

- 3) Standardizing conditions of incubation
- 4) Quantitative and qualitative evaluation of the assay

Production of antibody with a high affinity (avidity of antibody for antigen) and specificity was required for the GIP assay. Immunization of both guinea pigs and rabbits with GIP emulsified in Freunds Complete Adjuvant, yielded antisera of low affinity and titer (final dilution usable in the assay). Conjugation to bovine serum albumin resulted in a 5,000% increase in titer (Figure 1). It can no longer be assumed that below a molecular weight of 5,000, proteins are not antigenic (Yalow and Berson, 1971a). This does not mean, however, that conjugation will not be required. The increase in response to GIP following conjugation indicated that it was necessary for this polypeptide although its molecular weight is 5105.

Polypeptide antigenicity is predominantly a function of species variation in structure (Odell <u>et al</u>, 1971). It follows that the larger the polypeptide the greater the likelihood of alterations in amino acid composition amongst species. The GIP used for immunization was of porcine origin. The lack of production of adequate antibody in rabbits

as compared to guinea pigs could be due to a greater similarity between porcine and rabbit GIP, than between porcine and quinea pig GIP.

Limited quantities of GIP excluded the use of a large series of animals for antibody response evaluation. Thus age and breeding could also have limited the GIP antisera production. If the animals were not immunologically competent (5.5 to 8.5 months for rabbits and 55 to 70 days for guinea pigs), injection could result in tolerance (Hurn and Landon, 1971). Also since the immune response is inherited, random breeding is essential when utilizing a small number of animals to increase the probability of obtaining a good antisera (Yalow and Berson, 1971a). With these limitations in mind rabbits cannot be ruled out as unsuitable for GIP antisera production.

There appears to be confusion in the field as to whether pure or impure preparations of substances should be used for immunization. Both were used for GIP antibody production. Yalow and Berson (1971a) indicated that crude preparations may yield higher affinity antisera than pure. This was based upon practical experience. Parker (1971) suggests that as long as pure peptide is available for labelling, an impure material can be used for immunizing. To improve sensitivity of the assay, however, a pure material should be used. This is explained on the basis of heterogeneity of antibody response. All antibody in a single antiserum will not have equal affinity for the antigen. As the amount of antibody of low affinity increases the concentration of antibody required in the assay also increases, thus resulting in a decrease in sensitivity. Crude preparations tend to increase the production of low affinity antibody.

The effect of the dose of antigen used for immunization can be explained on the basis of high and low affinity antibody production. Parker indicated that a decrease in antigen concentration stimulated production of high affinity antibody while the converse was true for low affinity antibody. This would explain an increase in titer seen 20 days post immunization; since at that time the concentration in the animal would be much lower than immediately following immunization.

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The quality of the labelling is important in determining the specificity, sensitivity and precision of a radioimmunoassay (Hunter, 1969). A high concentration of iodinated molecular fragments and contaminants will increase the amount of labelled material that must be added to an assay, assuming that these do not interact with the antibody. If they do contain the immunologically competent part of the molecule they will combine with the antibody but at a different K value than the intact polypeptide. Either condition would yield decreased assay sensitivity.

GIP was a difficult peptide to label due to its sensitivity to chloramine-T. Fragments produced were a particular problem during the early developmental stages. Recent evidence (Greenwood, 1971) indicated that the addition of plasma in the eluting buffer used during the iodination purification, selectively adsorbs small peptide fragments. This is one variable that was not investigated during development. But if this were the case, then an increase in the human plasma concentration in the eluting buffer should increase the purity of the labelled product. Greenwood also suggests that saturation should be attempted with different proteins such as human serum albumin, bovine serum

albumin and globulins, to determine which is the most efficient at removing labelled fragments.

Early in the development of the assay it was found necessary to perform labelling in a siliconized tube to prevent adsorption of material to the glass (Yalow and Berson, 1968a). Iodinated fragments and unreacted ¹²⁵I are not adsorbed while intact hormone is. This would result in a reduced concentration of labelled peptide in solution, making purification more difficult.

The degree of ¹²⁵I substitution in a molecule must be evaluated by comparing the improved assay sensitivity with the increased probability of decay catastrophe found following high specific activity iodinations. Decay catastrophe is the radioactive production of labelled fragments following decay of one of the substituted iodines (in a di- or triiodinated preparation). GIP has two tyrosine molecules and thus is a possible candidate for decay catastrophe. At the present ¹²⁵I/GIP ratio of 1:3, double iodination should be reduced to a minimum (Yalow and Berson, 1968a).

The diluent buffer provides the environment for the antibody/antigen reaction and thus is extremely important. Although typically radioimmunoassays seem to be run at a pH of 7 to 8, the GIP assay was most sensitive at a pH of 6.5. This pH was first utilized because GIP's isoelectric point is approximately 7.5 and it is an anionic protein.

More difficult to determine than pH or ionic strength is the concentration of plasma and the amount of protection from the plasma required by the polypeptide. GIP was found to be very sensitive to protease activity, a property shared with glucagon (Assan, et al, 1971) and parathyroid hormone (Arnaud <u>et al</u>, 1971) but not with gastrin (Yalow and Berson, 1971). Yalow and Berson (1971b) indicate various substances used to protect against protease activity. Trasylol was chosen for GIP. Trasylol is a competitive inhibitor of protease activation and a noncompetitive inhibitor of protease activity (Dubber <u>et al</u>, 1968).

Below a Trasylol concentration of 250 KIU/ml of diluent buffer, GIP was not protected adequately from protease activity. This was reflected in a decrease in sensitivity and an increase in the damage component of the assay (tubes containing only GIP and diluent buffer). Above that concentration there was a suppression of the standard curve (Figure 6). Although other individuals use Trasylol at double the concentration selected for GIP (Assan <u>et al</u>, 1971 and Arnaud <u>et al</u>, 1971), no reference in the literature could be found of a suppressive effect of Trasylol at high concentrations. Since the antibody/antigen reaction is not affected by Trasylol (Forster, 1969), the suppression must be due to a direct action on the GIP molecule.

Plasma added to diluent buffer serves two purposes; to eliminate adsorption to glass and to maintain a constant assay environment. Ideally the only difference between the standard and the unknown, should be the concentration of the actual peptide being measured. In practice, however, the hormone is usually assayed in samples containing serum proteins which may introduce an effect of non-specific competitive binding. Many methods have been proposed to correct for this interference at the assay and/or analysis stage. Yalow and Berson (1971b) stress the importance of evaluating plasma dilutions thus providing an index of the susceptibility of an assay to protein interference. Corrections at the

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assay level are numerous. Arnaud <u>et al</u>, (1971) routinely analyze their samples at three dilutions, discarding samples that failed to duplicate within preestablished limits. They also equalized the concentration of plasma in the standard with that in the unknown tubes by the addition of hormone devoid plasma. Protein interference can also be reduced by adsorbing the antibody onto the incubate tube (Thorell, 1968). Nadeau and Zahnd (1971) correct for interference by incubating each different plasma evaluated with excess antibody. They reason that non specific competitive binding will depend on the amount of protein present. Thus corrections based on incubating antigen and unknown alone will yield falsely high values when compared with the true assay situation in which antibody is also present. The use of an excess antibody tube in conjunction with the damage tube allows for a more complete evaluation of the extent of non specific interference.

The GIP assay was developed to minimize the protein interference and also to correct for any interference that was still present. Ten percent human plasma was added to the buffer to minimize the plasma concentration difference between unknowns and standards. The plasma had undergone prior charcoal treatment to remove any polypeptides. It was outdated plasma from the blood bank and thus should be devoid of protease activity. Evaluation of protein interference was originally via the damage tube, but an excess antibody correction was incorporated.

Since the conditions can never be totally equalized throughout, the GIP assay was set up to incorporate reasonable control for protein interference. The method of Arnaud et al, (1971), as previously mentioned, is certainly closest to the ideal but it would be totally impractical for evaluating the large number of samples required during feeding studies involving numerous sequential determinations.

Although an incubation time was established as optimum for the assay this was based on one particular antibody. The affinity or association constant (K) is a function of the antibody and thus with changes in antibody affinity there will be consequent changes in optimum incubation times. Each new antiserum must then require reevaluation. Incubations were carried out at 4°C because K is greatest at the lower temperature.

GIP antibody is used at high dilutions which necessitate longer incubation times. This approach is supported on theoretical grounds by Parker (1971). He states that during long incubations the antigen will redistribute itself to high affinity antibody. This reduces the amount of low affinity antibody/antigen complexes which more readily disassociate during separation. A decrease in disassociation will yield an increase in assay sensitivity.

The antibody/antigen complexes do not spontaneously precipitate because of the small quantities of antibody and antigen employed in radioimmunoassays. As a result some means must be adopted for precipitating either the free antigen or antibody/antigen complexes. Daughaday and Jacobs (1971) described six methods of separating antibody/ antigen complexes from free antigen:

1) electrophoretic and chromatoelectrophoretic methods

2) gel filtration

3) nonspecific precipitation of hormone-protein complexes

4) immunoprecipitation of soluble hormone-protein complexes

- 5) solid-phase adsorption of hormone
- 6) solid-phase adsorption of antibody

The choice of method for a particular assay must involve consideration of completeness of separation, reproducibility, flexibility of conditions and general convenience with respect to time and effort. Methods 2 to 5 inclusive were evaluated for use with the GIP assay. Dioxane, double antibody and charcoal-dextran were found to provide a degree of separation. Charcoal-dextran was the method of choice. The concentration of charcoal-dextran presently employed was found to yield optimum exclusion of antibody/antigen complex and relative lack of interference from nonspecific proteins in the incubation volume (Figure 10 and 11),

Coating the charcoal with dextran was initially developed by Herbert (1968) to improve exclusion of the antibody/antigen complex. Palmieri, Yalow and Berson (1971) and Raptis (1971) found no increase in effectiveness of separation with charcoal-dextran as opposed to charcoal alone. GIP separation was improved with dextran coating, supporting the findings of Herbert (Lau, Gottlie and Herbert, 1966). The use of coating is likely dependent upon the particular peptide assay system, Herbert (1968) indicates that the charcoal-dextran separation is <u>`</u>• almost instantaneous. The half hour time delay between addition and centrifugation in the GIP assay allows for some charcoal precipitation prior to centrifugation. The major reason for this incubation period is to allow for standardization of separation time. If centrifugation was accomplished immediately following addition, the time delay would vary with the size of the-assay.

The charcoal-dextran technique was a rapid and simple method of

separation, both important considerations for a routine clinical test. It was also considerably more economical than the double antibody method, and as compared to methods one and six, required very basic equipment. Charcoal-dextran had therefore satisfied the criteria previously mentioned by Yalow and Berson for separation in radioimmunoassays. It provided the most complete separation (Figure 10), was reproducible, was very convenient and reasonably flexible.

Once the techniques had been established, the assay had to be evaluated quantitatively and qualitatively. Quantitation involves determinations of specificity, sensitivity, accuracy and precision. A qualitative appraisal comprises determination of plasma effect by measuring samples at different dilutions (Yalow and Berson, 1971b).

The extreme specificity of the antibody was determined by comparing a GIP standard curve to the curves obtained with gastrin, CCK-PZN, secretin, glucagon and motilin. At levels up to 10 ng there was no concross reactivity of any of the peptides with the GIP antibody (Figure 20). As mentioned earlier this was extremely important in view of the structural similarities between GIP, glucagon and secretin. Equally important is the origin of GIP, glucagon, secretin, CCK-PZN and motilin from the endodermal tissues of the fore- and mid-gut regions. The likelihood of all these substances being released simultaneously is high and thus many sera measured for GIP content would have a high gut peptide content. The specificity exhibited by the antibody provides assurance that the competitive binding exhibited by unknown samples is, in fact, a measure of GIP concentration.

The sensitivity as measured by the lowest level of standard signif-

icantly different from zero competition was 25 pg. The particular curve (Figure 22) was not as sensitive (evaluated visually as the slope of the curve) as compared with the results presented in Figure 19. This was due to the increasing age of the labelled antigen which was nearing the three week expiry date. Twenty five picograms was thus the minimum sensitivity expected with the GIP assay and therefore 25 pg/100 μ l or 250 pg/ml was the minimum routinely readable serum level.

Replotting the data by the method of Nadeau and Zahnd (1971) vielded the curve indicated in Figure 22. This was preliminary data as the method requires a 1/200 dilution of antibody to be tested with each separate plasma. Since normal dilution is 1/40,000, a large stock of antibody would be required before this could be evaluated with a large number of samples. A comparison of the sensitivities of curves in Figures 21 and 22 based on slope would indicate a marked improvement in Figure 22 due to the protein interference correction. Feldman and Rodbard (1971) have indicated an additional criteria of sensitivity. The slope should be steep in the area of zero competition and yield a high intercept. Comparing Figures 21 and 22 again indicates a much steeper slope in Figure 22 thus yielding larger changes between different ordinate values. This would allow for easier differentiation between unknown values within this area of the curve. Neither curve satisfies the criteria for a high intercept. Figure 19, which represents data from an assay utilizing newer antigen, comes much closer to satisfying this criteria,

Improvements in standard curve sensitivity represented in Figures 15 to 19 are due mainly to improvements in labelling of antigen and antibody production. As the labelling procedure was standardized,

further assay sensitivity increase would require improved antibody affinity. This could be achieved by immunizing a new series of animals as indicated previously.

Accuracy as determined by measuring plasma to which a known amount of GIP had been added was 100%. This indicates that the assay was capable of measuring all the free GIP present in serum unknowns.

Precision of intra assay measurements were all under the 10% maximum acceptable limit, and usually under 5%. Inter assay precision as evaluated by repeat 10% CCK-PZN measurements was 11.9%. These determinations were made during stages of assay improvement, and thus deviated more than would be expected with assay stability. The precision of the assay was still considered adequate for unknown evaluations.

Qualitative validation of the assay was assessed by the method of Yalow and Berson (1971b). The results depicted in Figures 23 and 24 did not indicate identical reactivity of the GIP standard and unknown. Yalow and Berson mention two possible reasons for this: differences in hormonal cross-reactivity factors yield plasma dilution values that parallel the standard curve. This indicates decreased competitive binding similar to the curve obtained with 10% CCK (Figure 25). Since the GIP dilutions resulted in inconsistant reactivity (a decrease with lower dilutions) it must have been due to non specific binding effects.

Of the non specific factors indicated by Yalow and Berson (L971b), ionic environment, pH, presence of heparin and differential effect of protein; only the effect of serum proteins could account for an apparent decrease in concentration with decreased dilution. This decrease is measured as an increase in the B/F ratio; ie, Serum proteins (albumin

and gamma globulin) will competitively inhibit the antibody/antigen reaction (Hunter,1968); yielding a decrease in antibody/antigen formation. There will be simultaneous formation of albumin/antigen complexes that will precipitate with the antibody yielding a false B elevation. This can be corrected by an excess antibody evaluation (Yalow and Berson, 1971a). Alternatively, the serum to be investigated could be rendered peptidefree by extraction with charcoal and added to the standard curve. This is impractical in routine assays.

Figure 25 indicates another qualitative validation of the assay apart from serum dilution. The parallel nature of the curve, implies competitive binding of the antibody with GIP in both standards and 10% CCK-PZN. This validates the ability of the assay to measure the GIP content of tissue samples.

All the quantitative evaluations of the GIP radiommunoassay supported its use for serum analysis. The qualitative plasma dilution did indicate a problem with competitive protein binding, however, methods for controlling and/or correcting it were suggested. More important was the fact that the test was done only once. The results should be repeated on a greater number of samples before definite conclusions are made. Prior to further evaluations the unknown could be analyzed routinely at multiple dilutions.

Once these parameters had been determined and the assay accepted as suitable for unknown measurements, a quality control procedure had to be established. A means had to be developed for evaluating each new assay prior to reporting results. To accomplish this 1 ng samples of a

single batch of 10% CCK-PZN were assayed in a series of experiments. The mean and standard deviation of the repeat values were calculated and a chart was set up indicating the mean and establishing boundary lines at \pm 2 S.D. Each subsequent assay included a 10% CCK-PZN measurement which was plotted on the graph. Any values falling outside the standard deviation indicated a possible assay failure. If two successive assays yielded 10% CCK-PZN values outside of the boundary lines the unknowns in those assays must be reevaluated prior to reporting. Two successive assays are necessary because 1 in 20 assays would be expected to yield "out of bounds" 10% CCK-PZN values due to chance alone $(\pm 2$ S.D. yields a 95% confidence level).

The chart can supply information on shifts in procedures as well as information on individual assays. A shift occurred between assays 1 to 5, and is indicated by the gradual increase in 10% CCK-PZN values during this period. Shifts can be due to many causes. Particularly suspect with radioimmunoassays would be antibody affinity change or labelling efficiency change. Once a shift like this was noted the technique must be reevaluated until the particular cause was discovered. Shifts were difficult to determine if the results were not available for visual inspection. The values indicated on the chart in Figure 26 were obtained during a period of assay improvements and thus the results were not as consistent as one would expect from a stable assay.

Once the assay is fully established further controls with control charts should be incorporated in each assay. Included among these should be two patients pooled serum; one a pooled normal and the other a pooled abnormally high. These three samples would evaluate the assays' accur-

acy (10% CCK-PZN), precision (10% CCK-PZN and normal patient pool) and ability to determine abnormal sera (abnormal high pool). Thus the 10% CCK-PZN was an initiation of an established quality control program for the assay.

The normal range was evaluated by the method of Hoffman (1971), The basis of the calculations involves the assumption that a large number of determinations of serum GIP levels of different individuals should yield a normal distribution. Thus a plot of the percentage of values falling within a frequency distribution should yield a sigmoid curve. The probability graph paper is a log transformation to convert the sigmoid curve into a straight line. If a straight line is obtained by plotting all the values, with the exclusion of the obviously abnormal, the data can be considered normally distributed and the normal range can be read directly from the probability plot as ± 2 S,D,. The estimate was based on 45 determinations of the serum fasting level of GIP from patients without gastro-intestinal disorders. A straight line was obtained and thus the normal range was determined to be ± 2 S,D,.

The range should be re-evaluated on a larger sampling (150 samples) when the data is available. This is particularly important for GIP as the normal value decreased considerably from November to February. A further reduction would be expected from the observations with the gastrin radioimmunoassay (Yalow and Berson, 1972), as indicated earlier. This would follow increased assay sensitivity resulting from improvements in any of the assay stages, particularly increased antibody affinity.

Normal ranges are traditionally taken as \pm 2 S.D. because the in-

dividual determinations are affected by a large number of extraneous factors. Included among these factors are: sex, age, exercise, diet, emotion, posture, tourniquet techniques, drugs and fluid intake (Hoffman, 1971). Age has been found to be a particularly important source of variation with other gastro-intestinal polypeptides, yielding increased fasting levels with age (Yalow and Berson, 1972). With these limitations in mind the normal range is necessary for clinical evaluation to provide guidelines for distinguishing between normal and abnormal results.

Following determinations of the normal range and establishment of at least one control for evaluating individual assays, preliminary investigations were undertaken to evaluate GIP serum levels during feeding studies, glucose tolerance tests and in certain pathological states. None of the studies attempted to accurately quantitate responses as the number of subjects included was too small. Results and conclusions were therefore of a preliminary nature. The intent was one of sampling the areas available for study following development of the assay.

The feeding study provided evidence of GIP release following food intake. No specific secretagogues could be determined from the mixed meal ingested. It does support the position of GIP as a human gastrointestinal peptide, released into the blood stream by a feeding stimulus. This is part proof of GIP's hormonal status. Final hormonal proof would require measurement of a concomittant decrease in acid and pepsin secretion following increased serum levels of GIP.

A study of GIP release following ingestion of a hypertonic solution was provided by the glucose tolerance tests (Figure 29). Not only did the results reveal the stimulatory effect of hypertonic solutions with

respect to GIP but also the possible insulin secretagogue activity of GIP itself. Involvement of a gastro-intestinal polypeptide in insulin release has long been suspected due to the increased insulin response with oral as opposed to IV glucose administration (McIntyre <u>et al</u>, 1965). Attempts to determine which polypeptide was responsible have to date been inconclusive (Rehfeld, 1972, and Yalow and Berson, 1972). None of the gastro-intestinal polypeptides (gastrin, CCK-PZN, secretin and gut glucagon) tested have shown increased insulin secretion in either invivo or invitro studies.

Dupre (personal communication to J. C. Brown) studied the effect of pure CCK-PZN, 10% CCK-PZN and GIP on the invitro release of insulin from pancreatic slices. Pure CCK-PZN did not stimulate insulin release while 10% CCK-PZN did. Pure GIP was found to be a potent stimulator of insulin release. The assumption from this was, of course, that the action of 10% CCK-PZN was due to GIP contamination. Following these results invivo studies were initiated. Subjects were glucose loaded to yield a blood level of 160 mg% at which time serum insulin levels were 40-50 uU/ml. An intravenous infusion of 1.0 mcg of GIP/min was continued for 30 minutes and yielded insulin levels up to 100 uU/ml.

With the development of the radioimmunoassay it was possible to measure GIP levels following oral glucose tolerance tests (Figure 29). The data indicated a GIP response similar in temporal sequence to the blood glucose increase. Cataland (personal communication) has measured serum GIP, glucose and insulin levels following glucose ingestion. The peak GIP response occurred at 15 to 30 minutes (Figure 29) while the insulin and glucose peaks were at 30 minutes. Also included in Figure 29

is a total lack of GIP response to oral glucose in a patient who was insulin dependent. All the data supporting GIP as an insulin secretagogue were of a preliminary nature. Final support awaits further investigation but the positive findings with both invivo and invitro bases tests were certainly more promising than that found with the other gastro-intestinal polypeptides.

Cellular localization of GIP was recently reported using the antibody supplied from this laboratory and presently used in the radioimmunoassay (Polak <u>et al</u>, 1973). The cells were of the APUD (gastrointestinal endocrine polypeptide) type and found predominantly in the midzone of the duodenum, and to a lesser extent in the jejunum. The areas of high GIP immunofluorescence corresponded best with the DI endocrine cell of the small bowel. The areas of GIP immunofluorescence were distinct from the S cell (secretin) and the L cell (enteroglucagon) and considerably more abundant than the S cell.

Considering GIP's known role as an exogenous inhibitor of acid secretion it was logical for GIP to be implicated in disorders with high and low acid secretion. Duodenal ulcers are usually associated with high acid secretion. Polak <u>et al</u> (1973) were unable to identify GIP cells in patients with duodenal ulceration. Decreased or absent gastric acidity is a symptom of pernicious anemia and gastric carcinoma. Biopsies of small bowel from patients with gastric carcinoma were examined by Polak <u>et al</u> (1973) and found to have increased GIP immunofluorescence. Preliminary data on two pernicious anemia cases yielded serum GIP levels four times greater than normal fasting levels. These data were extremely tentative due to the small number of cases examined

but they did support the possibility that GIP may be involved in pathological conditions associated with alterations in gastric acidity. Cause and effect will have to be investigated thoroughly but it is reasonable to consider that GIP may be valuable as a therapeutic agent in cases of duodenal ulceration to reduce gastric acidity.

GIP's role as a stimulator of intestinal secretion has implicated it in disorders associated with increased intestinal secretion. Two important examples are vibrio cholera and pancreatic cholera. Both conditions are associated with profuse watery diarrhea yielding decreased serum potassium and acid base inbalances. Pancreatic cholera is due to an endocrine secreting pancreatic tumor that has been identified by immunofluorescence to contain GIP or a GIP like material (Elias <u>et al</u>, 1972). This condition is usually associated with achlorhydria and alterations in the glucose tolerance curve. Both of these symptoms could be explained on the basis of known GIP actions mentioned previously. Preliminary attempts to measure serum GIP levels in these patients, however, have been unsuccessful.

Following the development of the radioimmunoassay for GIP, preliminary studies support GIP's position as a human circulating polypeptide produced primarily in the APUD cells of the duodenum. GIP serum levels increase in response to a feeding stimulus, indicating it may be functionally important in the feeding response. Pathological conditions in which GIP may be involved include: duodenal ulceration, pernicious anemia, vibrio and pancreatic cholera. Much work will be required in all of these areas in order to establish GIP's actions: physiological and pathological.

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