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IMMUNOLOGICAL TECHNIQUES IN THE INVESTIGATION OF THE PHYSIOLOGICAL FUNCTIONS OF GASTRIC INHIBITORY POLYPEPTIDE AND MOTILIN

by

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ABSTRACT

A radioimmunoassay was developed, specific for the gastrointestinal polypeptide, motilin. Antisera were raised in guinea pigs and rabbits. The immunogen was porcine motilin, conjugated to bovine serum albumin by the carbodiimide condensation reaction. The routine antiserum behaved identically towards endogenously-released motilin and the pure standard preparation. A radioactive tracer of high specific activity was obtained after incorporation of ^{125}- iodine into the motilin molecule by the chloramine-T method. The optimum conditions for all other assay variables were established to produce the most sensitive displacement (standard) curve. Motilin antiserum, coupled directly to an agarose matrix, retained full antibody activity and sensitivity. It is a feasible technique for use in both the radioimmunoassay and in the extraction of motilin from both serum and tissue extracts.

The fasting serum levels of IR- motilin was 190 $\pm$ 131 pg/ml in men and 294 $\pm$ 44 pg/ml in dogs (mean $\pm$ SD). The increase in motor activity in the extrinsically denervated fundic pouch of the dog after duodenal alkalinization was associated with a concomitant elevation in serum IR- motilin levels. This increase in serum IR- motilin was in the same range as that achieved by the exogenous administration of the porcine polypeptide which produced the same motor response. Duodenal acidification produced an apparent increase in serum IR-motilin with no associated increase in gastric motor activity. Only one peak of motilin immunoreactivity was detected when serum containing alkali-stimulated motilin or a partially purified duodenal extract were subjected to gel filtration on Sephadex G-50. The distribution of motilin throughout the hog gastrointestinal tract, determined by radioimmunoassay on partially purified extracts, agreed with

the immunocytochemical findings that motilin was predominantly located in the duodenum and jejunum, with traces in the upper ileum.

Virtually the intact molecule was required for the expression of full biological potency. The individual amino acids were important inasmuch as they contributed to the charge distribution and conformation of the molecule.

The physiological release and function of motilin have yet to be determined. Elevated levels of circulating IR- motilin have not been associated with any gastro-intestinal function, although they appear to be depressed by feeding. Motilin has been implicated in the control of the interdigestive phase of gastric motor activity. It may be acting in a local or paracrine manner. Motilin has not been implicated in any clinical state as yet.

The hormonal status of gastric inhibitory polypeptide (GIP) has been studied with the existing radioimmunoassay, modified to improve the label specific activity (by ion exchange chromatography). Direct coupling of GIP antisera to agarose beads was unsatisfactory, antibody activity and sensitivity being greatly reduced by the close proximity of the solid matrix. The postulated role of GIP as the enterogastrone of Kosaka and Lim, suggested by studies with exogenously-administered polypeptide, was confirmed by experiments in the dog. Pentagastrin-stimulated gastric acid secretion was inhibited by intra-duodenal infusion with glucose or fat; this inhibition being associated with a significant elevation in the circulating serum IR- GIP levels, within the range produced by ingestion of a mixed meal. GIP does not appear to be involved in the inhibition of gastric acid secretion produced by duodenal acidification.

Endogenous GIP stimulated by either fat or glucose exhibited at least 3 immunoreactive components after column chromatography. The IR- GIP eluting in the void volume appeared to represent a non-specific complex between GIP and a serum protein and is possibly biologically inactive. A second IR-GIP component with a molecular weight of 7500-8000 (ProGIP), eluted ahead of the established form of GIP (molecular weight = 5105). ProGIP has been found to be relatively unstable. ProGIP and GIP_{5000} have also been detected in extracts of hog duodenal mucosa. The established insulinotropic effect of GIP correlates best with that percentage of the total IR- GIP composed of ProGIP and GIP_{5000}. The relative proportions of IR- GIP_{5000} and IR- ProGIP in serum samples taken at different times after ingestion of either fat or glucose, suggest that ProGIP is either a precursor of GIP or that the ProGIP-producing cells occupy a more distal region of the duodenal and jejunal mucosa than the GIP- producing cells.

Exogenous administration of synthetic somatostatin in dogs and man will inhibit both GIP release by either fat or glucose and the insulino-tropic action of GIP at the level of the β-cell. Naturally-occurring intestinal or pancreatic somatostatin may contribute to the control of GIP release and serve to modulate the GIP- mediated response of the gastric parietal or pancreatic β-cell.

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This thesis is dedicated to the memory of my father, John Dryburgh, who all unknowingly started me along this path.

LIST OF ABBREVIATIONS

APUD - amine precursor, uptake and decarboxylation

BGP - brain gastrin immunoassayable peptide

BSA - bovine serum albumin

CDI - carbodiimide

CE plasma - charcoal extracted plasma

CCK-PZ - cholecystokinin-pancreozymin

CNBr - cyanogen bromide

FCA - Freunds Complete Adjuvant

GIP - gastric inhibitory polypeptide

GLI - glucagon-like immunoreactivity

IR- - immunoreactive

IRP - insulin-releasing polypeptide

KIU - Kallikrein inhibitor units

LDD - least detectable dose

M_5 - pure porcine motilin

ND - non-detectable

NSB - non-specific binding

PTH - parathyroid hormone

PITC - phenylisothiocyanate

PLC - proinsulin-like component

PZn - Pancreozymin (commercial)

RIA - radioimmunoassay

Sn - secretin

VIP - vasoactive intestinal peptide

Sources of Reagents

^{125}I-sodium (carrier free)	Amersham/Searle	1MS 30
Chloramine-T	Eastman Kodak Co. Rochester, N.Y. 14650	#1022
Sodium metabisulphite	Fisher Scientific Co. Fair Lawn, New Jersey	S-244
Lactoperoxidase (from milk)	Sigma Chemical Co. St. Louis, Mo. 63178	#L-2005
Hydrogen peroxide (30%)	Fisher Scientific Fair Lawn, New Jersey	H-325
Bovine serum albumin (fr V)	Sigma Chemical Co. St. Louis, Mo. 63178	
Trasylol (10,000 KIU/ml)	FBA, Boehringer Ingelheim Canada	
Microfine silica (Quso G32)	Philadelphia Quartz Co. Valley Forge, Pa. 19482	
AG 1-X10 resin (200-400 mesh)	Biorad Laboratories Richmond, California	140-1541
Freunds Complete Adjuvant	Difco Laboratories Detroit, Michigan	
1-ethyl-3-(3-dimethyl)-amino propyl carbodiimide	Calbiochem San Diego, California	341006
Dimethyldichlorosilane	Biorad Laboratories Richmond, California	
Charcoal (Carbon decolourizing Neutral)	Fisher Scientific Fair Lawn, New Jersey	C-170
Dextran T 70	Pharmacia Uppsala, Sweden	
Insulin RIA Kit	Amersham/Searle	1M 39

Sephadex gels	Pharmacia Uppsala, Sweden	
Sepharose resin	"	
CM celluloses	Whatman, England	
Glacial acetic acid) Hydrochlorid acid) Ammonia solution)	Aristar BDH Chemicals Poole Dorset, England	
Cyanogen bromide	Eastman Organic Chemicals Rochester, N.Y.	919
Trypsin - TPCK	Worthington Biochemicals Freehold, New Jersey	230 u/mg
Carboxypeptidase A	Worthington Biochemicals Freehold, New Jersey	
Phenylisothiocyanate	Eastman Organic Chemicals Rochester, N.Y.	
Trifluoroacetic acid	Eastman Organic Chemicals Rochester, N.Y.	
Dansyl chloride	Sigma Chemical Co. St. Louis, Mo. 63178	
Acetic anhydride	Eastman Organic Chemicals Rochester, N.Y.	13004
Succinic anhydride	Eastman Organic Chemicals Rochester, N.Y.	868
Ethanolamine	Sigma Chemical Co. St. Louis, Mo. 63178	A-5629
Acrylamide	Eastman Organic Chemicals	X 5521
N N'-methylenebisacrylamide	" " "	8383
NNN'N'-tetramethylene-diamine	" " "	8178
Fluothane -Halothane B.P.	Ayerst Laboratories, Montreal	#3125
Lipomul	Upjohn Co. of Canada Don Mills, Ont.	#1984N
Pentagastrin(Peptavlon injectionable)	Ayerst Laboratories Montreal, Canada	#3290

INTRODUCTION

In 1905 Starling adopted the term "hormone", first coined by W.B. Hardy, to describe a chemical substance, released by some physiological stimulus from its cell of origin and carried to its target organ by the circulation. Over the last decade the facts which have emerged about endocrine control in general, and gastrointestinal control in particular, would indicate that this classic paradigm must undergo revision and re-evaluation and that the role of a chemical messenger may be more subtle than was originally envisaged.

The three generally accepted hormones with gastrointestinal activity, secretin, gastrin and cholecystokinin-pancreozymin have known chemical structures and physiological importance. They have been joined over the last ten years by numerous other peptides of both gastrointestinal and extra-gastrointestinal origin. These substances have had their structures confirmed but their true hormonal status is uncertain, e.g., motilin, gastric inhibitory polypeptide (GIP), vasoactive intestinal peptide (VIP), bombesin and somatostatin. Other workers have introduced impure extracts with biological activity, whose active moiety may be identical with other, already identified polypeptides, e.g., insulin-releasing peptide (IRP), bulbogastrone, enterogastrone and chymodenin. These candidate hormones were described succinctly by Grossman (1974) as being either "peptide mimickers of physiological events or pure peptides seeking physiological roles". The results obtained when the classical physiological methods of assessing humoral status were applied to these peptides were equivocal, in many cases. The infusion of sufficient exogenous pure polypeptide into the circulation, usually accepted as the D_{50}, in order to mimic a physiological event, or the traditional cross-circulation experiments were no longer

enough to establish true physiological function. The effective levels achieved by exogenous administration of the polypeptide should be in the range of the serum levels measured during endogenous polypeptide release. This requirement is complicated by the fact that the biological activity observed during the endogenous release of any gastrointestinal polypeptide is being modulated by the activity of other polypeptides released at the same time. Before the hormonal status of a biologically active principle can be evaluated it must be available in a chemically pure form, vide the confusion which has arisen around the biological activity of cholecystokinin-pancreozymin, due to the studies which have been performed with a preparation of this polypeptide which was only 10-14% pure. Some method for the measurement of serum and tissue levels of the putative hormone is also essential.

Some of the candidate hormones which fall into the second category of Grossman, i.e., polypeptides extracted from biological tissues, which have been purified and characterized, include VIP, somatostatin and motilin. These substances, infused intravenously, demonstrated varied biological activities, but their endogenous release cannot be measured in association with any of these activities by any of the methods developed for the estimation of that substance in the circulation. In 1953 Feyrter described his concept of the paracrine cell. He postulated the existence of secretory cells, scattered throughout the gastrointestinal mucosa, adjacent to their target cells. The structures he thought responsible, the "helle Zellen" or clear cells were later recognized as being identical with the APUD (amine precursor uptake and decarboxylation) cells of Pearse (1968). Under normal circumstances, he postulated, the secretions of these cells would pass to the target cell via the extracellular fluid and never "spill" into the circulation in any significant amounts. The paracrine system

has yet to be proven to exist but it is a plausible concept and the gut mucosa, which may be regarded as the single largest endocrine organ in the body, with its multitude of secretory and receptor cells scattered over an immense area, would be ideally suited to such a mechanism. Some evidence for the existence of the modification of endocrine function has been obtained with somatostatin.

This tetradecapeptide was originally isolated from the hypothalamus of the sheep by Brazeau et al (1973) during their search for a pituitary growth hormone releasing factor. Instead they were repeatedly able to demonstrate the existence of a growth hormone release inhibitory factor. This material was extracted, purified, sequenced and later synthesized. It was originally named somatotropin-release inhibitory factor (SRIF) or growth hormone-release inhibitory hormone (GRIH) but the findings that it was able to inhibit the release of insulin (Alberti et al, 1973), glucagon (Gerich et al, 1975), gastrin (Bloom et al, 1974) and GIP (Pederson et al,1975) have favoured the use of a less specific name, somatostatin. Studies with gastrin and GIP have indicated that somatostatin did not only inhibit endogenous release of these polypeptides but also blocked their action at the level of the target cell. If effective levels of hypothalamically-released polypeptide were to occur in the circulation all these systems would be inhibited simultaneously, and this is an unacceptable premise. Somatostatin-producing cells have been localized by immunochemical means in hypothalamic and pancreatic islet tissue, and in gastric and intestinal mucosa (Dubois, 1975). The location of somatostatin in the pancreas is distinct from the insulin-producing β cell, and the glucagon-producing α cell. It is postulated to be the D cell, common to the pancreas, stomach and intestine.

A teleologically more acceptable concept would involve the modulation of hypothalamic, gastric, intestinal or pancreatic function by hypothalamic, gastric, intestinal or pancreatic somatostatin, released locally by an appropriate stimulus, where it acted in a paracrine manner.

A second type of secretory process, postulated to exist and capable of acting in the gastrointestinal tract, is the neurocrine system, whose nerve cell axons extend to the target organ. Their secretions therefore have only to cross the synaptic junction, in a manner analogous to that of the neurotransmitter, acetylcholine. Peptides with gastrointestinal activity such as somatostatin, substance P, VIP and gastrin have all been detected in normal neural tissue. Although somatostatin has been detected in both the hypothalamus and pancreas the possibility that some neural connection exists between these two areas seems unlikely in view of the fact that no nerve fibres to the adult pancreas have been shown to contain IR- somatostatin. Somatostatin immunoreactivity has also been demonstrated in the foetal pancreas by the third month of gestation. It is more likely that this peptide is being synthesized in both regions and is not merely being absorbed by pancreatic tissue after synthesis in the brain. The evidence so far available favours a paracrine, rather than neurocrine role for somatostatin. On the other hand, immunoreactive substance P has been demonstrated in both central and peripheral neural tissues, in association with primary sensory neurons in the dorsal horn, and in non-myelinated free nerve endings in the skin, sweat glands and gut wall (Hökfelt et al, 1975,1976). It has been suggested that substance P not only has a direct stimulatory effect on smooth muscle cells in the gastrointestinal tract, but that it also enhances the effect of nerve stimulation. As these same doses of substance P appear not to enhance the response to applied acetylcholine to any significant degree, it

may be acting prejunctionally to modulate the response of the gut musculature to cholinergic stimulation, and be neurocrine in its action.

Two polypeptides with established gastrointestinal properties, gastrin and VIP, have also been located in neural tissue. VIP was found in the gastrointestinal tract of several mammals (Said and Mutt,1970) and has been extracted from pancreatic tumours associated with the Werner-Morrison syndrome (Bloom et al, 1973). As it has also been extracted from tumours of neural origin it was logical to look for VIP in cultured neuroblastoma cells from mice, as well as in normal canine neural tissue. In the normal extracts the highest concentrations were found in the cerebral cortex, the hypothalamus and hippocampus. IR-VIP was also detected in sympathetic ganglia, the adrenal gland and in extracts of the vagus nerve (Said and Rosenberg, 1976: O'Dorisio et al, 1976). Extracts from both normal cortex and tumour tissue showed VIP-like activity when assayed in vitro on rat stomach or guinea pig gall bladder strips. No physiological functions have been assigned to this polypeptide, but the relatively larger concentrations in the central and peripheral nervous system, compared to that in the intestine, suggest that it might function as a neurocrine transmitter. Immunoreactivity to gastrin has also been detected in the brain, predominantly in the cortical grey matter. Extracts from this region, however, showed a lower affinity for the antiserum used than did heptadecapeptide gastrin, and eluted behind this gastrin from Sephadex G-25. Vanderhaeghen et al (1975) have named this material brain gastrin immunoassayable peptide (BGP). It remains to be seen if BGP corresponds to a smaller molecular form of gastrin, e.g., the tridecapeptide found in sera from Zollinger-Ellison patients (Rehfeld and Stadil, 1973a), and to examine the range of its biological activity.

A model specific for the actions of the gastrointestinal polypeptides, combining features from both the endocrine and paracrine systems, has been proposed by Wingate (1976). His Eupeptide system is based on the following facts. Most gastrointestinal polypeptides influence both motor and secretory activities of the gut, and most gastrointestinal polypeptide-producing cells are situated in close proximity to the cell whose secretions they influence. However, it has been well established that several of these factors are released into the systemic circulation in significant amounts. Wingate therefore postulated a dual action for the gut peptides and suggested that they acted at a local target cell as a paracrine, to promote secretion or local muscle activity, and at a distant target cell to modulate the motor control of the digestive tract.

Immunological techniques such as radioimmunoassay, immunocytochemistry and affinity chromatography, have been applied to the physiology of polypeptides and although providing some of the answers about their function, they have also raised many more problems. The advent of the radioimmunoassay in particular (Berson and Yalow, 1958) has proved a valuable tool in monitoring polypeptide responses in various situations, but the results require careful evaluation. Some long-held beliefs have had to be re-examined. Secretin had been postulated to be the polypeptide responsible for the inhibition of gastric acid secretion when acid passed into the duodenum. Exogenously administered secretin did, in fact, mimic this response. However, the development of a radioimmunoassay, specific for secretin, proved that the minimum effective circulating IR- secretin levels achieved after infusion of the polypeptide were much higher than those produced by duodenal acidification (Ward and Bloom, 1974). When secretin was administered to produce serum IR- secretin levels within the physiological range, no effect was observed on acid secretion or gastric motor

activity although a significant effect was seen on the exocrine pancreas, (Bloom, 1975). This failure to confirm the role of secretin as the acid-stimulated gastric inhibitory agent, by radioimmunological means, has re-stimulated interest in the work of Anderson et al, (1967) on the impure extract of the bulbar region of the duodenum, bulbogastrone. They were able to show that acidification of the bulbar region resulted in a profound inhibition of gastric acid secretion, which was not observed if the acid was installed into the lower duodenal regions. However, until this material has been purified and its endogenous release can be stimulated by physiological secretagogues, any suggestions that it is the major inhibitory agent released by duodenal acidification must be purely speculative.

The use of immunological techniques in the measurement of circulating polypeptide levels in serum, plasma or tissue extracts has demonstrated that several polypeptides existed in more than one molecular form. Gregory and Tracy (1964), using classical peptide extraction techniques, described two forms of heptadecapeptide gastrin, differing only in the presence of a sulphated tyrosine residue, but had the foresight to state "there may be present in antral mucosa other gastrin composed of part of the peptides we have isolated, or indeed incorporating them, or the active parts, within a larger molecule". In 1971 Yalow and Berson confirmed this supposition by demonstrating that gastrin in human plasma did, in fact, exist in more than one molecular form. This heterogeneity was shown immunologically when different antisera crossreacted in differing degrees with the endogenously occurring polypeptide and the pure standard preparation. Fractionation by gel filtration, electrophoresis or ion exchange chromatography, followed by monitoring of the fractions obtained, by radioimmunoassay, allowed comparison of size or

charge distinction between components sharing immunoreactivity. By 1973 Rehfeld and Stadil had isolated four components of immunoreactive gastrin from the sera of Zollinger - Ellison patients by the technique of high resolution gel filtration. Component I eluted in the void volume of the column and corresponded to the "big, big" gastrin of Yalow and Berson, (1972); component II (big gastrin) had a molecular weight of 7000; component III correlated well with heptadecapeptide gastrin; component IV (minigastrin) was a tridecapeptide. The different gastrins have been found to vary in location, response to stimuli, and also perhaps in function. Big, big gastrin is a major component of the fasting sera in normal subjects but occurs in insignificant amounts in normal or pathological tissue extracts, and its serum levels are not elevated by feeding. Treatment of the sera with 8.0M urea, or solutions of increasing ionic strength, significantly depressed the size of this fraction of IR- gastrin, suggesting that this largest molecular form may be a protein/peptide complex (Rehfeld et al, 1975). The physiological function of such a complex is not understood, although the suggestion has been made, with respect to insulin, that this type of protein-peptide binding may act as a limiting factor in the transport of a peptide across a cellular membrane (Simon and Antoniades, 1975). Component II or big gastrin would appear to be the major IR- component in the circulation after stimulation, whilst the heptadecapeptide form predominates in antral tissue (Dockray et al, 1975). Evaluation of the importance of the immunoreactive forms of gastrin under different conditions is further complicated by the varying half-lives of the heterogeneous forms of gastrin.

Prior to this work, Berson and Yalow (1968) had demonstrated that human parathyroid hormone (PTH) in tissue extracts had a molecular weight of 9000, whilst

that in serum was mostly composed of a smaller form with a molecular weight of 7000. In 1972 Canterbury et al isolated a third PTH with a molecular weight of 5000. The 7000 molecular weight form was found to represent the C-terminal portion of the larger molecule (Segre et al, 1972) and as Aurbach et al (1971) had postulated that the biologically active portion of the larger molecule resided at the N- terminal, the possibility was raised that a significantly large proportion of the total immunoreactive PTH in sera was biologically inactive. Habener et al (1971) have isolated a still larger immunoreactive molecule from slices of parathyroid tissue. Biosynthesis studies, measuring the uptake time of tritiated amino acids into this component and the 9000 molecular weight form are suggestive of a precursor-polypeptide relationship (Cohn et al, 1972) and the largest molecular form of PTH can be enzymatically degraded by trysin to produce a polypeptide with increased biological and immunological activity.

The best illustration of a precursor-hormone relationship so far comes from the studies on proinsulin and insulin. In 1967 Steiner provided evidence that the synthesis of insulin involved production of a precursor which was synthesized in the rough endoplasmic reticulum of the β cells in the pancreatic islets, and transferred to the Golgi apparatus (Steiner et al, 1969). Approximately 95% of the proinsulin was converted to insulin within the secretory granules in the cytosol, the remainder being secreted into the circulation along with the insulin. At least two enzyme systems, one trypsin-like (Nolan et al, 1971) and one similar to a carboxypeptidase (Kemmler et al, 1971), appear to be necessary for the conversion of the proinsulin to insulin and a chymotryptic-like cleavage has been implicated by the work of Tager et al, (1973) in <u>in vitro</u> studies in the rat islet preparation. The proinsulin

molecule has been found to vary only slightly from species to species, the average molecular weight being around 9000. Most studies on the biosynthesis of proinsulin have been performed on the isolated islet preparation (Steiner, 1967) and conversion to insulin has been found to be strictly intracellular. Glucose is an important regulator of proinsulin synthesis, which is favoured under hyperglycaemic conditions over other cellular proteins (Permitt and Kipnis, 1972a, 1972b). Many antisera to insulin also cross-react with proinsulin and other intermediate forms, which are together designated proinsulin-like-component (PLC), and therefore measurement of IR-insulin alone would be possible only after gel filtration. Proinsulin has been shown to have most of the biological properties of insulin but only 3 - 5% of its biological potency (Narahara, 1972) and as the IR- PLC in fasting sera approximates 15% of the total IR- insulin it must be taken into account when correlating immunological activity with biological activity.

The ratio of PLC: insulin declines in the first hour after glucose stimulation and then gradually increases. Elevated PLC: insulin ratios have been found in hypokalaemias of different aetiologies (Gorden et al, 1974), severe diabetes and chronic renal failure (Mako et al, 1973), and are diagnostic of β cell adenoma (Rubenstein et al, 1974).

A different problem has arisen in the studies investigating the relationship between the immunoreactive forms of glucagon. Different components of the total material which crossreacts with antisera to glucagon were found to respond differently when challenged by the same stimulus. Samols et al (1965,1966) noted that the circulating levels of immunoreactive glucagon (IR- glucagon)appeared to increase after oral administration of glucose and

that this immunoreactivity originated from the gut rather than the pancreas. This was confirmed by Buchanan et al (1967) who infused (intrajejunally) glucose into pancreatectomised dogs and measured a significant increase in IR- glucagon levels. Valverde et al (1968) monitored IR- glucagon after gel filtration of dog duodenum mucosal extracts and found two glucagon-like-immunoreactive (IR-GLI) components, one with a molecular weight of 3500 (small GLI) and a second, much larger molecule, with a molecular weight of 12,000 (large GLI). A fraction similar to the second component of Valverde was purified from a crude extract of pig ileum by Murphy et al (1973) and was found to possess little biological resemblance to pancreatic glucagon. Sasaki et al (1975) further purified the small GLI component from porcine duodena by gel filtration on Biogel P-10 and separated two peaks of protein, one with a molecular weight around 3500, which crossreacted completely with antisera raised to pancreatic glucagon and appeared identical in its spectrum of biological activity. The second fraction eluted behind the ^{125}I-glucagon marker, had a molecular weight of 2900, and showed varying degrees of crossreactivity depending on the antisera used in the assay. Histochemical studies of the secretory cells in the gastrointestinal mucosa initially indicated a cell very similar to the α - cell of the pancreatic islets. Ultrastructural studies now suggest that, whilst these A- cells in the fundic mucosa are identical in morphology with the pancreatic α -cell, those in the intestinal mucosa show slight structural differences. These have been designated A- like and may correspond to the EG cell of Polak et al (1971). The distribution of the 3500 and 2900 molecular weight components throughout the gastrointestinal mucosa correspond closely to the distribution of A and A- like cells respectively. It is strongly suggested that GLI, originally defined by Unger et al (1968) to encompass any immunoreactive material origina-

ting from the gut, is not a single entity, but is made up of true glucagon of gut origin and several other peptides, more correctly called GLI or enteroglucagonoid, which share a degree of immunological and possible biological identity with true glucagon. An oral glucose load causes a decrease in pancreatic glucagon plasma levels and an increase in circulating IR-GLI levels and therefore an antiserum specific for pancreatic glucagon must be used to measure the true pancreatic response to this stimulus.

The conclusion must be drawn that any comparison between biological and immunological activity of any polypeptide must take into account the presence of immunoreactive but possibly biologically less potent precursor forms or complexes between the peptide and a larger protein, as well as immunologically similar but functionally different molecules. All antisera should be screened for differences in their crossreactivity with the biologically occurring forms of each peptide and in the possibility of raising antibodies to a specific region of the active molecule considered.

An obvious, though sometimes experimentally ignored, observation is that no physiologically functional peptide acts in isolation, and that its biological effect is modulated by the hormonal milieu at that time. A simple example of this fact is shown by the combined effects of secretin and cholecystokinin-pancreozymin on the exocrine pancreas. In the intact animal there is no measurable bicarbonate response to an infusion of cholecystokinin-pancreozymin, but a combination of this peptide with secretin will produce a greater secretory response than infusion of secretin alone. The increased enzyme output which follows cholecystokinin-pancreozymin is ineffective unless it is washed from the pancreatic ducts into the duodenum by the secretin-induced aqueous secretion

(Brown et al, 1967a). It is also logical to correlate the activity of gastrointestinal polypeptides to the stage of the digestive cycle during which they are normally released, and to measure that activity in an environment of the circulatory digestion products that would pertain at that stage. Physiological levels of IR- gastrin have no effect on insulin release in the fasting man, but in the presence of a degree of hyperglycaemia the initial insulin response after gastrin administration is longer and more pronounced than that seen after intravenous glucose alone (Rehfeld and Stadil, 1973b). In the isolated, perfused rat pancreas Pederson and Brown (1976) were able to demonstrate a threshold glucose level, below which GIP had no effect on insulin release.

The major functions of the gastrointestinal hormones so far discovered relate to their effect on the secretory capacity or motor activity of the gastrointestinal tract. Another property was suggested by the discovery that gastrin had a trophic effect on the cells of the gastric mucosa. Patients treated for duodenal ulcer by antrectomy showed atrophy of the gastric mucosa (Lees and Grandjean, 1968) which was not the case if the treatment was vagotomy only (Melrose et al, 1964). Disuse atrophy could not be the cause of this as the acid secretion was initially depressed to the same degree in either case. In contrast, subjects with Zollinger-Ellison Syndrome showed hyperplasia of both the gastric and duodenal mucosa (Ellison and Wilson, 1967). The role of gastrin was confirmed in rats, when increased RNA and DNA synthesis resulted from single shots of gastrin, and chronic gastrin treatment produced a thickening of the gastric mucosa (Johnson, 1976). Mainz et al (1973) found that exogenous CCK-PZ caused an increase in both cell mass and cell number in the exocrine pancreas, and postulated a role for CCK-PZ in maintaining pancreatic function and integrity. Physiological levels of the synthetic octapeptide of CCK-PZ were found to have a trophic effect on duodenal mucosa but had no effect on

gastric mucosa. These same levels would, however, inhibit the trophic effect of gastrin on the gastric mucosa, and the same result was found with secretin (Johnson and Guthrie, 1974).

In 1930, Kosaka and Lim used the term "enterogastrone" to describe a humoral agent, released from the duodenal mucosa by fat or fat digestion products, the function of which was to inhibit both gastric acid secretion and gastric motility. This definition was later expanded to require that any candidate polypeptide must inhibit gastric acid secretion stimulated by histamine and insulin-induced hypoglycaemia, as well as gastrin and its analogues. Secretin, cholecystokinin-pancreozymin, VIP and GIP have all been considered at some time to fill this role, they all appeared to inhibit the acid secretion produced by some or all of these stimuli, and cholecystokinin-pancreozymin is also released by the presence of fat in the duodenum, but the only polypeptide which satisfies all these criteria is GIP.

Brown and Pederson (1970) showed that the ability of the 10% pure preparation of cholecystokinin-pancreozymin of Jorpes and Mutt to produce gastric acid inhibition was significantly reduced by a simple purification step, involving gel filtration on Sephadex G-50, although its effect on gall bladder contractility was unaltered. A side fraction, produced in the purification of cholecystokinin-pancreozymin was found to contain inhibitory activity but had no effect on the gall bladder, (Brown et al, 1969). A polypeptide was isolated and purified, (Brown et al, 1970), sequenced (Brown, 1971: Brown and Dryburgh, 1971) and was found to be a straight chain polypeptide with 43 amino acids and a calculated molecular weight of 5105.

The amino acid sequence was :-

NH_2-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-Lys-
Ser-Asp-Trp-Lys-His-Asn-Ile-Thr-Gln

A radioimmunoassay for GIP has been developed and antibodies to GIP raised in guinea pigs did not crossreact with glucagon, gastrin, motilin, cholecystokinin-pancreozymin, secretin, VIP or insulin. Studies performed on peptide fragments obtained by cyanogen bromide cleavage at the methionine residue, or on synthetic peptide fragments, suggest that the immunoreactive site lies within the sequence 21-38. Antiserum to GIP has also been used in the immunohistological localization of the GIP-producing cell in the duodenum and jejunum of man, dog and baboon. It was tentatively identified as the D1 cell, (Polak et al, 1973) in the APUD series defined by Pearse (1968,1970,1974) but is now known to be the K cell (Solcia et al, 1973).

Sera from human volunteers, taken before and after ingestion of a normal meal, were subjected to radioimmunoassay. Fasting levels of immunoreactive GIP (IR-GIP) ranged from non-detectable to 400 pg/ml and rose after eating to a mean peak of 1200 pg/ml, remaining elevated for at least 3 hours (Kuzio et al, 1974). The major physiological secretagogues for GIP release were oral glucose (Cataland et al, 1974) and oral fat (Brown et al, 1974).

The discovery that circulating IR-GIP levels were elevated when glucose came into contact with the duodenal and jejunal mucosa suggested that this polypeptide might be a factor in the entero-insular axis, i.e. might be a hormone of intestinal origin which contributed to the regulation of the endocrine

pancreas. Dupre et al (1973) infused porcine GIP intravenously in man and showed a significant enhancement of the IR- insulin response to an intravenous infusion of glucose, associated with an improvement in glucose tolerance. The circulating levels of IR- GIP achieved during this exogenous administration of GIP were comparable to those released endogenously by ingestion of glucose. Studies in dogs suggested that lower levels of circulating IR- GIP were insulinotropic only in the presence of a degree of hyperglycaemia, although higher, possibly non-physiological levels stimulated insulin release in the fasted animal.

The existence of the humoral gastric motor-activity stimulating principle, later named motilin, was first suspected when Brown et al (1966) perfused the duodenum of the dog with alkali or fresh pig pancreatic juice and demonstrated an increase in gastric motor activity in extrinsically denervated or totally transplanted pouches of the fundus of the stomach. Earlier, Shay and Gershon-Cohen (1934) had described increased gastric emptying of a barium sulphate test meal after instillation of 1% bicarbonate into the duodenum. This observation coupled with the work of Thomas et al (1934), who diverted the gastric (acidic) contents away from the duodenum and recorded an increase in the rate of gastric emptying, suggested a pH sensitive, duodenal reflex that contributed to the control of gastric motor activity. Weisbolt et al (1969) proposed that a relationship existed between the rate of gastric emptying and the motor activity of the gastric musculature which would ensure that the contents of the stomach were delivered to the duodenum at a rate, and in a consistency that would allow optimal duodenal and jejunal digestion and absorption.

A crude duodenal extract (Pancreozymin, Boots Pure Drug Co.), administered intravenously in dogs, produced similar changes in the motor activity of the fundic pouches, whilst the purer GIH preparation of CCK-PZ did not (Brown, 1967). Gel filtration of the crude extract on Sephadex G75 produced five protein peaks. Fractions 4 and 5 were inhibitory for gastric motor activity and fraction 5 was a potent stimulant of pancreatic enzyme output, i.e. it corresponded most closely to the GIH preparation. Fraction 2 represented a 20-fold purification of the original stimulatory material (Brown and Parkes, 1967). Motilin was eventually purified from a side fraction produced in the purification of secretin (Brown et al, 1972). The amino acid sequence was determined by the subtractive dansyl-Edman's technique on peptides produced by cleavage of the molecule with cyanogen bromide, trypsin, chymotrypsin and thermolysin. Porcine motilin was found to be a 22 amino acid residue polypeptide with the sequence :-

NH_2-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-
Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln

and a molecular weight of 2700 (Brown et al, 1973).

The porcine polypeptide produced a significant increase in motor activity in the extrinsically denervated fundus and antrum of the canine stomach in doses as low as 50 ng/kg. It had no significant effect on gastric acid secretion, but did elevate pepsin output to a higher degree than could be explained by a simple washout phenomenon (Brown et al, 1972). The method of measuring motilin activity required bioassay in the chronic dog, prepared with an extrinsically denervated pouch of the fundus and a Mann-Bollman fistula into the duodenum. In vitro preparations of muscle strips from the ileum, colon and circular layers of the stomach of the rat, guinea pig and rabbit were examined

but the sensitivity of every preparation decreased immediately after the first exposure to motilin and the initial response could not be duplicated. Development of a radioimmunoassay was deemed desirable to confirm the role played by motilin in the increase in gastric motor activity after duodenal alkalinization and for further investigation of its physiological function.

It is the purpose of this thesis to study the hormonal status of the gastrointestinal polypeptides, GIP and motilin, and to look more closely at their possible physiological roles, bearing in mind the following points:-

1. Biological activity seen after exogenous administration of a polypeptide can only be considered physiological if that same response can be elicited by that polypeptide when it is released by a physiological stimulus, and the circulating levels of the peptide are comparable.
2. Inability to measure an increase in polypeptide levels in the systemic circulation does not necessarily preclude that polypeptide from having a physiological role.
3. If immunological techniques are used to measure the polypeptide levels in the circulation, then it is essential to determine what percentage of the total immunoreactivity represents the true biological activity.
4. The biological activity of a polypeptide will depend on the hormonal and nutrient milieu pertaining at that time and results obtained in an isolated situation, e.g., in vitro, or by infusion of a polypeptide associated with digestion,in a fasting animal, may not represent its true physiological activity.
5. Gastrointestinal polypeptide activities need not be restricted to influencing the secretory or motor activities in the gastrointestinal tract. They may also play an important metabolic role in regulating the growth and responsiveness of the target organ.

METHODS

DEVELOPMENT OF A RADIOIMMUNOASSAY FOR MOTILIN (MOTILIN RIA)

A. Rationale

The concept of a radioimmunoassay is based upon the specific relationship that exists between an antibody and its antigen. Unlabelled antigen competes with labelled antigen for the binding sites on the antibody. The percentage of a fixed initial amount of labelled antigen bound to the antibody gives an index of the amount of unlabelled antigen present in the mixture. The concentration of antigen in an unknown sample may be determined by comparing the displacement of labelled antigen it produces with that produced by a series of standard solutions. The commonest label which can be incorporated into polypeptides containing tyrosine or histidine residues is an isotope of iodine, ^{125}I or ^{131}I.

A successful polypeptide radioimmunoassay is dependent on three absolute requirements. Firstly, the ratio of isotope to polypeptide in the radio-active tracer must be high enough so that sufficient tracer may be added to ensure an efficient counting rate without adding significant amounts of polypeptide and obscuring the upper limits of sensitivity of the curve. The second essential is an antibody of high affinity. This affinity is expressed as a constant K. The relationship between K and the upper limit of sensitivity of the radioimmunoassay can be developed as follows:-

From the 1st order Law of Mass Action

$$B/F = K([A^\circ] - B)$$

Where K is the equilibrium (affinity) constant, $[A^\circ]$ is the concentration of antibody binding sites and B & F are the concentrations of bound and free hormone

If b is the fraction of bound hormone and [H] is the total hormone concentration, then

$$B = b[H]$$

$$\therefore \quad B/_F = \frac{b}{1-b} = K([Ab°] - b[H[)$$

As the hormone concentration is increased to $[H^1]$ then $B/_F \downarrow$ and $b[H^1] \uparrow$.

By definition b has a maximum of 1.

$\therefore$ The most sensitive assay condition prevails when

$$[Ab°] \geq [H^1] \text{ for a } B/_F \text{ of } 1.$$

Assuming a $B/_F$ of 1 with minimal tracer and no unlabelled hormone, then $[H] \rightarrow 0$

$$\text{i.e.,} \quad 1 \leq K[Ab°].$$

When [Ab°] approximates $[H^1]$

$$1 \leq K[H^1] \quad \text{or} \quad K \geq \frac{1}{[H^1]}$$

i.e., the greater the value of K, the lower the concentration of total hormone that is detectable.

The final requirement is that the antibody should react identically with the unlabelled antigen, whether it be in the form of standard or endogenous polypeptide. Ideally, the labelled and unlabelled peptide should also behave identically in the system but this is not an absolute necessity. If these conditions are satisfied then the optimal values for all other variables may be established.

B. Iodination of Motilin

(I) Chloramine - T Method

A modification of the original method of Hunter and Greenwood (1963) was used in routine isotopic labelling of motilin. The polypeptide contains only

1 tyrosyl and no histidyl residues. The ratios of µg polypeptide: mCi $Na^{125}I$ essayed were 4:1 and 2:1.

The following reagents were prepared freshly for each iodination:-

a) Motilin (M_5) - 2 or 4 µg in 50 µl 0.2M sodium phosphate buffer, pH 7.5

b) $Na^{125}I$ - 1 mCi in 10 µl carrier-free sodium hydroxide

c) Chloramine-T - 40 µg in 10 µl deionized water

d) Sodium metabisulphite - 100 µg in 20 µl deionized water

Reagents a, b & c were added in quick succession, with bubbling to ensure rapid mixing, in a 10 x 75 mm siliconized glass culture tube. Reagent d was added, in like manner, after a 15 sec. delay. Motilin contains 1 methionine residue but no tryptophan and appeared fairly stable in the presence of the oxidizing agent, withstanding exposures to chloramine-T of 2 mins. without undue fragmentation occurring, as shown by polacrylamide gel electrophoresis (Fig. 1).

The reaction mixture was immediately transferred to a column of Sephadex G25 fine (0.6 x 30 cms.) and eluted in 0.2 M. acetic acid, containing 0.5% Bovine Serum Albumin (BSA) and 100 Kallikrein Inhibitor Units (KIU) Trasylol per ml. Fractions of approximately 400 µl were collected and 10 µl aliquots were counted for 0.1 min. in an automatic γ counter. The resultant column profiles are illustrated in Fig. 2 & Fig. 3, showing the separation of peptide-bound and free iodide. Aliquots of the appropriate fractions, diluted to contain ~5000 cpm/100 µl, were incubated for 24 hours at 4°C, with or without antiserum to estimate the specific versus non-specific binding (N.S.B) for that fraction. Those fractions showing the highest,

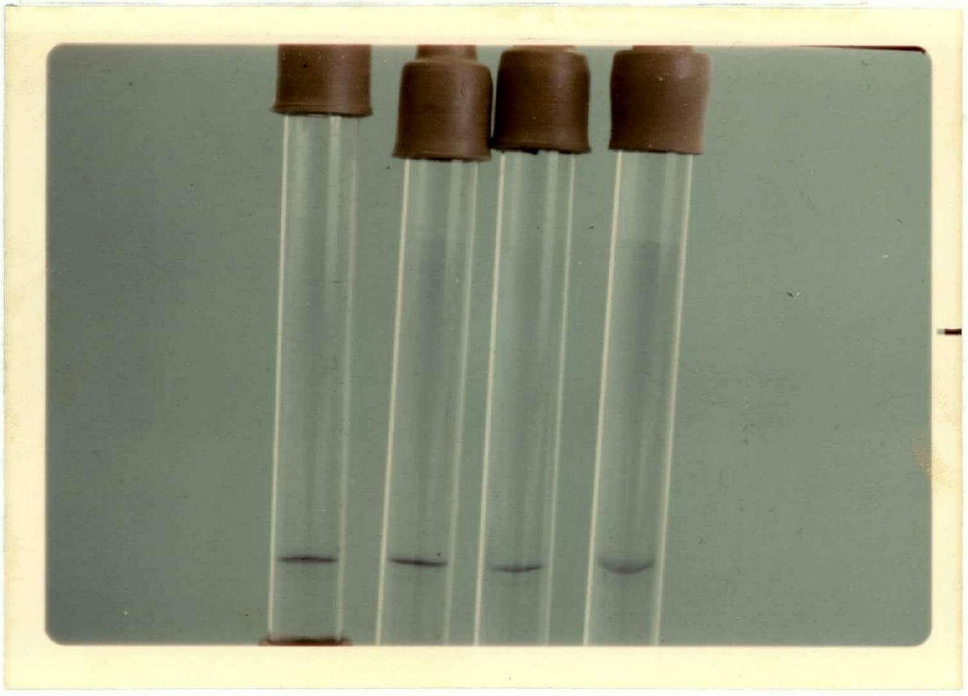

Fig. 1 Polyacrylamide gel electrophoresis of motilin after exposure to chloramine-T for 15, 30, 60 and 120 sec. No polypeptide fragmentation is observed after 60 sec exposure but is visible at 120 sec. The method for polyacrylamide gel electrophoresis is detailed on p. 72.

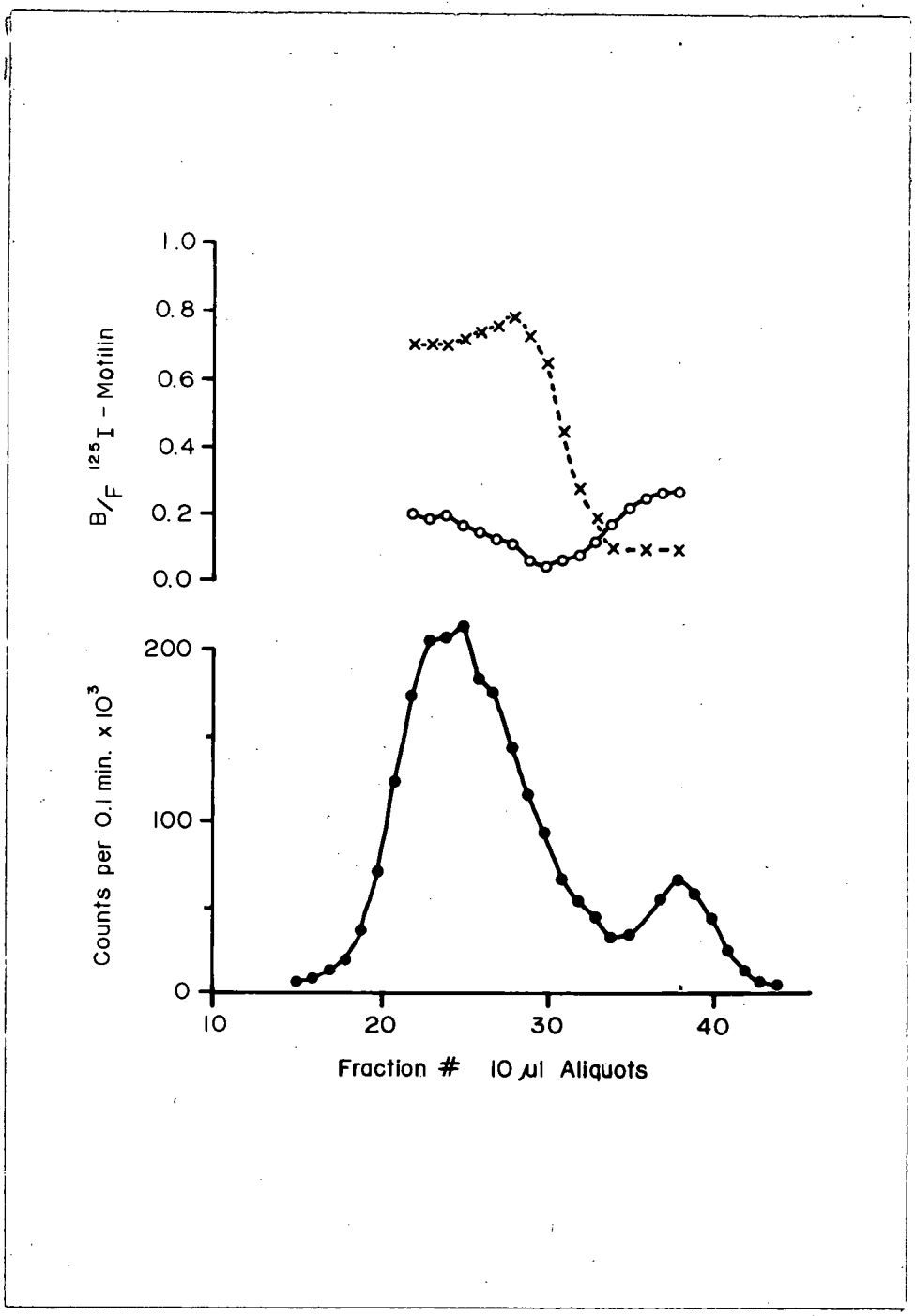

Fig. 2 Chloramine-T iodination of motilin at a peptide:iodine ratio of 4 µg:1 mCi. Separation of labelled motilin from free iodide on Sephadex G25 in 0.2M acetic acid. Counts per 0.1 min (●●); maximum binding (x--x); NSB (o—o).

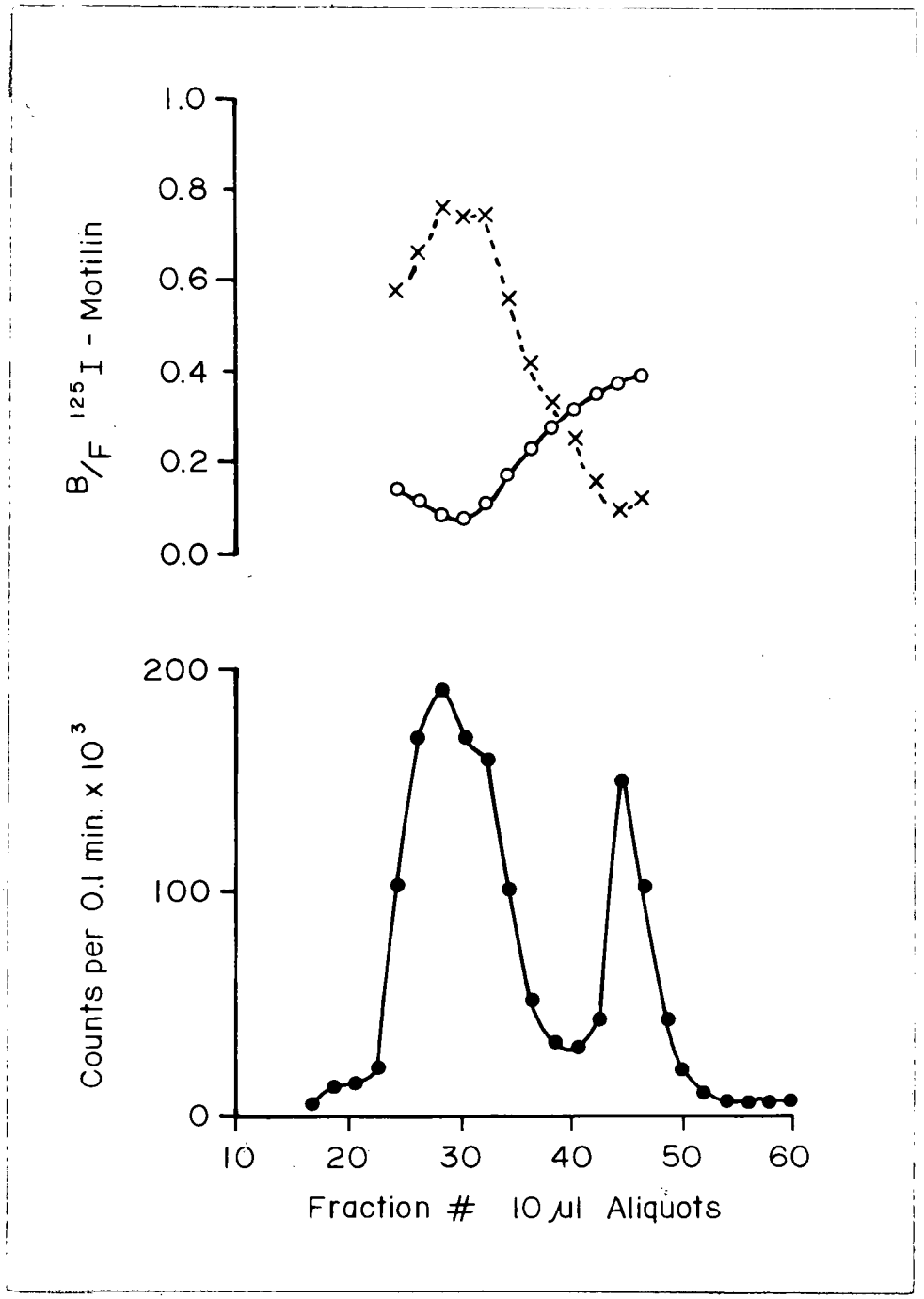

Fig. 3 Chloramine-T iodination of motilin at a peptide:iodine ratio of 2 µg:1 mCi. Separation of labelled motilin from free iodide on Sephadex G25 in 0.2M acetic acid. Counts per 0.1 min (●—●); maximum binding (x–x); NSB (o—o).

specific binding and lowest, non-specific binding were pooled, diluted in the eluant buffer and aliquotted for storage at - 20°C, so that each aliquot contained ~ 2 x 10^6 cpm./ 2 mls. This was presumed to contain monoiodinated motilin.

Label stored in this manner was stable for periods of up to 3 months. Lyophilization of label proved feasible but was not routinely performed.

(2) Lactoperoxidase Method

An alternative, gentler and more easily controlled method of oxidizing the iodide to iodine involves the use of lactoperoxidase (Miyachi et al, 1972). The following procedure follows the method of Holohan et al (1973). The reagents were mixed in the following order in a 10 x 75 mm. siliconized, glass, culture tube.

a) Motilin (M_5) - 4 µg in 50 µl 0.05 M sodium acetate, pH 5.0

b) $Na^{125}I$ - 1 mCi in 10 µl carrier-free sodium hydroxide

c) Lactoperoxidase - 500 ng in 10 µl sodium acetate, pH 5.0

d) Hydrogen peroxide - 0.86 nM in deionized water

3 x 10 µl at 5 min. intervals

After 15 mins. the reaction mixture was transferred to a Sephadex G25 fine column and eluted, monitored, assayed and stored as previously described on p. 21. A typical column profile is shown in Fig. 4.

(3) Estimation of Specific Activity of ^{125}I - Motilin

Dose - response curves were obtained (a) by increasing the concentration of unlabelled motilin and measuring the displacement of a constant amount of radioactive tracer (routine standard curve) and (b) by adding increasing

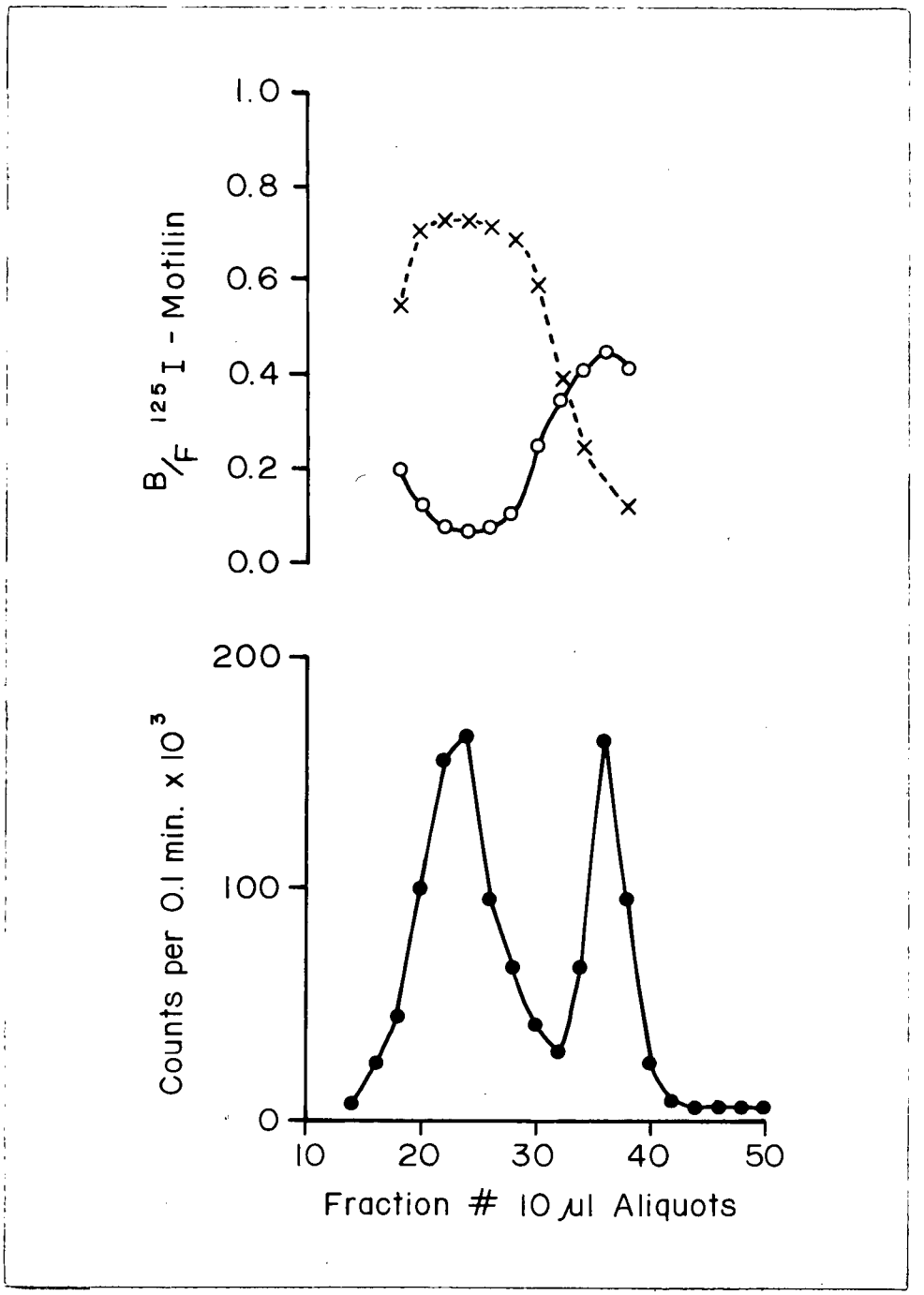

Fig. 4 Lactoperoxidase iodination of motilin at a peptide:iodine ratio of 2 µg:1 mCi. Separation of labelled motilin from free iodide on Sephadex G25 in 0.2M acetic acid. Counts per 0.1 min (●—●); maximum binding (x-x); NSB (o—o).

amounts of labelled motilin only and measuring the different ratios of tracer bound to antibody.

One point from the label dilution curve was arbitrarily placed on the standard curve and the other points fitted accordingly. Fig. 5 illustrates that the curves obtained when 3 different fractions of labelled motilin (fractions 24, 25 and 26 from the column profile shown in Fig. 3) were plotted on a standard curve they could be superimposed upon that standard curve. It can be concluded that the binding kinetics of the antiserum were virtually identical for both labelled and unlabelled antigen. The number of counts per minute (cpm) producing the same displacement as a standard amount of motilin can be read directly from this curve and the value converted to mCi/mg as an index of specific activity.

Example (from results shown in Fig. 5)

28 pg motilin $\equiv$ 18,000 cpm

$\therefore$ 1 mg motilin $\equiv$ $\frac{18,000}{28} \times 10^9$ cpm

$\equiv$ 643×10^9 cpm

---

1 Curie = 3.7×10^{10} dps (disintegrations/second)

$\therefore$ 1 mCi = 2.2×10^9 dpm (disintegrations/min.)

From the efficiency of the γ counter - 81%

1 mCi = 1.78×10^9 cpm

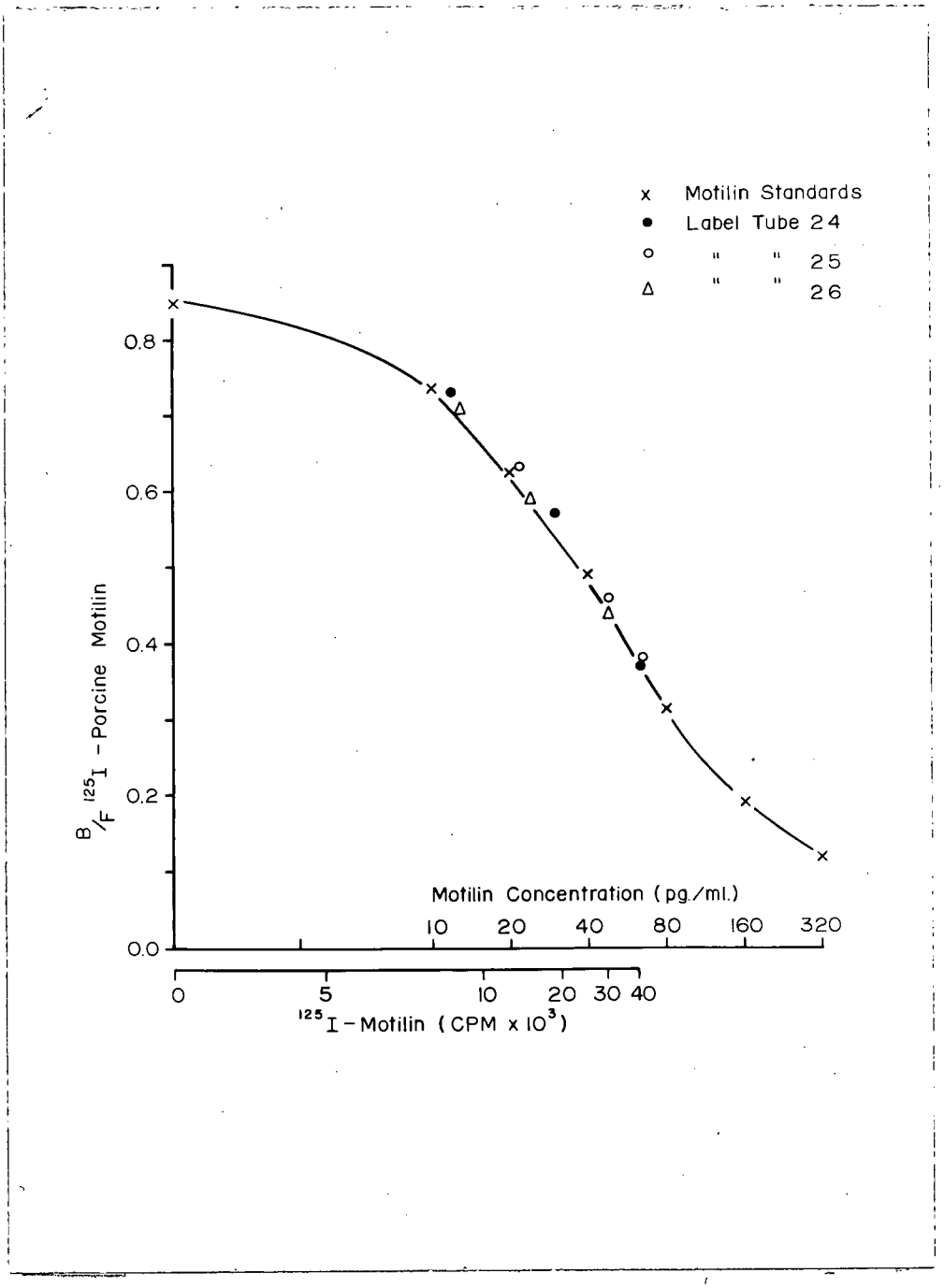

Fig. 5 Standard curve for motilin (x–x) in comparison with label fractions 24, 25, 26. Dilutions of each fraction from 8 x 10^3 cpm to 40 x 10^3 cpm were added and the dilution of Fraction 26 containing 25 x 10^3 cpm was fitted to the standard curve, the other fractions being fitted accordingly. (Dryburgh and Brown. Gastroenterology 68:1169-1175, 1975).

1 mg. motilin $= \frac{6.43 \times 10^{11}}{1.78 \times 10^{9}}$ mCi

$= 361$ mCi

∴ Specific activity of this iodination was 361 mCi/mg and the addition of 5000 cpm to each assay tube entailed the addition of 7.5 pg motilin.

The specific activity was not measured after every iodination but was checked at intervals and on every occasion when the routine iodination was varied in any way.

A rough estimate of specific activity may be calculated from the percentage of the radioiodine incorporated into the polypeptide,

Example (from column profile in Fig. 3)

$\%^{125}I$ incorporated into polypeptide = 71%

Specific activity of ^{125}I = 14 mCi/mg

∴ 1 mCi ^{125}I = 72 ng. Iodine

∴ 72 ng iodine was reacted with 2 µg motilin

i.e., 0.57 nM iodine was reacted with 0.74 nM motilin

∴ 0.40 nM iodine was incorporated into 0.74 nM motilin

i.e., 50 ng iodine was incorporated into 2 µg motilin

i.e., 0.7 mCi ^{125}I was incorporated into 2 µg motilin

i.e., 350 µCi ^{125}I was incorporated in 1 µg motilin

∴ Specific activity = 350 mCi/mg.

However, it must be remembered that this calculation depends on the assumption that iodine was incorporated into all the available polypeptide.

C. Production of Antisera to Motilin

(I) In guinea pigs

A series of guinea pigs (6) were immunized with pure porcine motilin (M_5). Conjugation of motilin to a large molecular weight protein was deemed advisable because of its low molecular weight.

Motilin was conjugated to bovine serum albumin by means of the carbodiimide condensation reaction (Goodfriend et al, 1964) using 1 - ethyl - 3 - (3 - dimethyl) - amino - propyl - carbodiimide (CDI) as follows: - 20 - 200 µg motilin per animal, 80 mg BSA and 100 mg CDI were dissolved in 100 µl, 10 mls and 1 ml deionized water, respectively. 0.5 mls each BSA and CDI were added to the motilin, mixed gently and left at room temperature for at least 1 hour. The reaction was terminated by dialysis of the reaction mixture against distilled water overnight at 4°C. The volume was corrected by addition of deionized water and then emulsified with Freund's Complete Adjuvant (FCA) at a 1:1 ratio. The final volume was selected to allow 0.5 ml emulsion per animal.

The animals were immunized subcutaneously, in several sites on the abdomen and inner thigh. An early observation suggested that better, more specific antisera were produced if the immunization with conjugated material was preceded by an initial "priming" dose of polypeptide alone in a FCA emulsion. The schedule followed is shown in Table I.

(2) In rabbits

Ten rabbits were immunized with conjugated motilin. The route of immunization was intradermally, in several sites, in the supra-scapular region.

TABLE I

MOTILIN GUINEA PIGS - IMMUNIZATION SCHEDULE

DATE	IMMUNIZATION	BLEEDING	TYPICAL TITRE
4.6.74	50 µf M_5/FCA		
20.6.74	100 µg M_5/BSA/FCA		
20.7.74	200 µg M_5/BSA/FCA	23.8.74	$1:40x10^3$
31.2.75	50 µg M_5/FCA	29.9.75	$1:20x10^4$
23.1.76	50 µg M_5/FCA	3.3.76	$1:10x10^5$
10.8.76	50 µg M_5/FCA	24.8.76	$1:10x10^5$

M_5 - the purest preparation of natural motilin

BSA - Bovine Serum Albumin

FCA - Freunds Complete Adjuvant

Blood was obtained by marginal ear vein venepuncture. The schedule followed is tabulated (Table II)

(3) Storage of Antisera

The whole blood samples were allowed to clot at 4°C for 20 mins., then centrifuged. The antiserum was stored frozen at - 20°C until it could be assessed. Usable antiserum was aliquotted in 200 - 500 µl portions, and lyophilized for storage at - 20°C. No detectable loss of specificity or affinity for at least 3 years has been observed.

As required, the lyophilized aliqots were reconstituted in assay diluent buffer at a dilution of 1:10 and stored at - 20°C in 100 µl aliquots. This material was viable during the period of its use, usually 2 - 3 months.

(4) Effect of varying antibody titre

Titre, in this connotation, is defined as the final dilution of antiserum in the incubation mixture. This allows for direct comparison between different antisera in different assay protocols where the final incubation volumes may differ. It must be established for each antiserum and checked after each immunization and its subsequent bleeding.

The initial procedure was to establish a serial dilution curve for the antiserum. Varying titres of the antiserum were incubated under routine assay conditions with ^{125}I - motilin and the maximum binding obtained with each dilution plotted as % bound versus the reciprocal of that titre. A typical dilution curve is shown in Fig. 6. From this the range of titre producing

TABLE II

MOTILIN RABBITS - IMMUNIZATION SCHEDULE

DATE	IMMUNIZATION	BLEEDING	TYPICAL TITRE
14.10.75	20 µg M_5/FCA		
13.11.75	50 µg M_5/BSA/FCA	24.11.75	
15.12.75	50 µg M_5/BSA/FCA	29.12.75	$1:10x10^2$
23. 1.76	50 µg N_5/FCA	13. 2.76	$1:10x10^4$
		8. 4.76	$1:20x10^4$

M_5 - the purest preparation of natural motilin

BSA - Bovine Serum Albumin

FCA - Freunds Complete Adjuvant

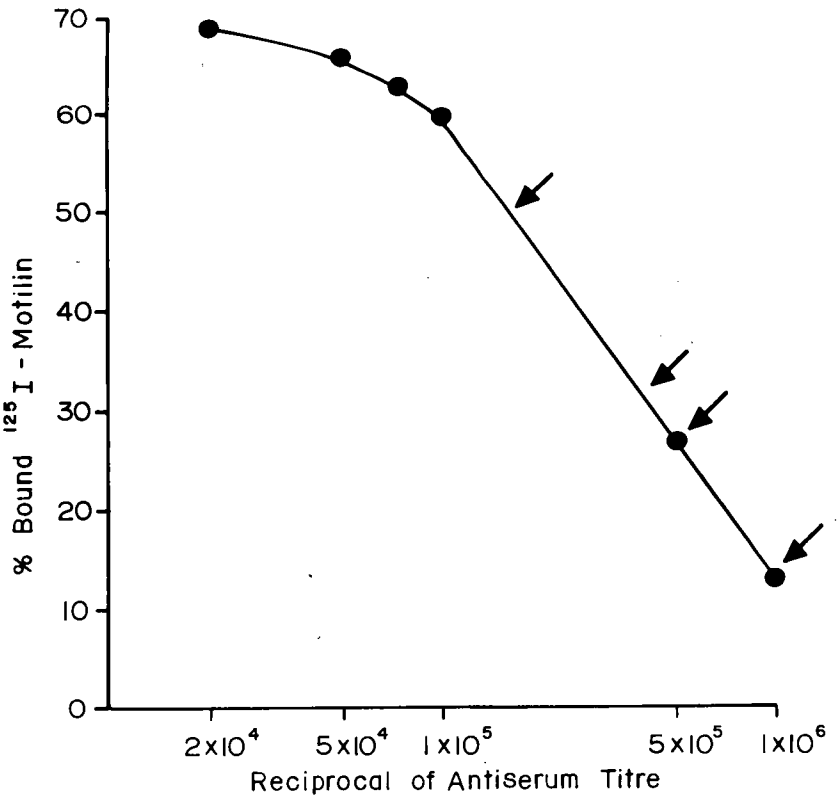

Fig. 6 Curve obtained with serial dilutions of motilin antiserum GP 71. Arrows indicate the titres selected for use in the standard curves shown in Fig. 7.

the most sensitive assay may be roughly estimated.

Various authorities have stipulated that the most effective assay is produced at the titre resulting in a maximum binding of 50%, or 33% (Berson & Yalow, 1958). However, it is becoming clear that no such hard and fast rule can be followed and that the optimum titre should be established for each RIA individually. Fig 7 is a comparison of the standard curves obtained with varying titres of the same antiserum shown in the dilution curve. The titres selected were those which resulted in binding 50%, 33%, 27% and 13% of the label. The results were plotted as $B/_{Bo} \times 100$ against the motilin standards and the curves evaluated by the criteria, slope at zero dose, midrange value and least detectable dose. The results are presented in Table III. The most effective titre was $1:5 \times 10^5$ - i.e., that producing a maximum binding of 27%. The most sensitive standard curves for the motilin RIA under the routine conditions were obtained when the maximum binding was 25 - 30%.

(5) Measurement of comparative immunoreactivity of antiserum

The comparative immunoreactivities of natural gastric inhibitory polypeptide, natural porcine secretin, natural (10% pure) cholecystokinin - pancreozymin, synthetic glucagon and synthetic human gastrin with motilin antisera were investigated. On a different date the comparative immunoreactivity of motilin antiserum with vasoactive intestinal peptide was examined. No significant cross-reactivity between the antiserum and any of these polypeptides was detected even when concentrations of up to 10 mg per incubation volume were employed. The results are illustrated in Figs. 8 & 9.

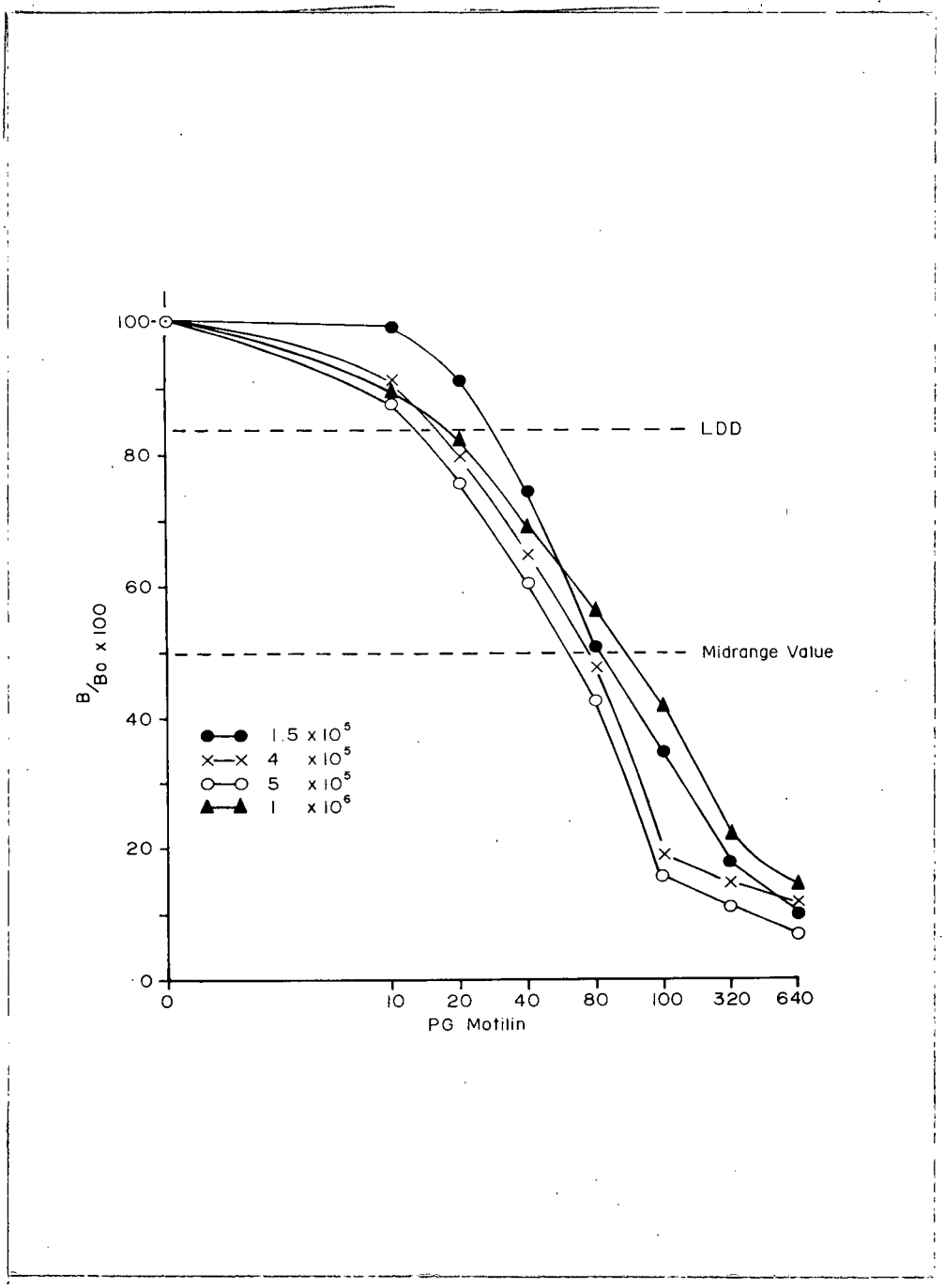

Fig. 7 Standard curves for motilin, demonstrating the effect of varying the antibody titre on the assay sensitivity. LDD = least detectable dose.

TABLE III

EFFECT OF VARYING ANTIBODY TITRE ON ASSAY SENSITIVITY

CONDITION:- TITRE	MAXIMUM BINDING(%)	SLOPE AT ZERO (L/MOLE)	MIDRANGE VALUE (PG MOTILIN)	L.D.D. (PG MOTILIN)
$1:1.5x10^{5}$	50	$0.6x10^{-13}$	82	40
$1:4\ x10^{5}$	33	$2.6x10^{-13}$	73	20
$1:5\ x10^{5}$	27	$4.0x10^{-13}$	60	20
$1:1\ x10^{6}$	13	$2.5x10^{-13}$	105	20

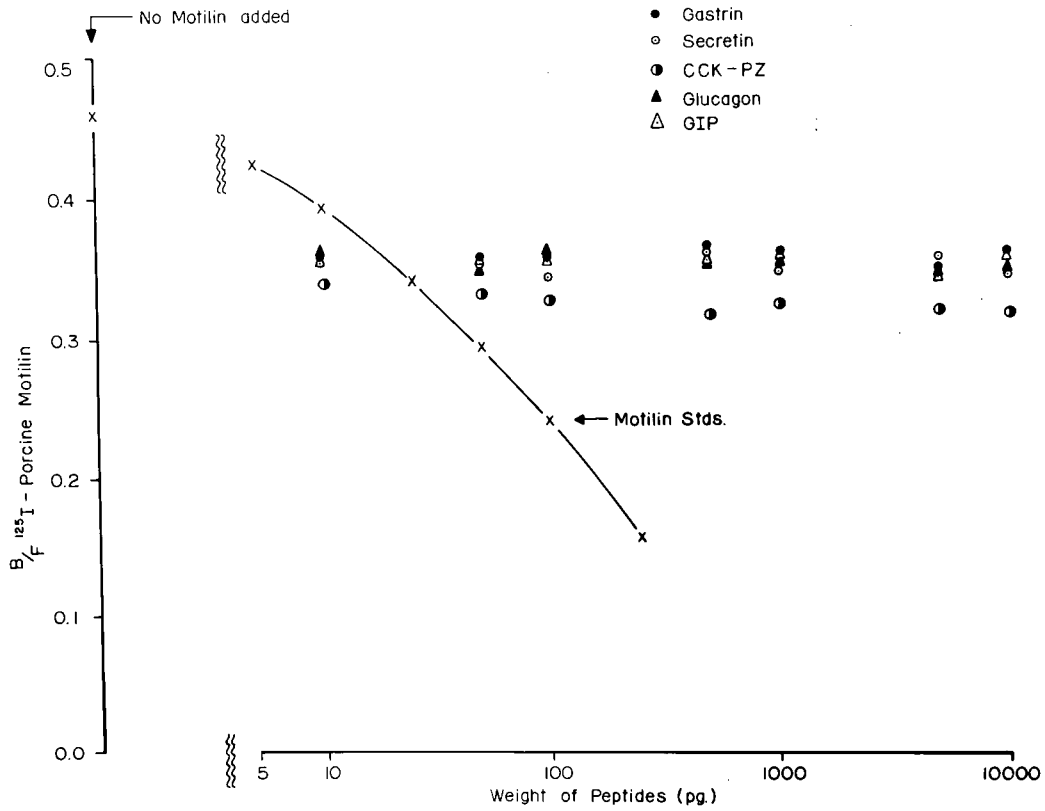

Fig. 8 Comparative immunoreactivities of natural motilin, synthetic human gastrin, synthetic glucagon, natural secretin, cholecystokinin-pancreozymin (10%) and natural gastric inhibitory polypeptide with antiserum to motilin. (Dryburgh and Brown, Gastroenterology 68 : 1169-1175, 1975).

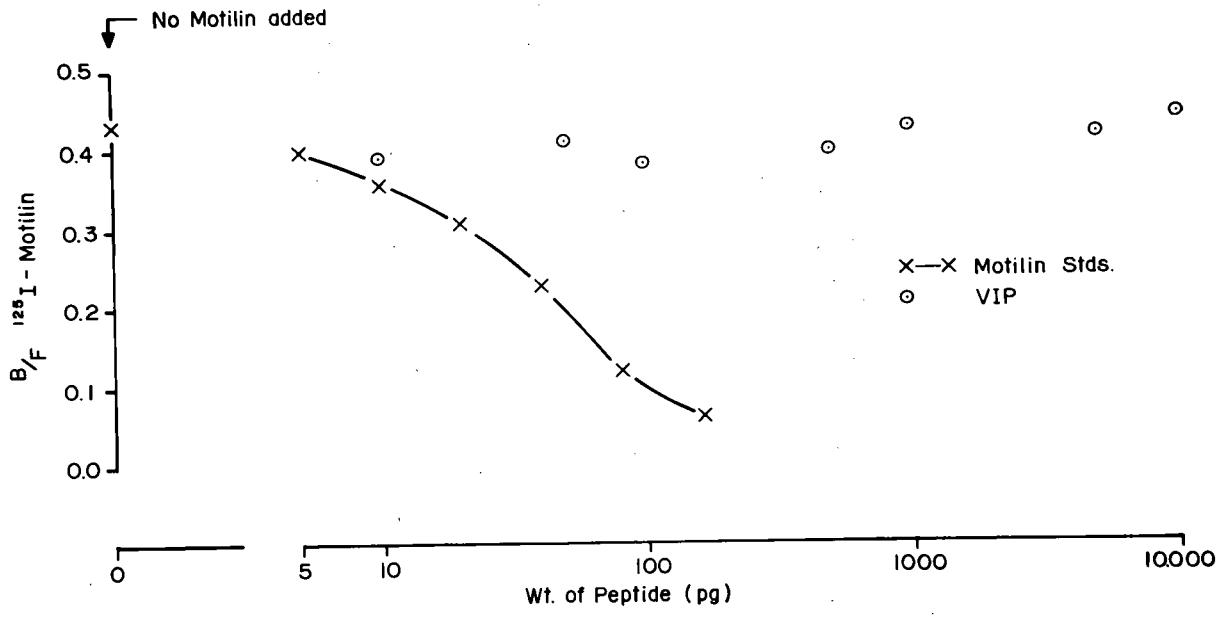

Fig. 9 Comparative immunoreactivities of natural motilin and vasoactive intestinal peptide with antiserum to motilin.

(6) Antibody recognition of antigen in standards and unknowns

Peak IR - motilin samples from dog experiments in which motilin was either endogenously released after duodenal alkalinization or exogenously administered by an intravenous infusion were serially diluted in assay diluent buffer or charcoal-extracted plasma respectively. After RIA, one value from each series was fitted to the standard curve and the remaining values plotted accordingly (Fig. 10).

Both serum dilution curves could be superimposed upon the standard curve indicating that the relationship between the antibody and the unlabelled antigen is unchanged whether the antigen is the isolated polypeptide in the standard preparation or the naturally occurring form in the unknown, i.e., in sera.

(7) Measurement of affinity of antiserum

From the 1st order Law of Mass Action the following equation was developed by Scatchard (1949):

$$B/_F = K([A^\circ] - B^\circ)$$

Where $B/_F$ is the ratio of Bound labelled antigen to Free labelled antigen $[A^\circ]$ is the concentration of total antibody; B° is the fraction of total antigen bound and K is the constant of the antibody - antigen reaction in the direction $Ab + Ag \rightarrow AbAg$.

In any individual assay K and $[A^\circ]$ are constant $\therefore$ $B/_F$ and B° may be expressed linearly in a Scatchard plot.

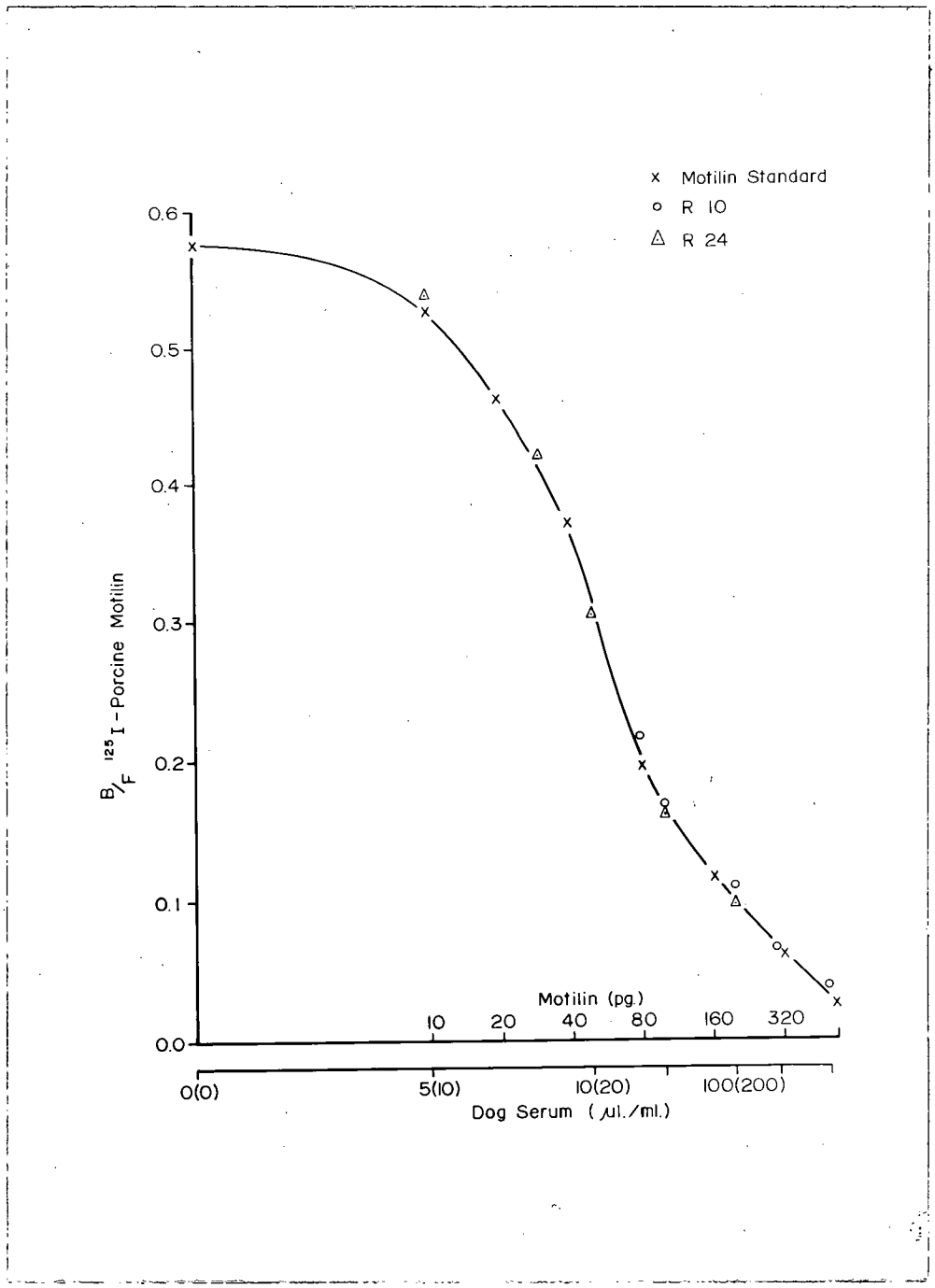

Fig. 10 Serum samples R 10 (exogenous motilin) and R24 (endogenous motilin) incubated at several dilutions in diluent buffer or charcoal-extracted plasma. The dilutions of R 10 and R 24 respectively, at 50 (100) µl/ml were fitted to the standard curve and the other dilutions fitted accordingly. (Dryburgh and Brown, Gastroenterology 68 :1169-1175, 1975).

In fact, any antiserum represents a population of antibodies of varying affinities and therefore the Scatchard plot is a curve composed of several straight lines. The highest affinity antibodies are represented by the line with the steepest slope.

The routine RIA standard curve was replotted as a Scatchard plot. The standard values were converted to absolute antigen values by addition of the amount of polypeptide incorporating the added radioactive tracer, calculated from the specific activity of that tracer. B° is the product of this value, in moles, and the concentration of label bound by it, i.e., B. K is the slope of the line produced by plotting $B/_F$ against B°. Figs. 11) shows the Scatchard plot of a typical antiserum of reasonable affinity.

D. Conditions of Radioimmunoassay

(1) Methods of standard curve evaluation

The criteria for evaluating the sensitivity of a standard curve are many and various. Three have been selected and a combination of at least two of these have been used in all comparisons.

The standard curves obtained were never linear so the "slope at zero dose" was estimated as a Scatchard plot and its slope measured as the plot approached zero (Feldman & Rodbard, 1971).

The least detectable dose (L.D.D.) was taken as that concentration of unlabelled antigen which produced a displacement of binding $\equiv$ 2 x the standard deviation at maximum binding.

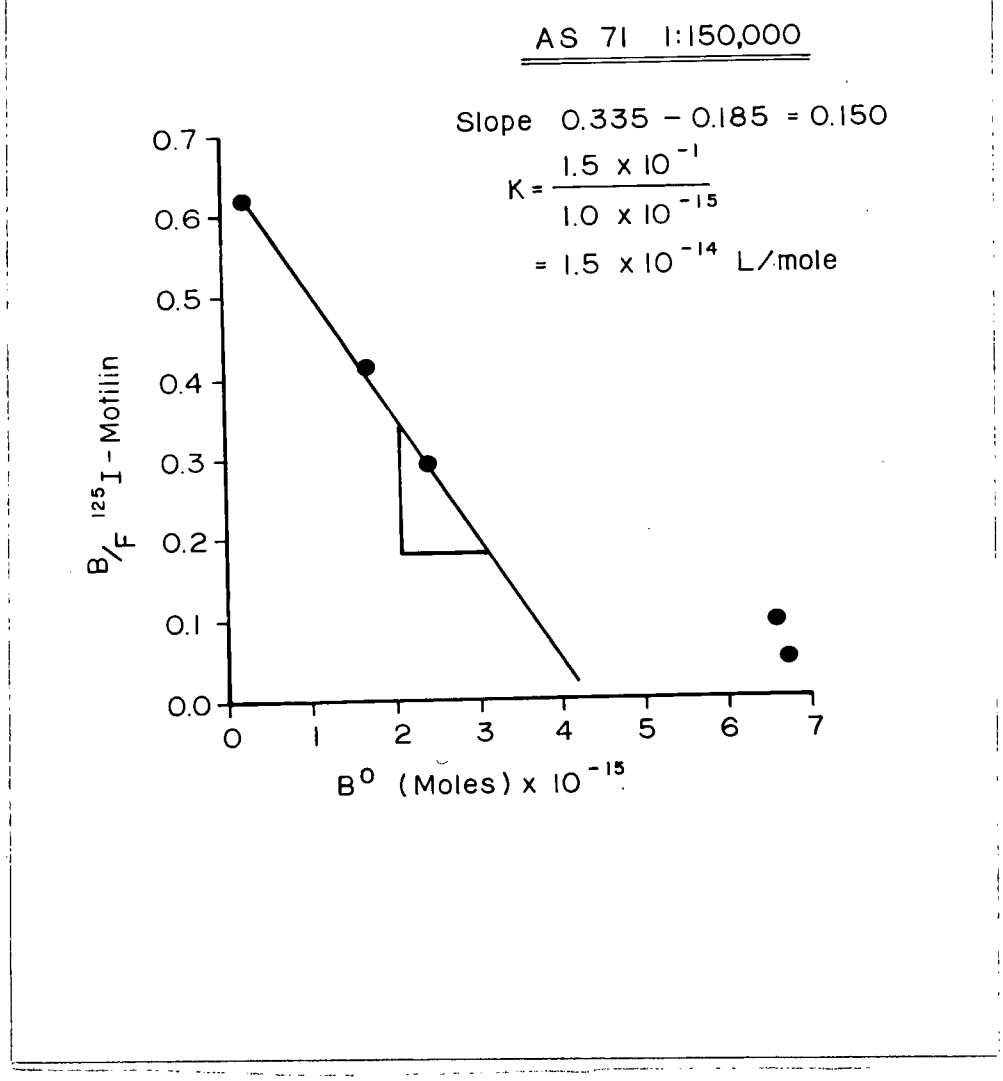

Fig. 11 Standard curves with motilin antiserum (GP 71) represented as a Scatchard plot, B/F being plotted against the fraction of total antigen bound (B°). The slope of the line gives the affinity constant of this antiserum (K).

Both these criteria evaluate the sensitivity of the standard curve at its upper limit. The third parameter, the midrange value, allowed comparison of standard curves in the region where they were most likely to approach linearity and was that concentration of unlabelled motilin which displaced 50% of the maximum label bound.

(2) pH of the diluent buffer

Motilin standards in the range 12.5 - 400 pg were incubated with antiserum 75A at a final dilution of $1:80x10^3$ for 48 hours at 4^oC in 0.04M sodium phosphate buffer at either pH 6.5 or 7.5, and in veronal buffer, 0.05M at pH 8.5. There was no significant difference in the displacement observed either at the midrange value or the L.D.D.

(3) Trasylol concentration in the diluent buffer

Trasylol is a broad spectrum proteolytic enzyme inhibitor, containing 10,000 kallikrein inhibitor units (KIU) per ml. Standard curves were incubated in 0.04M sodium phosphate buffer, pH 6.5, containing 0%, 0.25%, 0.5% or 1.0% Trasylol. The resultant displacement is shown in Fig.12 and Table IV and 0.25% Trasylol was selected as the optimum concentration.

TABLE IV - Effect of varying the Trasylol concentration in the diluent Buffer

Condition	Midrange (pg M5)	L.D.D. (pg M5)
0.0% Trasylol	70	25
0.25% Trasylol	24	12.5
0.5% Trasylol	47	25
1.0% Trasylol	135	50

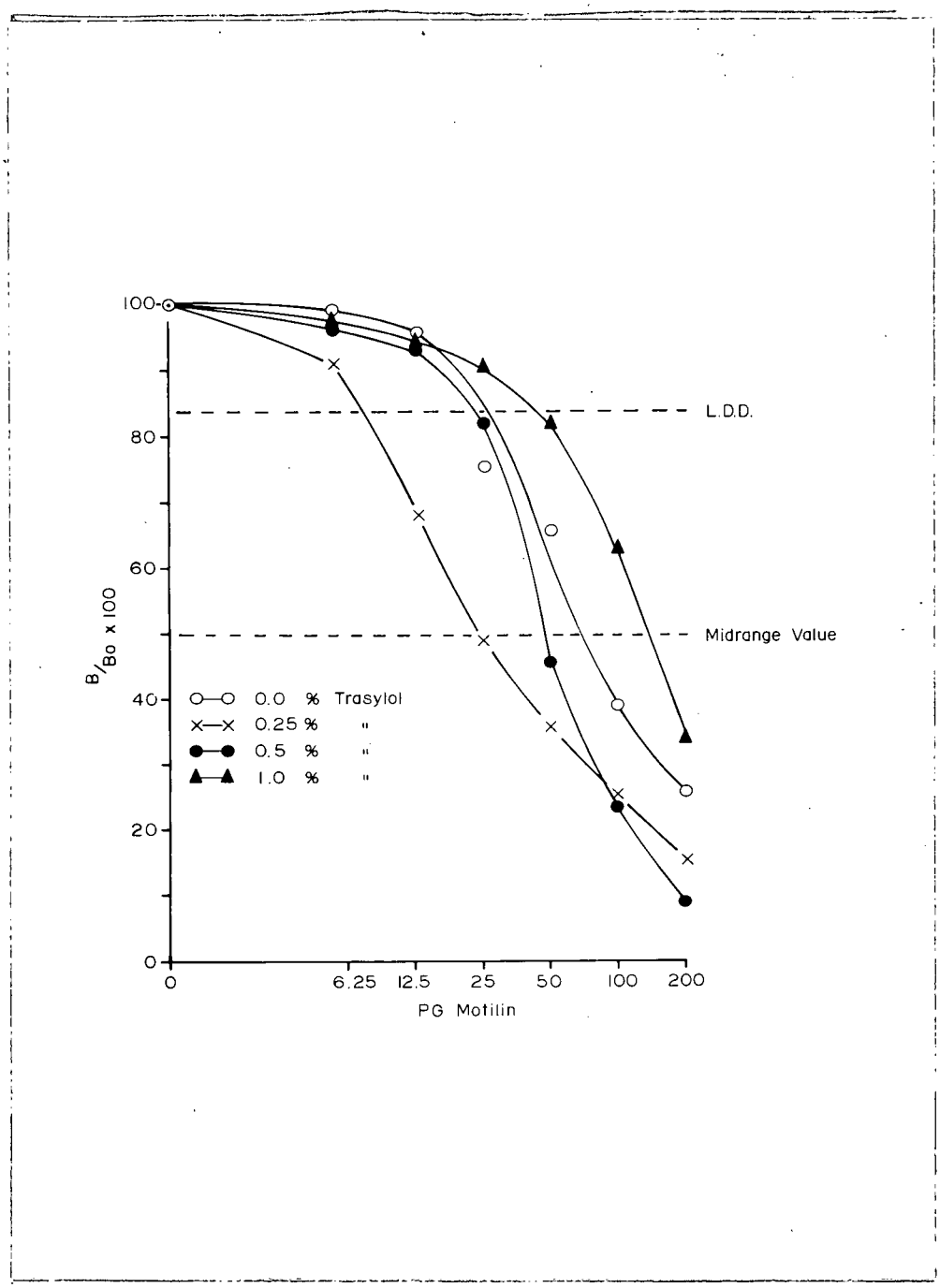

Fig. 12 Effect of varying the Trasylol concentrations in the diluent buffer on the sensitivity of the routine standard curve for motilin.

(4) Plasma concentration in the diluent buffer

Standard curves were set up to compare the effect of varying the plasma concentration in the diluent buffer, from 2 - 10%

The plasma was outdated blood bank stock which had been extracted twice with 1% (w/v) charcoal for 1 hour at 4°C to absorb any small peptides still present. The charcoal was removed by centrifugation at 5000 r.p.m. for 20 mins. The charcoal-extracted plasma was screened for detectable polypeptide levels and stored at -20°C. The greatest sensitivity was achieved when the buffer contained 5% charcoal-extracted plasma (shown in Table V).

TABLE V - Effect of varying the plasma concentration in the diluent buffer

Condition	Midrange (pg M5)	L.D.D. (pg M5)
2% plasma	55	25
5% "	55	12.5
10% "	105	25

(5) Concentration of labelled antigen added

Labelled antigen at concentrations varying from 2000 c.p.m./100 µl to 20,000 c.p.m./100 µl was added to standard curves in the routine assay. The concentration of~ 5000 c.p.m./100 µl was selected as giving the most sensitive curve at the most efficient counting rate (Table VI, Fig. 13)

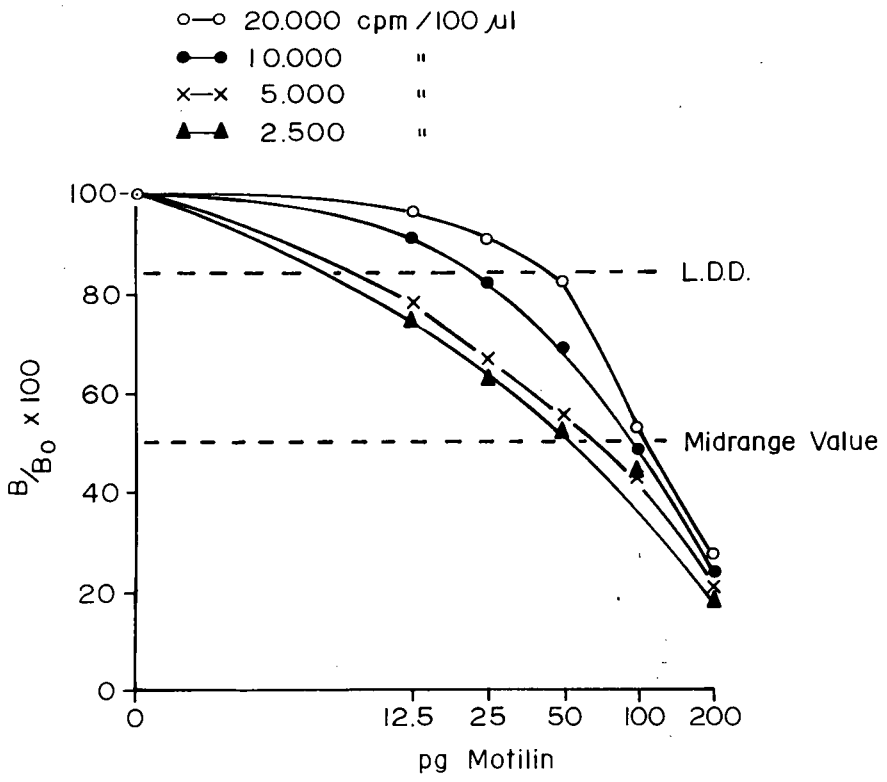

Fig. 13 Effect of varying the concentration of labelled antigen on the sensitivity of the routine standard curve for motilin.

TABLE VI - Effect of varying the antigen concentration on the assay sensitivity

Condition	Midrange	L.D.D. (pg M5)
20,000 cpm/100 µl	105	50
10,000 "	94	25
5,000 "	67	12.5
2,500 "	54	12.5

(6) Period of incubation

Standard curves, a, b and c were set up and iodinated antigen, containing ∿ 5000 cpm/100 µl was added immediately to a and b which were then incubated for 24 hours and 48 hours respectively. Standard curve c was incubated with antibody alone for 24 hours. After label addition the incubation continued for a further 48 hours.

A 48 hour incubation period was deemed to give a more sensitive assay, from the results in Fig. 14 and Table VII. There was no advantage gained by prior incubation of cold antigen with antibody, i.e., under disequilibrium conditions. Longer incubation periods of 3 - 4 days were also satisfactory.

TABLE VII - Effect of varying the incubation period and type

Condition	Midrange (pg M5)	L.D.D. (pg M5)
24 hour equilibrium	145	50
48 " "	53	25
24/48 " dis-equilibrium	53	25

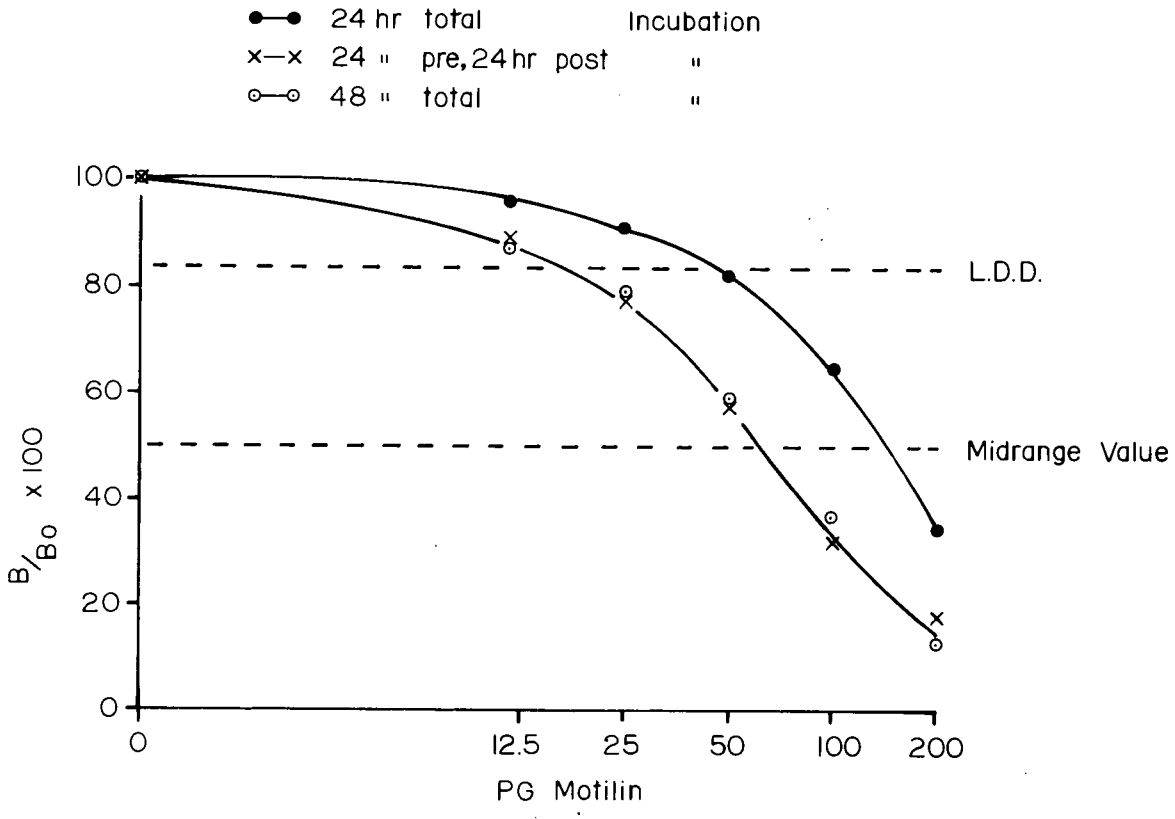

Fig. 14 Effect of varying the length and type of incubation on the sensitivity of the routine standard curve for motilin.

(7) Protection from adsorption to glass

Adsorption of peptide and iodinated material on to the glass tubes, used in the assay, can be a problem. The plasma content of the diluent buffer did reduce the counts adsorbing to the glass but siliconization of the assay tubes with 1% (v/v) dichlorosilane in benzene was also performed to see if any further improvement could be achieved.

It was also essential to determine whether it was necessary to compensate in the standard curve for the extra protein added in the monitoring of plasma or serum samples. Standard curves were incubated in siliconized tubes, non-siliconized tubes and in non-siliconized tubes with the addition of 100 µl of charcoal - extracted plasma. The results obtained suggested that siliconization of the tubes was not necessary. The standard curves obtained after incubation with and without plasma were corrected for their individual non-specific binding (see sections 7 and 10) and no significant difference was detected - i.e., the addition of plasma was unnecessary. (Table VIII, Fig. 15)

TABLE VIII

Effect of siliconization or plasma addition on assay system

Condition	Midrange (pg M5)	L.D.D. (pg M5)
Siliconized tubes	59	25
Non-siliconized tubes	57	125
Non-siliconized tubes & plasma	56	25

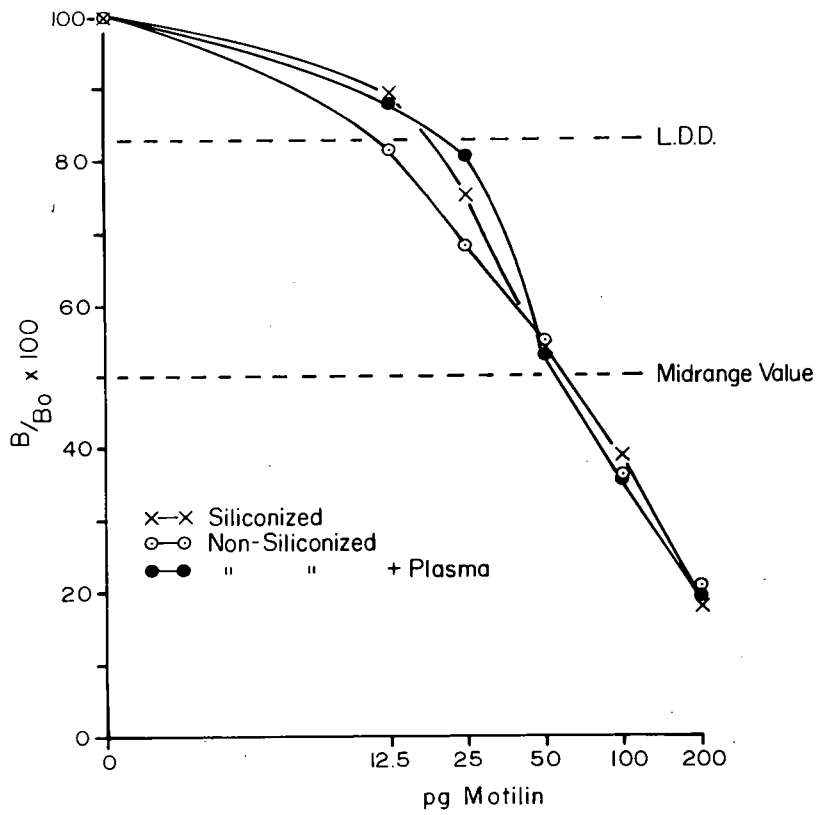

Fig. 15 Effect of siliconization of the incubation tubes or the addition of plasma on the sensitivity of the routine standard curve for motilin.

(8) Routine assay conditions

The diluent buffer, 0.04 M sodium phosphate, pH 6.5, containing 5% charcoal - extracted plasma and 0.25% trasylol, was used in all dilutions and for correcting the final volume to 1.0 ml. The composition of the incubation mixture was:-

100 µl ^{125}I-motilin containing ~5000 c.p.m.

100 µl standard motilin, range 12.5 - 400 pg.

or

100 µl interassay control

or

50 - 200 µl unknown

100 µl antiserum at the appropriate initial dilution

Diluent buffer to a volume of 1.0 ml

All assays were set up, with standards in triplicate and unknowns in duplicate, in 10 x 75 mm glass culture tubes at 4°C. and incubated at 4°C for 48 - 72 hours.

Non-specific binding (N.S.B.) was measured by setting up tubes, minus antiserum, for the standard curve, the interassay controls, each group of sera from one subject and all other unknowns.

In assays where only one separated component was to be counted (see section 9) total count tubes, containing 100 µl ^{125}I - motilin only, were set up in quadruplicate. Table IX illustrates the assay layout.

LAYOUT FOR ROUTINE RADIOIMMUNOASSAY

	D.B.	LABEL	ANTIBODY	STD	CONTROL	UNKNOWN
Total counts	-	100 *	-	-	-	-
Standard curve NSB	900	100	-	-	-	-
Maximum binding	800	100	100	-	-	-
STDS	700	100	100	100	-	-
Interassay control NSB	800	100	-	-	100	-
Interassay control	700	100	100	-	100	-
Unknown NSB	800	100	-	-	-	100
Unknown	700	100	100	-	-	100

* refers to volume in µl

(9) Separation procedures

Both specific and non-specific methods exist for the separation of the antigen/antibody complex (Bound) from the Free antigen. The specific methods include the double antibody technique and the use of a solid phase antibody matrix where the antibody is coupled to an immunologically inert material - e.g., Sephadex, Sepharose or Polyacrylamide. The use of Sepharose-coupled antibody in the motilin R.I.A. is described in the section on affinity chromatography.

The non-specific methods include the addition of alcohol resulting in the precipitation of large molecular weight proteins, including the antigen/antibody complex and the use of dextran-coated charcoal which will adsorb the free antigen, leaving the antigen/antibody complex in solution. This last method is that most commonly used in this laboratory.

Phosphate buffer, 0.04M, pH 6.5, containing 2% plasma, was cooled to 4°C. The dextran was mixed well to ensure a complete suspension before the addition of charcoal. The suspension was mixed gently at 4°C for at least 1 hour prior to the addition of 200 µl to each assay tube, excluding the total count tubes. After being vortexed briefly, the tubes were centrifuged at 2800 rpm for 20 min. The supernatant was then decanted into a separate tube (for $B/_F$ estimations). Each tube was sealed with wax and counted in an automatic γcounter.

Various charcoal concentrations were examined, each coated with 10% (w/w) dextran. The results are graphed in Fig. 16 and evaluated in Table X.

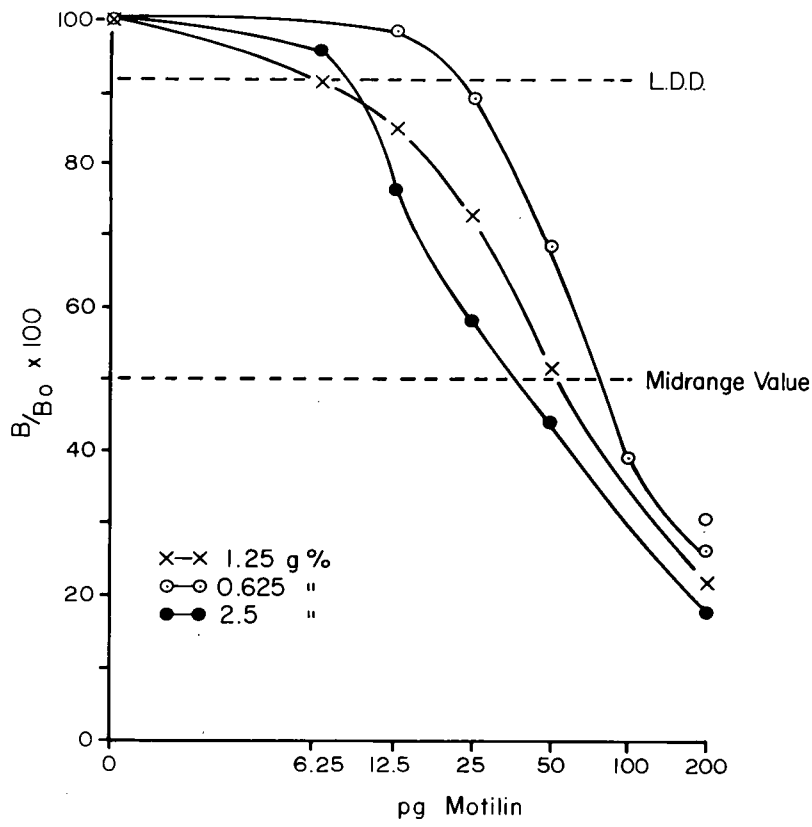

Fig. 16 Effect of varying charcoal concentrations in the separation procedure on the sensitivity of the routine standard curve for motilin. At each concentration the charcoal was coated with 10% (w/w) dextran T-70

1.25g% (w/v) charcoal was finally selected because it was the concentration producing the greatest sensitivity at the upper limit of the curve.

TABLE X

Effect of varying the charcoal concentration in the separation procedure

Condition	Midrange (pg M5)	L.D.D. (pg M5)
0.625g% charcoal	70	25
1.25 g% "	52	6.25
2.5 g% "	35	12.5

Recently, the dextran-coated charcoal suspension has been prepared, 1 litre at a time, in phosphate buffer only, and mixed for 3-4 hours. As required, an appropriate volume was removed, the plasma added, and the suspension mixed for ~ 15 mins. before use. This suspension keeps well at 4°C for 1-2 weeks and provides a more homogenous suspension, as demonstrated by an improvement in the replication of triplicate and duplicate values.

(10) Methods of data analysis

There are numerous methods used in the expression of RIA results. Those used in this study include $B/_{F}$, $B/_{TC}$ or % B, $B/_{Bo}$ x 100 and all include a correction to account for the non-specific binding of labelled antigen to glass or plasma protein.

The calculation of $B/_F$ requires the counting of both the Bound and Free antigen after separation and is obtained from the expression:

$$B/_F = \text{SAMPLE } \frac{(\text{Bound c.p.m.})}{(\text{Free c.p.m.})} - \text{NSB } \frac{(\text{Bound c.p.m.})}{(\text{Free c.p.m.})}$$

The other methods require that only one component is counted after separation but do require some method of estimating the Total Counts (TC) initially added to each tube. If dextran-coated charcoal is the method of separation used it is more convenient to count the Free antigen in the charcoal pellet. Therefore, %B is calculated from the expression:

$$\%B = \text{SAMPLE } \frac{(TC - \text{FREE c.p.m.})}{TC} - \text{NSB } \frac{(TC - \text{FREE c.p.m.})}{TC}$$

$$\text{i.e., } \%B = \frac{(\text{NSB}) \text{ FREE c.p.m.} - (\text{SAMPLE}) \text{ FREE c.p.m.}}{TC}$$

Results may also be expressed as a percentage of the maximum binding - i.e., $B/_{B_0} \times 100$, where B_0 is the binding of label achieved when no unlabelled antigen is added to the system.

Standard curves are prepared by plotting one of these values against the concentration of standard antigen, expressed either logarithmically or arithmetically. Fig. 17 is a routine standard curve for motilin obtained after all the conditions for a sensitive RIA had been established.

All these conditions were established for RIA with a specific antiserum to

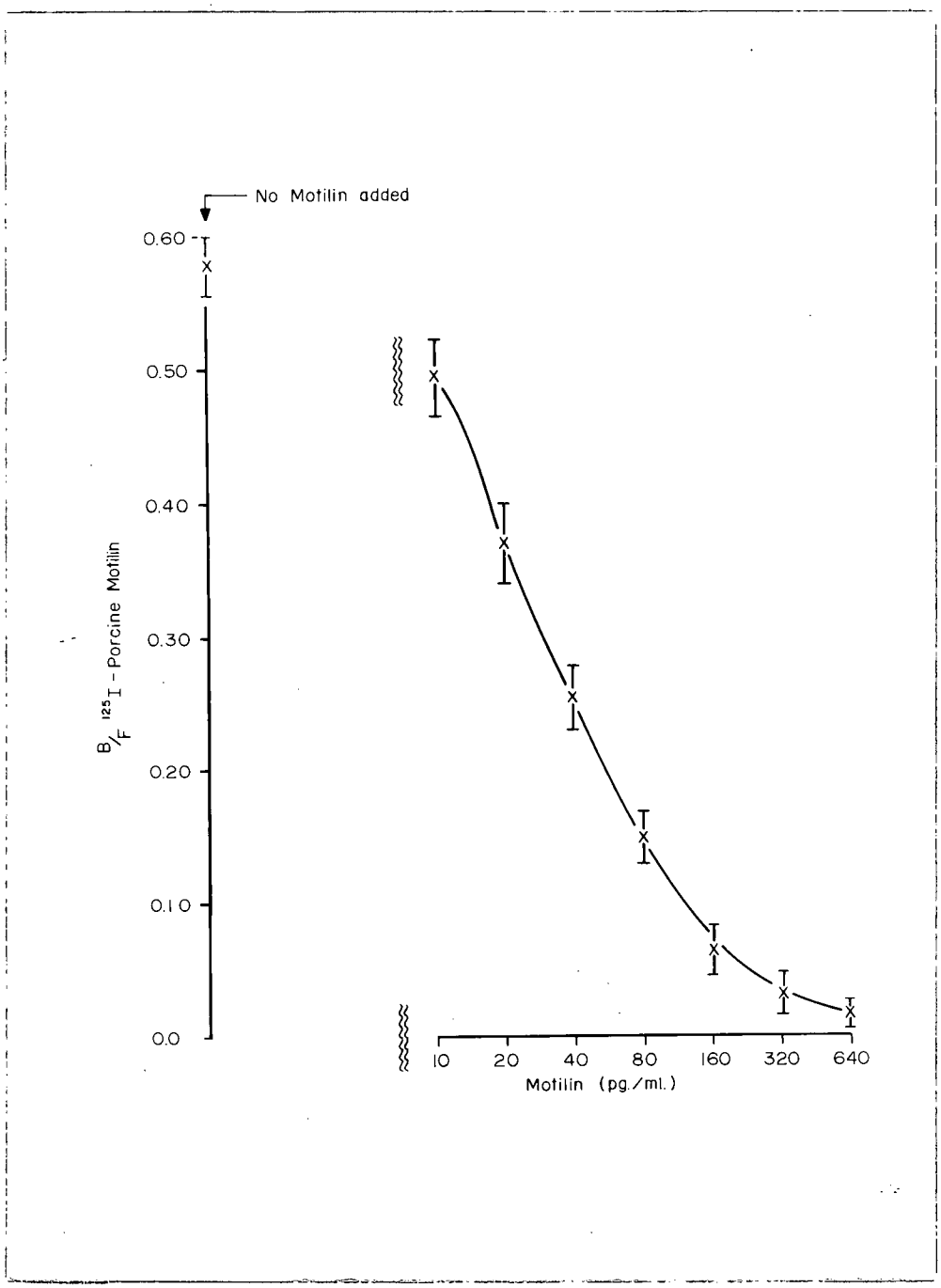

Fig. 17 Routine standard curve for motilin, obtained after the optimum conditions had been established. Each point represents the mean (± SD) for 7 observations; (Dryburgh and Brown, Gastroenterology 68: 1169-1175, 1975)

motilin; they do not necessarily hold true for all motilin antisera and should be re-evaluated for each antiserum.

E. Assay standards and interassay controls

(I) Preparation and storage of standards

Natural porcine motilin (M5) was used in all standard preparations. One - two mg were weighed accurately on a Cahn electrobalance, dissolved in deionized water to give a concentration of 1 µg/100 µl and aliquotted in 100 µl amounts into siliconized glass culture tubes for lyophilization and storage at -20°C.

Each month, or as required, a 1 µg aliquot was reconstituted in 0.04M sodium phosphate buffer, pH 6.5, containing 0.25% trasylol and 5% BSA, to a concentration of 80 ng/ml. This solution was stored at -20°C in 1.0 ml amounts in polyvinyl microtest tubes.

At the time of assay an aliquot was diluted 1:20 - i.e., 400 pg/100 µl and serial dilutions prepared over the range 6.25 - 400 pg/100 µl. Any remaining standard was discarded after thawing.

(2) Preparation and storage of controls

One µg aliquots of M5 were diluted in 0.04M phosphate buffer, pH 6.5, containing 0.25% trasylol and 5% BSA, to a concentration of 1 ng/ml. One ml aliquots were stored at -20°C in polyvinyl microtest tubes and 100 µl samples were assayed at the beginning and end of every assay. Any remaining control was discarded after being thawed.

(3) Inter-and intra-assay control

In 5 different assays, 20 duplicate determinations of the control value were made. The mean $\pm$ S.D. was 110 $\pm$ 23 pg motilin/100 µl. Results in any assay in which the control lay outside these values were discarded. Intra-assay variability was checked by having duplicate measurements of the control at the beginning and end of each individual assay and applying the same conditions to their evaluation.

PREPARATION OF SYNTHETIC AND NATURAL MOTILIN FRAGMENTS AND ANALOGUES

A. Synthetic motilin

(1) Preparation of 13 - norleucine - motilin

The synthetic analogue, 13 - norleucine - motilin was prepared in the laboratory by Dr. E. Wünsch, Max-Planck Institute für Eiweiss und Lederforschung, Munich, W. Germany (Wünsch et al, 1973). The RIA was used to monitor the final purification stages.

The initial crude synthesis product, MoA was separated by column chromatography on QAE Sephadex A-25 into MoB_1 and MoB_2, the latter being found to represent a failed synthesis, lacking 2 amino acid residues (-THR -TYR). Further purification of MoB_1 on SP - Sephadex C-25 resulted in MoC_1 and MoC_2, both synthetic products being identical to natural motilin with respect to amino acid composition and sequence.

(2) Preparation of synthetic motilin fragments

During the preparation and purification of the synthetic analogue, the fragments containing residues 9-22 and 13-22 were also isolated and purified.

B. Fragments of natural motilin

(I) Cyanogen bromide cleavage of motilin

Cyanogen bromide (CNBr) treatment of a polypeptide results in chemical cleavage of that polypeptide at the methionyl residue (Gross and Witkop, 1961, 1962). The reaction was performed in 70% (v/v) formic acid at a polypeptide concentration of 2.0 mg/ml and a CNBr concentration of 10 mg/ml. The reaction flask, foil-covered to exclude light, was left at room temperature for 6 hours, then the contents were diluted 1:20 with distilled water prior to lyophilization.

The immunological activities of the intact motilin molecule and the unseparated mixture of cleaved and non-cleaved CNBr-treated motilin were compared on an equimolar basis.

(2) Tryptic and chymotryptic digestion of motilin

Enzymatic cleavage of the polypeptide was performed in 1% ammonium bicarbonate at a polypeptide concentration of 0.2% (w/v) and an enzyme:substrate ratio of 1:50 (w/w). The reaction proceeded for 6 hours at room temperature and was terminated by lyophilization, redissolving in 0.5 ml water and boiling for 6 mins. in a water bath. The solution was centrifuged to remove any precipitation and the supernatant was lyophilized.

Biological and immunological activities were estimated for the unseparated digestion products and compared with the intact molecule on an equimolar basis.

C. Modifications of natural motilin

(I) C-terminal residue removal

The reaction involved treatment of 100 nM motilin in 0.1M ammonium bicarbonate with 200 µg carboxpeptidase A 'DFP' (Diisopropyl phospho-floridate treated) in 2.0M ammonium bicarbonate at a peptide: substrate ratio of 38:2 (v/v) for 6 hours. Kinetic studies had shown that after 6 hours at 22°C 80% of the C-terminal glutamine and 20% of the penultimate C-terminal residue, glycine had been removed. The reaction was terminated by lyophilization of the mixture.

(2) N-terminal residue removal

Removal of the N-terminal phenylalanine was achieved by one cycle of the Edman degradative procedure (Edman, 1956; Gray, 1967). Coupling of the phenylisothiocyanate (PITC) was accomplished by dissolving 100 nM of the polypeptide in 150 µl deionized water in an acid-washed 12 x 75 mm glass culture tube. Reagent (5% PITC in pyridine) was added and the tube was flushed with nitrogen to expel the air, and covered with parafilm prior to incubation at 45°C for 1.5 hours. At the end of this time the tube was uncovered after centrifugation, and dried over fresh phosphorus pentoxide, under vacuum at 60°C. The coupled phenylthiocarbamyl residue was cleaved from the peptide by treatment with 150 µl trifluoroacetic acid and incubation at 45°C for 30 mins. The mixture was evaporated to dryness in a vacuum

dessicator over sodium hydroxide pellets, then was redissolved in 200 µl deionized water. The free phenylthiocarbamyl phenylalanine was removed by extracting four times with 2 mls butyl acetate, the upper organic layer being discarded each time. The remaining aqueous solution, containing motilin 2-22 was evaporated to dryness under vacuum, over concentrated sulphuric acid, redissolved in 150 µl deionized water and a 10 µl aliquot removed for dansyl chloride determination of the new N-terminal residue, to check for completeness of the degradation cycle.

(3) Identification of N-terminal residue

The method of Gray (1967) was followed with the modification of Bruton and Hartley (1970) of using 5 x 5 cms polyamide plates.

The polypeptide, approximately 5nM, was transferred to a glass tube (pyrex) 0.6 x 50 mms, and lyophilized, 2 µl 1% sodium bicarbonate were then added and the tube was centrifuged and relyophilized. Deionized water and dansyl chloride, (dimethylnaphthalene - 5 - sulphonyl chloride, 2.5 mg/ml in acetone) 2.5 µl each were added to the tube which was centrifuged, covered with parafilm and incubated at 45°C for 20 mins. The contents were again lyophilized and redissolved in 50 µl 6M hydrochloric acid, the tube being then heat sealed and hydrolized at 110°C for 18 hours.

When acid hydrolysis was complete the tube was centrifuged, opened and dried under vacuum over sodium hydroxide pellets.

Thin layer chromatography on 5 cm^2 polyamide plates, in several solvent systems was performed. The hydrolysis products were dissolved in 2.5 µl

50% aqueous pyridine and ~ 0.5 µl spotted on each side of the plate. A standard solution (0.5 µl) containing the dansyl derivatives of phenylalanine, isoleucine, proline, glycine, glutamic acid, serine and arginine, 1 µM each acid/ml in acetone: 0.1M acetic acid (3:2 v/v) was spotted on one side only. The polyamide plate was subjected to ascending chromatography in two dimensions in the appropriate solvent systems.

Dimension		Solvent system
I	I	Water: 90% formic acid 200 : 3 (v/v) (Woods and Wang, 1967)
2	II	Benzene: glacial acetic acid 9 : 1 (v/v) (Woods and Wang, 1967)
2	III	Hexane: n-butanol: glacial acetic acid 3 : 3 : I (v/v) (Crowshaw et al,1967)
2	IV	0.1% Ammonia: Ethanol 9 : 1 (v/v)

After running in solvents I and II, the plates were viewed under short-wave ultra violet light and identification of the dansylated residue made. Dansyl serine/dansyl theonine, dansyl glutamic acid/ dansyl aspartic acid and

dansyl glycine/dansyl alanine may only be differentiated after chromatography in solvent III and solvent IV was used to separate the basic residues arginine, histidine and Σ-lysine.

If lysine or tyrosine was present at any position in the polypeptide, Σ-dansyl-lysine or O-dansyl tyrosine was always seen. Positive identification of either lysine or tyrosine as N-terminal requires the presence of bis-dansyl-lysine or bis-dansyl-tyrosine respectively.

If proline is the N-terminal amino acid, the hydrolysis must be limited to a 4 hour period.

(4) Acylation-acetylated derivative

Acetylation of the polypeptide with acetic anhydride was performed by a modification of the method of Riordan and Valee (1967). One mg motilin was dissolved in 1.0 ml 50% saturated sodium acetate. A thirty-fold molar excess of acetic anhydride was added in five portions over 1 hour and the reaction mixture was stirred continuously at room temperature. After a further hour the reaction mixture was frozen, lyophilized and desalted on Sephadex G15 fine (0.7 x 100 cms) in 0.2M acetic acid at a flow rate of 6 mls/hour and 1.2 ml fraction size. The acetylated derivative was compared with natural motilin, on an equimolar basis, for biological and immunological activity.

(5) Acylation-succinylated derivative

The method of Klotz (1967) was slightly modified. One mg motilin was dissolved in 2.0 mls deionized water, the pH brought to 7.0 with 0.1M sodium hydroxide and constantly monitored during the addition of a thirty-fold molar excess of succinic anhydride over 15 mins. The pH was maintained at 7.5 by addition of 0.1M sodium hydroxide and the mixture was stirred gently at room temperature for a further 1.5 hours. The solution was frozen, lyophilized and desalted on Sephadex G15 fine, as described for the acetylated derivative.

AFFINITY CHROMATOGRAPHY

A. Activation of Sepharose 4B

Equal volumes (~ 20 mls) of Sepharose 4B (Pharmacia, Uppsala, Sweden) slurry and deionized distilled water were mixed together gently over ice, in a fume hood. Cyanogen bromide (CNBr) at a concentration of 100 mg/g Sepharose 4B was added in a volume of deionized water, equal to the total, and the pH was immediately raised to pH 9 - 11 and maintained in this range by the addition of 4.0M sodium hydroxide. When the pH had remained stable for 10 mins. with no further addition of alkali, the reaction was considered terminated. Ice was added to the mixture and the gel was washed on a Buchner filter under gentle suction with at least 10 volumes of cold 0.1M sodium bicarbonate. The CN Br-activated Sepharose 4B could be stored as a moist slurry at 4° for 1-2 weeks (Cuatrecasas, 1970).

B. Coupling of ligand to activated Sepharose 4B

Activated Sepharose 4B was made up in an equal volume of 0.1M sodium bicarbonate. Antiserum to motilin or GIP was diluted in an equivalent volume of bicarbonate to a final concentration of 30 µl antiserum/g Sepharose 4B. The reaction mixture was stirred gently for 24 hours at 4°C. The coupled gel was washed well with 20 volumes cold deionized water on a Buchner filter. Aliquots of the diluted antiserum, prior to coupling, and the initial wash, after coupling, were put aside for RIA, to determine the efficacy of the coupling reaction. Figs. 18 and 19 illustrate the anti-serum dilution curves obtained in a typical procedure, with virtually no antibody activity detectable in the wash.

Unreacted active groups on the gel matrix were blocked by treatment with excess ethanolamine as follows: ethanolamine (MW 61.1, 16.4M, pH 12.7) was brought to pH 9.0 by addition of 5.0 hydrochloric acid. Sufficient ethanolamine was added to a known volume of coupled Sepharose 4B such that the final molarity, with respect to ethanolamine, was 1.0M. The reaction was complete after 4 hours at 4°C and the excess ethanolamine removed by washing the gel with 10 alternating cycles of 0.1M sodium acetate, pH 4.0, and 0.1M sodium phosphate, pH 8.0. The final product was stored in an equal volume of 0.1M sodium bicarbonate at 4°C. At this stage 0.01% sodium azide was added as a preservative.

COLUMN CHROMATOGRAPHY

A. Gel filtration

The technique of using a cross-linked dextran gel as a molecular sieve was

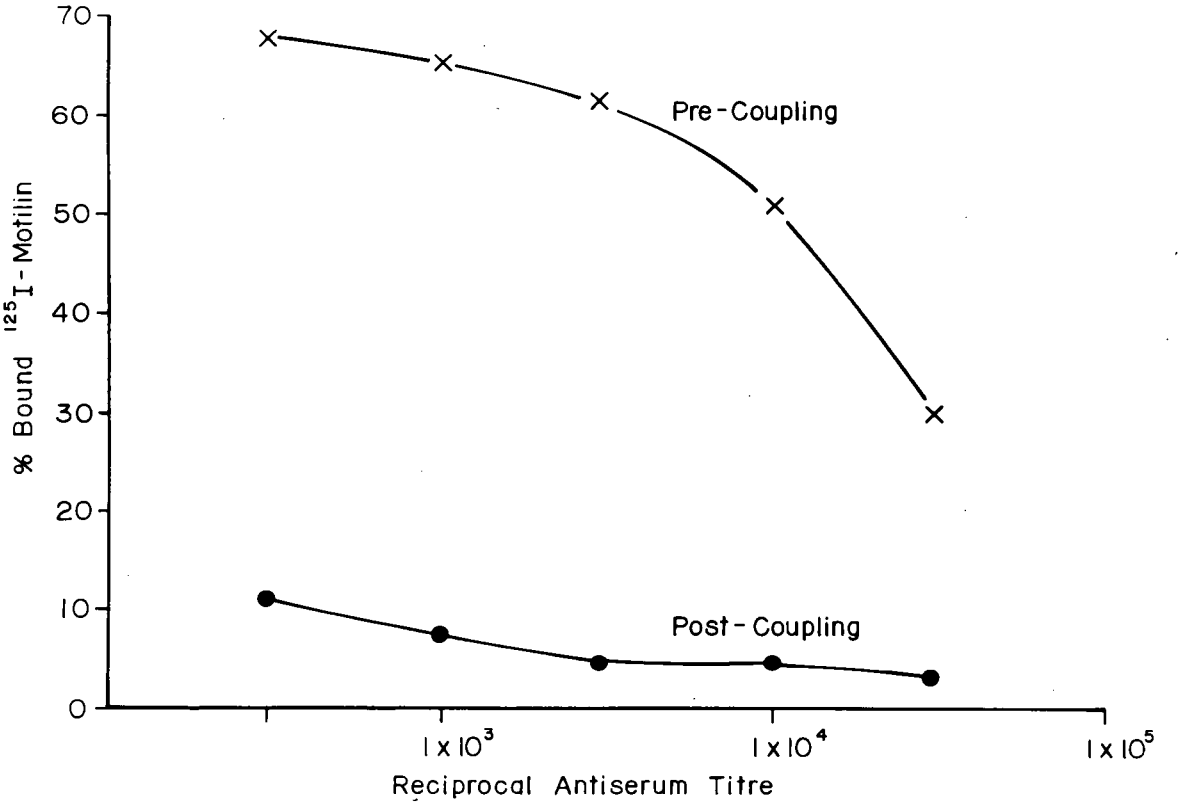

Fig. 18 Motilin antiserum dilution curves comparing the activity of the antiserum prior to coupling to Sepharose 4B with the activity remaining in the wash after completion of the coupling procedure.

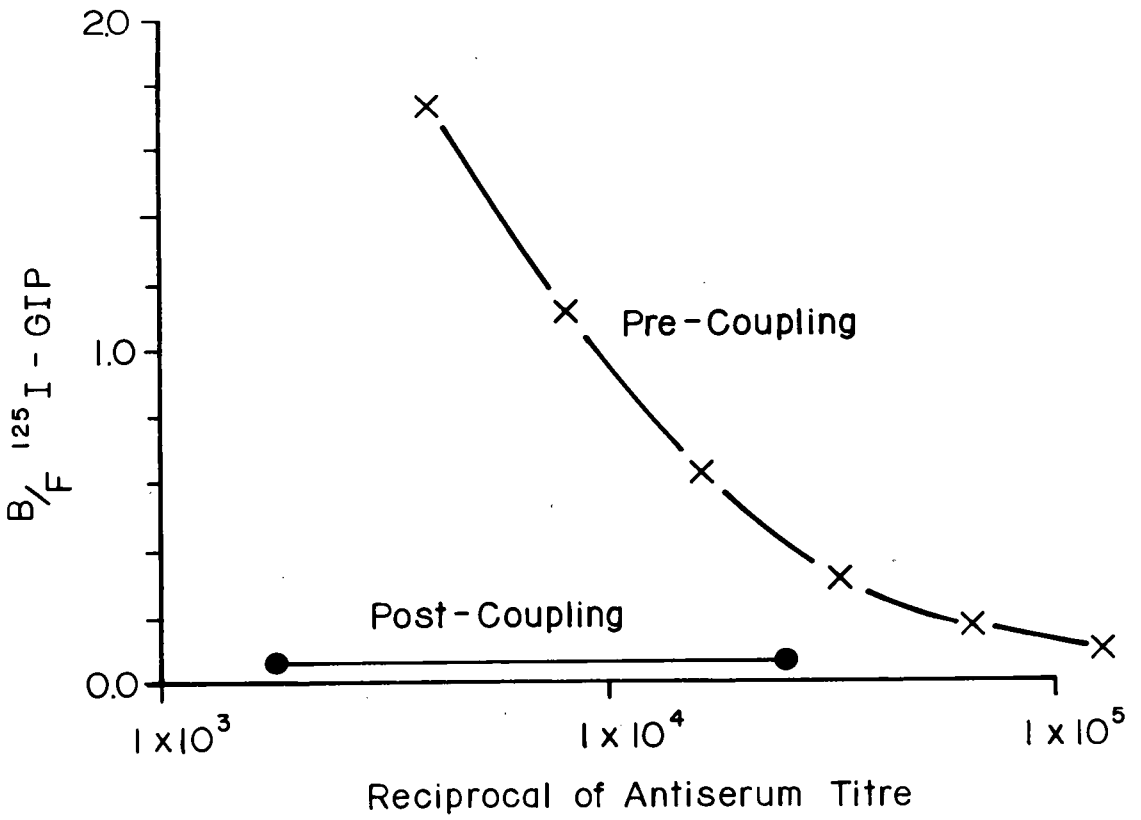

Fig. 19 GIP antiserum dilution curves comparing the activity of the antiserum prior to coupling to Sepharose 4B with the activity remaining in the wash after completion of the coupling procedure.

first described by Porath and Flodin (1959) and has become one of the most commonly used methods of separating the components of a mixture by molecular size.

The appropriate weight of the gel was stirred gently into excess buffer and allowed to swell overnight at room temperature. The fines were decanted before the gel was de-aerated under vacuum for 30 mins. The supernatant liquor was removed so that the final suspension was a slurry which would pour easily without trapping further air.

The column was mounted vertically, out of draughts and direct sunlight. Buffer was injected through the outlet tubing to fill the space beneath the bed support and to a level of approximately 10 cm above the support. The outlet was closed and the slurry poured gently down the column wall. If necessary, a gel reservoir was attached to the column to ensure all the gel being added at one time. The initial packing of the gel occurred under gravity until the gel reservoir could be removed, then the buffer reservoir was attached and the column packing was completed with the outlet open, at the hydrostatic pressure which would be used in subsequent operations.

A filter paper disc (Whatman's 3MM) was inserted to stabilize the gel-liquid interface and the gel equilibrated in buffer overnight.

The buffer above the level of the gel was removed and the sample, dissolved in a small volume of buffer, applied to the gel and allowed to sink to the level of the gel surface. A volume of buffer, roughly equivalent to the sample volume, was similarly applied, washing the sample well into the body

of the gel. Excess buffer was replaced on top of the gel and the column attached to the buffer. As the buffer flowed through the gel, fractions of eluant buffer of a pre-determined size were collected. Between runs, the column was stored in buffer containing 0.01% sodium azide as a preservative.

B. Ion exchange chromatography

Ion exchangers require precycling through acid and alkali to provide the necessary counter ion. An ion exchanger, e.g., Whatman's DE celluloses or Sephadex AE resins, were treated first in 0.5M hydrochloric acid for 30 mins. whilst cation exchangers (Whatman's CM or CE-Sephadex) were first treated in 0.5M sodium hydroxide. The exchangers were washed well with distilled water until the intermediate pH's were 4 and 8 respectively. The treatments were then reversed, the anion exchanger being washed in 0.5M sodium hydroxide and the cation exchanger in 0.5M hydrochloric acid for 30 mins. Both exchangers were well washed in distilled water until the effluent pH was near neutrality. The fines were decanted and the exchangers de-aerated under vacuum for 30 mins. Equilibration in the starting buffer was ensured by repeatedly stirring the exchanger into 15 volumes of that buffer and decanting the supernatant liquid after 10 mins. until the pH and conductivity of the effluant were identical to those of the buffer.

The columns were packed and the samples applied as described in Section A. Development of the column was achieved by either stepwise increases in buffer ionic strength or by establishing a gradient of ionic strength. The most strongly absorbed material was cleared by passage of 0.2M ammonia through the column, and the cellulose was stored in this buffer between runs. Further

pre-cycling was not necessary before re-use of the column, but it was essential that the column be well-equilibrated in the starting buffer to ensure replacement of the necessary counter ion.

POLYACRYLAMIDE GEL ELECTROPHORESIS

The method followed is a modification of that of Johns (1967). The gel solution was prepared by carefully mixing 10 mls of monomer (40% w/v acrylamide and 0.6% w/v N,N^1 - methylenebisacrylamide in distilled water) with 10 mls of catalyst I (0.5% v/v N, N, N^1, N^1 - tetramethylenediamine in 4.6M acetic acid) and 6 mls of catalyst II (0.6% w/v ammonium persulphate in distilled water). The mixture was de-gassed under gentle vacuum for 30 mins. and 10 ml gel solution placed in each prepared 5 x 75 mm gel column. Distilled water was layered on top of each gel and polymerization was accelerated under direct light for the first hour, then allowed to continue at room temperature. Gels were stored at least 3 days before use and could be kept for up to a month if dehydration was prevented.

Gels were equilibrated in a Shandon electrophoresis apparatus, Model 12734, modified to allow cooling of the system The buffer used was 0.01M acetic acid and current was passed at 320 volts, for 3 hours. The electrodes were placed with the anode uppermost.

Samples for electrophoresis were dissolved in 0.002M acetic acid, 1.0M with respect to sucrose. The apparatus reservoirs were emptied, the sample layered onto the gel surface from a Lang-Levy micropipette, and the reservoirs

refilled with fresh 0.01M acetic acid.

(A) Method for staining and destaining for qualitative determinations

The samples were settled into the gel by passing current through the gel at 320 volts for 15 mins. The dye, 1.0 ml amidoblack (0.5% w/v in 1.0M acetic acid) was mixed throughout the lower reservoir buffer, and the current run at 320 volts for a further 15 mins. The reservoirs were then carefully rinsed and refilled with 0.01M acetic acid, the apparatus re-assembled, and the gels destained by passing current at 320 volts until the gel was cleared of dye, except for the stained protein bands.

(B) Method for staining and destaining for quantitative determinations

The paired samples were allowed to settle into the gel as previously described, except that the time was extended to 25 - 50 mins. The gel tubes were removed from the apparatus, protected with plastic and the glass cracked in a vice. The gels were rinsed with tap water and the marker gels were stained in petri dishes, covered with 0.5% w/v amidoblack for 3 - 4 hours. These gels were destained electrically in an enamel or polyvinyl basin, in cotton wool saturated with 1.0M acetic acid, by passing current at 150 volts across the gels.

The remaining gels were stored moist at 4°C until the destaining process was complete. The marker gels were aligned along the unstained gels and the appropriate sections cut from the unstained gel with 000 silk. The gel section was then homogenized by passage through a 5 ml luer-lok syringe in 0.1M acetic acid or distilled water. The peptide was then either extracted

into the acetic acid overnight at 4°C for RIA determination or the water/gel mixture was emulsified with Freund's Complete Adjuvant and used in immunization. In the latter case the polyacrylamide acted as the carrier molecule for the hapten.

ANIMAL PREPARATIONS

(A) Chronic dog preparation

Labrador or labrador-cross breeds were selected for their size, nature and stamina. The weight range used was 20 - 25 kg. All surgery was performed aseptically. After an 18 hour fast, the dog was anaesthetized with a rapid intravenous injection of 5% sodium thiopental ("Pentothal"), given to effect, usually 9 - 15 mls. An endotracheal tube was placed in position and anaesthesia was maintained with "Fluothane" delivered from a Foregger open circuit anaesthetic apparatus at an oxygen flowrate of 3 litres/min. and a fluothane concentration of 2.5%.

(I) Bickel pouch

An extrinsically denervated pouch of the body of the stomach was constructed from the greater curvature. A stainless steel and teflon cannula was placed in the pouch and brought to the exterior through a stab wound in the dog's abdominal wall, in the left upper quadrant. Sectioning of the stomach wall removed vagal innervation and sympathetic denervation was achieved by stripping the nerve plexus around the splenic artery and vein; (the blood supply to the pouch) for approximately 1 cm and removing any mesentery from the pouch.

This is referred to in the following study as the fundic pouch.

The fundic pouch cannula was left open and draining at all times, except when fundic pouch motor activity was being monitered.

A stainless steel and teflon cannula (Thomas, 1941) was placed in the most dependent portion of the stomach remnant, with a purse-string suture. This cannula was brought to the exterior through a flank incision on the same side and ~ 5-6 cms posterior to the fundic pouch cannula. Except during experimental procedures, this cannula was kept closed with a teflon plug.

Gastrointestinal continuity was restored with a gastro-jejunostomy approximately 30 cm distal to the ligament of Treitz.

(2) Mann-Bollman fistula

A length of terminal ileum, approximately 10 cm long was removed and intestinal continuity restored with an end-to-end anastomosis. The distal end of the terminal ileum segment was attached to the duodenuum approximately 3 cm below the pylorus with an end-to-side anastomosis, and the proximal end brought to the exterior through a stab wound and stitched in place on the right abdominal quadrant to form a stoma.

A partial antrectomy was performed to remove gastrin-producing tissue with a resultant reduction in water and electrolyte loss through the continuously-draining fundic cannula.

(3) Gastric fistula

A gastric fistula was constructed in dogs when required by inserting a Thomas cannula into the most dependent portion of the whole stomach. The dogs were used in the control studies.

(4) Truncal vagotomy

Truncal vagotomy was performed by the thoracic route. After anaesthesia was induced, the animals were placed on a Bird respirator and the chest opened at the 8th rib interspace. The oesophagus was located and the left vagus identified, divided, and 2 cm removed. The right vagal branch was similarily treated. In a small proportion of the dogs an interconnecting branch lies between the right and left vagi. If present, this was also sectioned. After the air was expelled from the thoracic cavity, nylon sutures were used to approximate the intercostal muscles.

(5) Antrectomy

The antrum was removed in gastric fistula dogs. The pyloroduodenal junction was located and divided. The junction between the antrum and body of the stomach was identified by the subtle change in texture on the surface of the stomach and the antrum was excised by sectioning at this junction. All blood vessels supplying this region were ligated and sectioned.

(6) Vagotomy and antrectomy

In a separate operation the antrectomized dogs were vagotomized and vice

versa.

After all surgical procedures, dogs were maintained by intravenous therapy for 3 days post-operatively and allowed a 2 week recovery period before experimentation began.

A diagrammatic representation of the chronic dog preparation is shown in Fig. 20.

EXPERIMENTAL PROCEDURES

(A) In chronic dog with Bickel pouches and duodenal fistulae

Dogs were fasted overnight before use. They were harnessed in a stand which provided support whilst maintaining the dog upright. A polyethylene bottle was attached to the open Thomas cannula to collect drainage from the stomach and prevent passage of gastric secretions into the jejunum.

A Foley catheter (id. 3mms) was attached to a syringe barrel. Its tip was inserted into the duodenal fistula for the approximate length of the fistula or until saline in the syringe barrel flowed freely into the duodenum under gravity, and kept in that position with cords around the animal's body. Intra-duodenal infusion was performed under gravity from an open syringe barrel or at a pre-determined rate with the syringe driven by an infusion pump (Dual Infusion/Withdrawal Pump, Harvard Apparatus Co. Inc. Diver, Mass, U.S.A.).

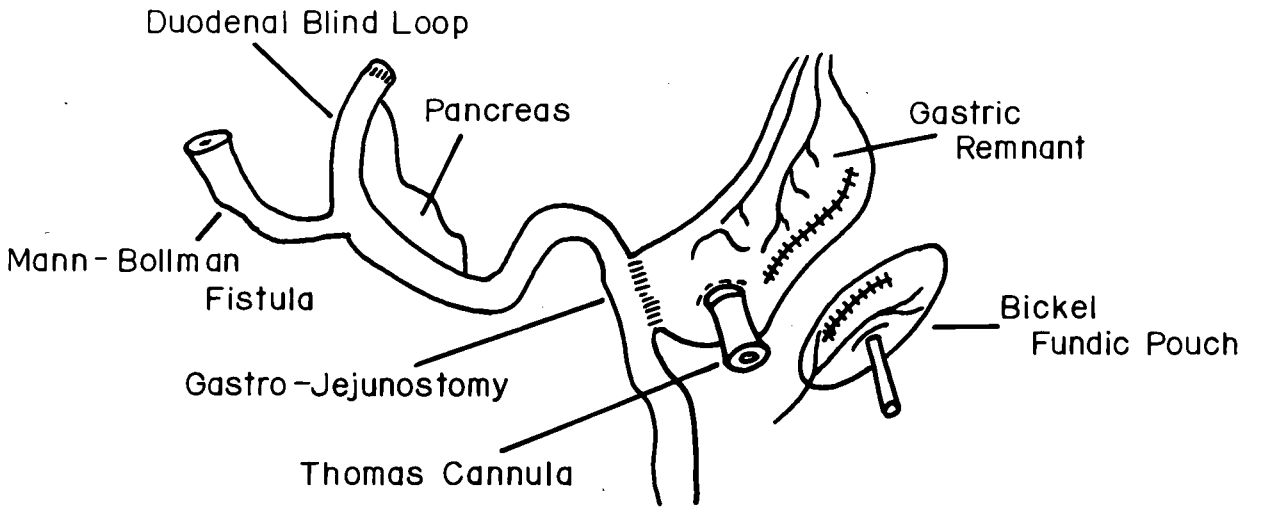

Diagram of Chronic Dog Preparation.

Fig. 20 Diagrammatic representation of the chronic dog preparation.

A 21 G $1^1/2$" hypodermic needle attached to polyethylene tubing (PE 60) was inserted into either the radial or saphenous vein for intravenous injection or infusion. Blood samples were taken from a permanently-indwelling (experimental duration) intravenous cannula on a 19G $^7/8$" needle (Argyle Venocut Infusion Set). Patency of both cannulae was maintained with a gravity-fed saline drip.

Blood samples were allowed to clot for 20 mins. at 4°C. After centrifugation for 10 mins. at 3000 rpm the serum was removed and stored at -20°C until required for RIA.

(I) Bioassay for motilin

The fundic pouch cannula was connected to a venous pressure transducer (Statham P 23 BB) via a water-filled tube with a side arm, allowing collection of fluid from the pouch.

The pouch was filled with ~25 mls tap water at the start of an experiment, the fluid being changed between test procedures. Test procedures, either intravenous infusion or injection or duodenal infusion, were not performed until the fundic pouch motor activity had established its basal rhythm. Recordings of fundic pouch motor activity were made continuously on a polygraph (Gilson pen recorder).

Motility indices were measured over a specific time period from the formula:

$$\text{M.I.} = \frac{\text{Amplitude (mm Hg)} \times \text{Duration (secs.) of each contraction}}{\text{Duration of period (mins.)} \times 10}$$

Unless otherwise stated, no two tests were performed less than 40 mins. apart and the response to any test was measured over the 10 min. period immediately following that test.

If required, serum samples were obtained and stored at -20°C until subjected to RIA for motilin, etc.

(2) Effect of GIP on acid secretion

A 15 ml graduated test tube was attached to the fundic pouch cannula to collect the output from the pouch over each 15 min.period. Acid secretion was stimulated by continuous intravenous infusion of pentagastrin or histamine dihydrochloride. If desired, exogenous GIP could also be administered via the same intravenous cannula.

The volume of gastric acid produced by the fundic pouch in each period was measured, diluted 1:10 with distilled water and titrated to pH 7.0 with 0.01M sodium hydroxide in a titrator assembly (Titrator II, Radiometer). The H^+ ion concentration was expressed as µEq. of H^+ ion per 15 mins.

In the appropriate experiments , glucose, fat or acid were infused intra-duodenally from a Harvard infusion pump, via a catheter, passed through the Mann-Bollman fistula. Glucose was administered as a 20% dextrose solution in distilled water at a rate of 1g/Kg over 30 min.whilst fat (Lipomul-Upjohn) and 0.15N hydrochloric acid were each infused at a rate of 1.91 mls./ min. over 30 min. A plateau of gastric acid secretion was considered established

after continuous intravenous infusion of pentagastrin had resulted in three consecutive periods during which the levels of H^+ secretion were within 10% of each other.

The Bickel pouches in different dogs varied in size and secretory capacity, and because of the trophic effect of gastrin on the gastric mucosa it was also possible for the secretory capacity of one Bickel pouch to vary during the duration of the study. Dose-response studies with pentagastrin were performed in each dog, and the dose of pentagastrin selected which resulted in a gastric acid output equal to 75% of the maximum output. The values of the H^+ secretion were expressed as ratios of the mean of three plateau periods. The IR-GIP response was plotted as the change in IR-GIP (Δ IR-GIP) from the mean of the three periods prior to the start of the duodenal infusion).

(B) In chronic dogs with gastric fistulae

The dogs were accustomed to being harnessed in the stand and were fasted for 18 hours prior to any study. Blood samples for RIA were obtained and intravenous infusions administered as described in Section A.

(I) Determination of the rate of gastric emptying of liquids

Sodium chloride, 0.15M, containing 60 mg/litre phenol red as an indicator, was instilled in the stomach via the gastric fistula and then drained at the end of a 10 min. period. Various cencentrations of motilin were administered as intravenous infusions.

The phenol red concentration in the fluid meal was determined in both the

initial meal prior to its instillation into the stomach and in the fluid drained from the stomach. Phenol red determinations were performed as follows:-

1 ml of the liquid meal plus 2 mls of sodium phosphate (27.5 g Na_3PO_4/litre) were made up to 10 mls with distilled water and mixed well. The O.D. of the solution was read at 550 nm in a 1 cm light path and used to calculate the rate of gastric emptying from the formula.

$$\text{Rate of emptying (mls/10 mms)} = \frac{(V_1P_1) - (V_rP_r)}{(P_i + P_r)\ /2}$$

Where V_i and V_r are the volumes of fluid instilled and recovered and P_i and P_r are the concentrations of phenol red in the instilled and recovered fluids respectively. This calculation is based on the assumption that the concentration of phenol red leaving the stomach is the mean of the initial and final concentrations.

(2) Determination of the role of gastric emptying of solids

Dogs were fed a proprietary canned dog food equivalent to a 3 g dry weight/kg. The amount of solid material that remained in the stomach at various time intervals (weighed after dessication) was expressed as a fraction of the initial weight.

Motilin was administered as an intravenous infusion of 1.0 µg/kg/hour

(C) In the intact dog

Dogs, in the weight range 30-35 kg, with no surgical interference, were trained to remain harnessed in the experimental stand. All animals were fasted for 18 hours. Blood samples for RIA and glucose determinations were collected and test infusions were administered as described in Section A. Test substances were given orally from a glass syringe fitted with a flexible catheter. When the catheter tip was held inside the dog's cheek by the posterior molars, any liquid deposited there induced swallowing without undue trauma to the dog.

Unless otherwise stated, glucose was administered orally as a 20% dextrose solution in distilled water at a dose of 1 g/kg. The oral fat used was Lipomul, a palatable emulsion containing 66g triglycerides per 100 mls.

Results were expressed as change from control (Δ), the control value being defined as the mean of three fasting serum values of that parameter, measured at 15 min. intervals at the start of the experiment. A control was only acceptable if the variation in serum glucose was less than 2%.

Statistical significance was measured using the Student t test.

SERUM ANALYSIS

(A) Radioimmunoassays

(I) GIP radioimmunoassay

A radioimmunoassay for GIP was developed by Kuzio et al (1974) but has undergone repeated modification since then in an attempt to improve the

specific activity and stability of the labelled tracer, the affinity of the antisera and the efficiency and reproducibility of the separation technique.

(1) Iodination of GIP

(a) Routine chloramine-T iodination and purification

Originally, when 6 µg GIP was reacted with 2 mCi ^{125}I-Na, the best tracer did not share complete identity with the peak of radioactivity but was associated with the descending limb of the radiochromatogram and therefore with reduced counts. If the ratio of polypeptide : iodine was increased to 6:1, the peak immunoreactivity coincided more closely with the radioactive peak, with no loss in specific activity.

The reagents were added in the following order in a siliconized 10 x 75 mm glass tube, mixing being ensured by bubbling air through the reaction mixture:-

6 µg GIP in 100 µl 0.4M sodium phosphate, pH 7.5

1 mCi ^{125}I-Na in 10 µl carrier-free NaOH

40 µg chloramine-T in 10 µl 0.4M sodium phosphate, pH 7.5

15 sec. exposure

252 µg sodium metabisulphite in 20 µl 0.4M sodium phosphate, pH 7.5

Separation of the free ^{125}I from the labelled peptide was routinely achieved by transferring the reaction mixture to a column of Sephadex G25 fine, (0.9 x 28 cm) and eluting the radioactive material with 0.2M acetic acid, containing 2000 KIU Trasylol/100 ml and 0.5% B.S.A. Approximately 40 x 400 µl fractions were collected and 10 µl aliquots were counted for 0.1 min. to produce the radiochromatogram in Fig. 21. A charcoal-binding assay was

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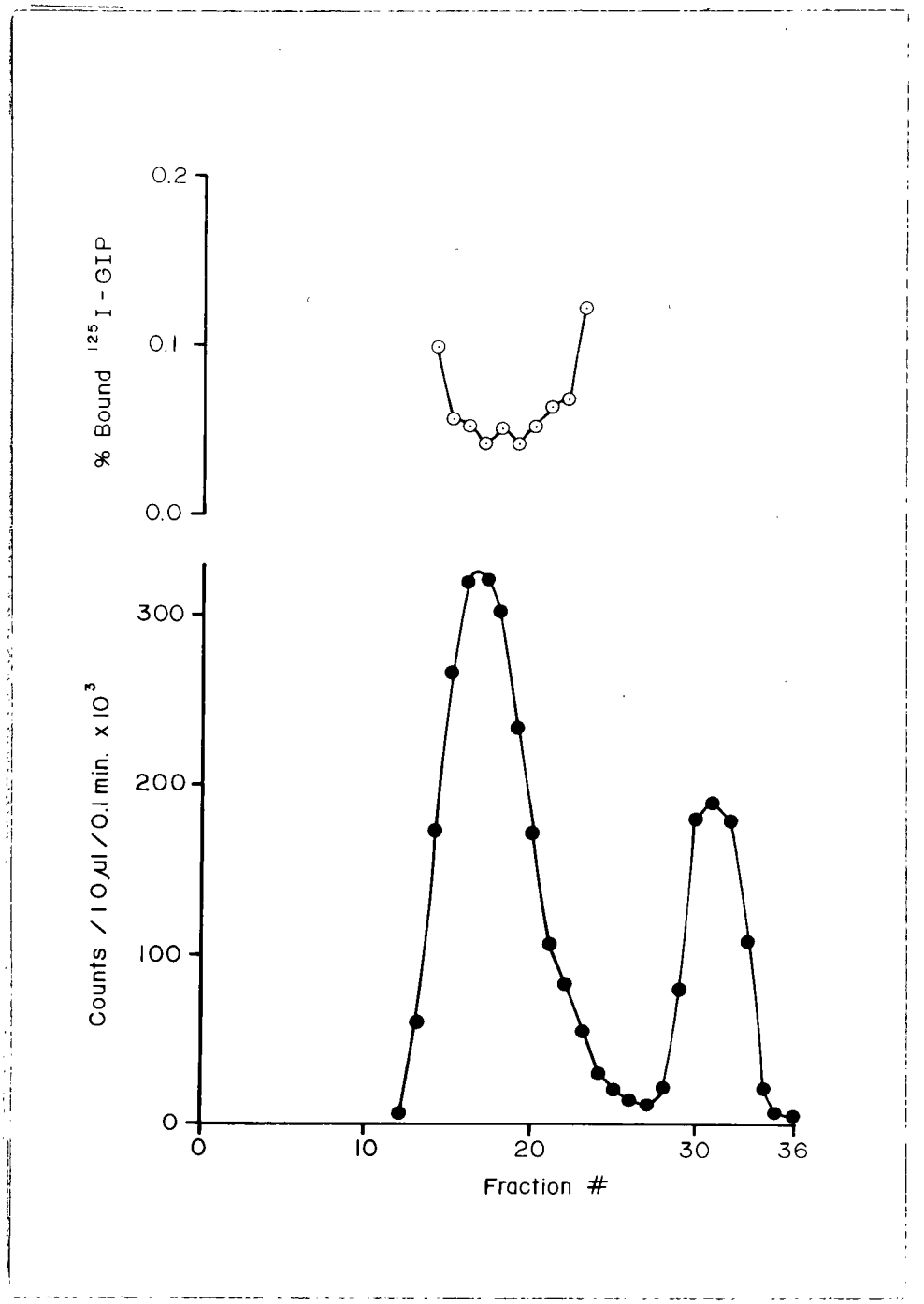

Fig. 21 Chloramine-T iodination of GIP at a peptide:iodine ratio of 6 ug: 2 mCi. Separation of labelled GIP from free iodide on Sephadex G25 in 0.2M acetic acid. Counts per 0.1 min (●—●) : NSB (o—o).

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performed by adding 200 µl of the routine dextran-coated charcoal suspension to tubes containing 5 x 10^3 cpm/100 µl in a total volume of 1 ml. The fractions displaying the lowest maximum binding to charcoal were pooled, diluted with column eluant buffer to a concentration of ~ 1.6 x 10^6 cpm/2 ml and stored at -20°C in siliconized glass tubes.

The average specific activity of labelled GIP purified in this manner was 50-90 mCi/mg and its shelf-life was 3-4 weeks.

(b) Variations of chloramine-T iodination

It had been shown that decreased concentrations of chloramine-T for the same time exposure only served to decrease the degree of incorporation of iodine into the peptide (Kuzio, 1973). The effect of decreased concentrations of chloramine-T on the degree of GIP iodination, when the exposure time was increased, was measured by reacting 2 µg porcine GIP with 1 mCi ^{125}I-Na in the presence of 4 µg chloramine-T. After 30, 60, and 120 sec, 20 µl aliquots were removed from the reaction mixture and added to 25 µg sodium metabisulphite in 20 µl phosphate buffer, along with 100 µl of the column eluant buffer. Each aliquot was purified in the routine manner on Sephadex G25 fine and the specific activity of each calculated approximately from the % incorporation of the iodine into the peptide, estimated from the appropriate radiochromatogram. After 30 sec exposure the specific activity was 45 mCi/mg, after 60 sec it was 112 mCi/mg and after 120 sec it was 350 mCi/mg.

(c) Lactoperoxidase method of iodination

As lactoperoxidase provided a gentler method of oxidation of iodide to

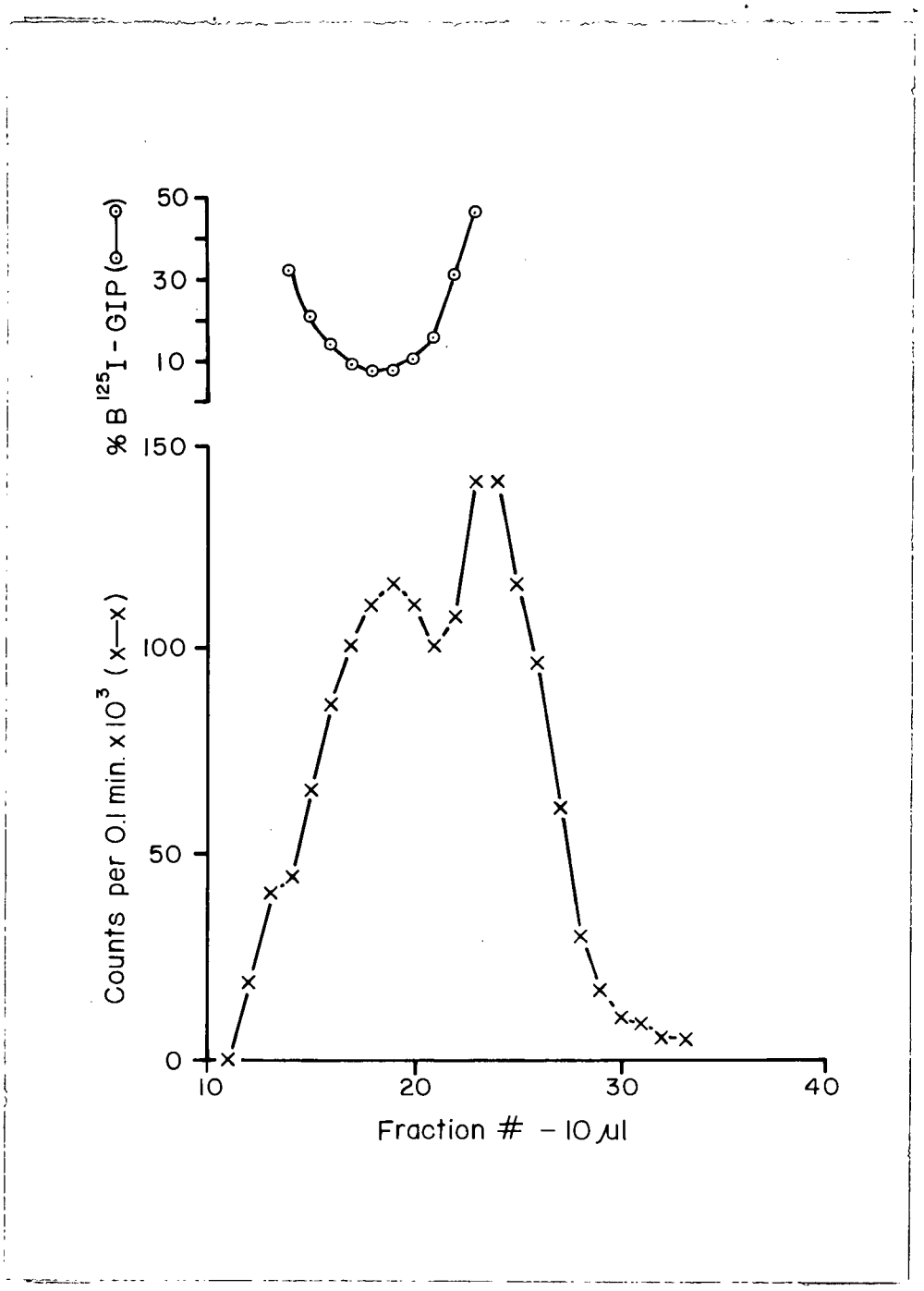


Fig. 2.2 Lactoperoxidase iodination of GIP at a peptide:iodine ratio of 6 µg:1 mCi. Separation of labelled GIP from free iodide on Sephadex G25 in 0.2M acetic acid. Counts per 0.1 min (x–x); NSB (o–o)

iodine, it was expected to be preferable as the oxidizing agent in the iodination of GIP. The procedure was performed exactly as was outlined for motilin. The radiochromatogram, in Fig. 22, was used to calculate the specific activity from the % incorporation of iodine into the polypeptide. The specific activity, calculated this way was 62 mCi/mg and therefore this method showed no improvement over the chloramine-T mediated oxidation.

(2) Purification of GIP

In an attempt to separate labelled from unlabelled GIP the fractions selected after gel filtration were pooled, lyophilized and subjected to ion exchange chromatography. The label was reconstituted in 2 ml 0.06M Tris buffer, pH corrected to 8.5 with 6.0M HCl, and applied to a column of QAE Sephadex A25, (0.6 x 15 cm), well-equilibrated with the Tris/HCl buffer, containing 1% Trasylol and 0.5% B.S.A. The column was developed with the same buffer and 1 ml fractions were collected at a flowrate of 20 ml/hour. The radiochromatogram obtained by counting these fractions in an automatic γ counter is shown in Fig. 23. The ^{125}I-GIP peak was located by determining the region of lowest charcoal-binding, as previously described. The appropriate fractions were pooled and diluted 1:4 in acid-ethanol, (15 ml ethanol : 5 ml distilled water : 0.3 ml concentrated HCl). The specific activity was determined by assaying serial dilutions of the labelled GIP.

(3) Extension of the shelf-life of ^{125}I-GIP

The charcoal binding (NSB)of the routine label preparation was 5 - 9 % B. Internal decay during the storage of this label raised this value to 20 % B

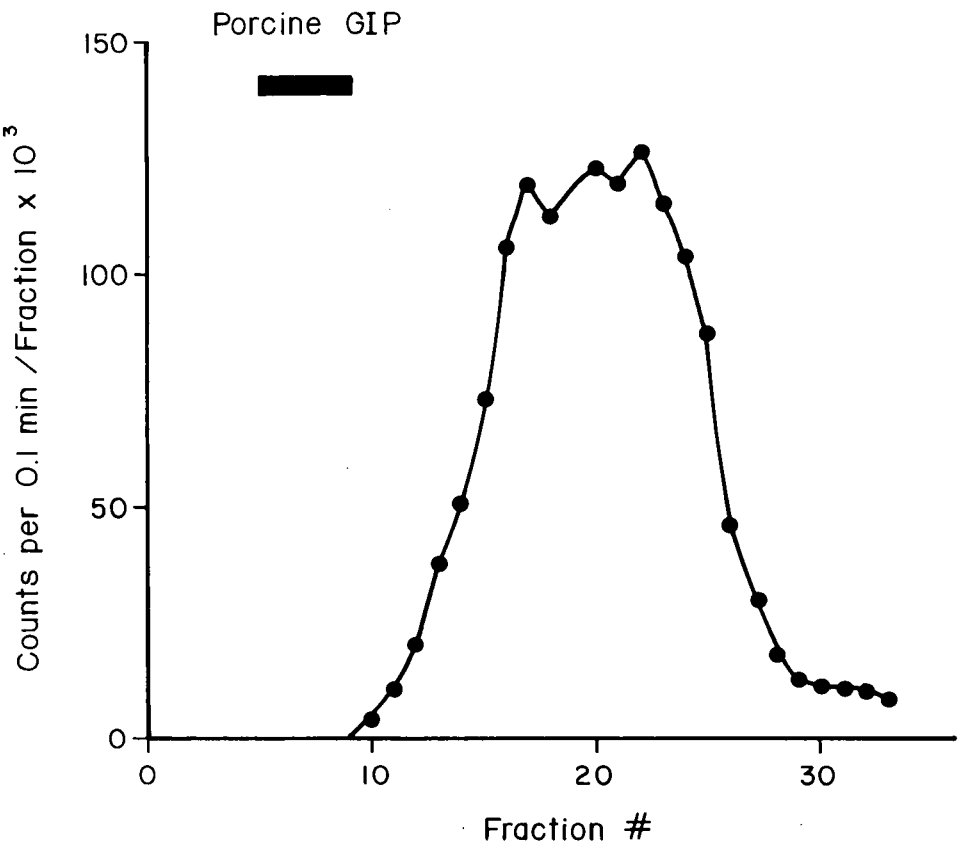


Fig. 23 Chloramine-T iodination of GIP at a peptide:iodine ratio of 5 µg: 1mCi. Separation of labelled GIP from unlabelled GIP on QAE Sephadex A25 in 0.06M Tris, pH8.5. Counts per 0.1 min (• •). Column calibrated with porcine GIP).

after 3 weeks at -20° and this was unacceptable. Repurification of the label was attempted by two different methods. A 2 ml aliquot of ^{125} 1-GIP containing 2.1 x 10^6 cpm, one month old and with a NSB of 24.2% B, was treated with 10 mg resin (AG I-XIO) to adsorb any free ^{125} 1 present. The mixture was vortexed well, centrifuged at 3000 rpm for 10 min. and the supernatant kept. A second label aliquot was treated with 10 mg microfine silica (QUSO) which absorbed the labelled antigen. After mixing and centrifugation, the supernatant was discarded and the pellet washed with distilled water. The labelled peptide was eluted from the silica into 2 mls 40% acetone: 1% acetic acid: 60% distilled water (v/v) and the supernatant was kept. Both supernatants and an untreated vial of the same label were diluted with diluent buffer to the same concentration of cpm/100 µl and standard curves prepared with each label. There was no significant difference in the standard curves obtained at either the LDD or midrange values but treatment with AG 1-XIO substantially reduced the NSB of the label and could be used to prolong the shelf life (Table XI; Fig. 24)

TABLE XI

Effect of treatment of ^{125} I-GIP on NSB of that label

Label treatment	LDD pg GIP	Midrange pg GIP	NSB % B
Untreated	25	160	24.2
AG I-XIO	25	200	14.1
QUSO	25	140	26.0

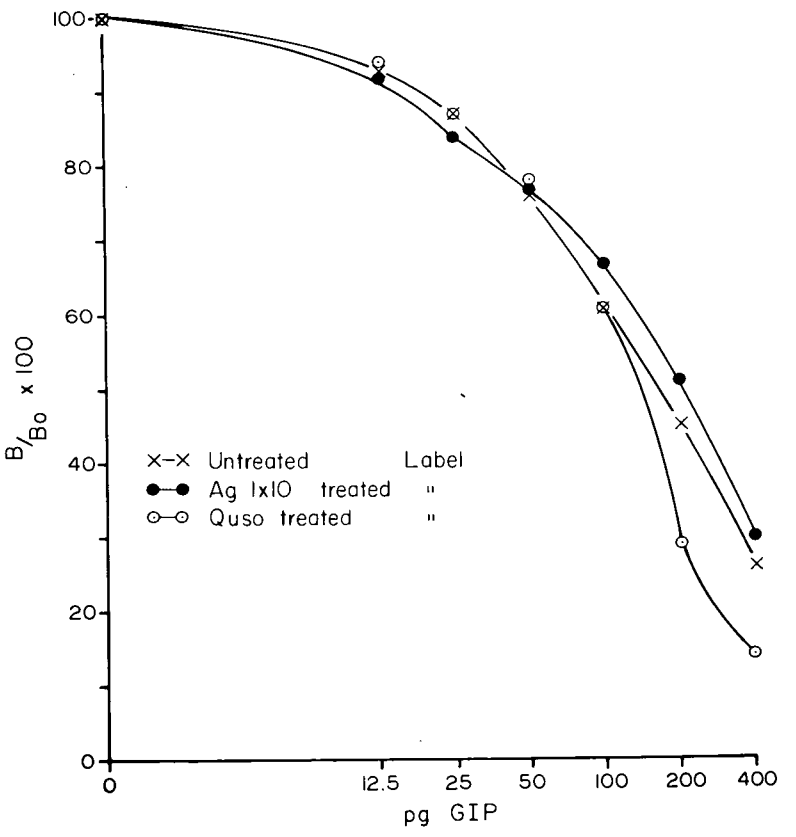


Fig. 24 Comparison of GIP standard curves obtained with untreated label, label treated with Quso and label treated with AG 1-x10 resin.

(4) Production of antisera to GIP

Antisera to GIP were raised in rabbits and guinea pigs by the methods previously detailed for motilin.

(a) Storage of antisera

Antisera attaining a usable titre was stored, lyophilized in 200 µl aliquots at -20°C. As required, an aliquot was reconstituted at a 1:10 dilution in diluent buffer and kept frozen in 50 - 200 µl amounts in siliconized 10 x 75 mm glass tubes at -20°C.

(b) Characterization of antisera

All antisera to GIP were checked for cross reactivity with other polypeptides, especially those of the secretin family, i.e., vasoactive intestinal peptide, glucagon and secretin itself, with which GIP shares structural similarities. The routine assay antiserum, Van 8, did not cross react with any of these, or with cholecystokinin - pancreozymin, gastrin, motilin or insulin at concentrations up to 10 ng/100 µl.

(5) Assay protocol

All dilutions and volume corrections were made with 0.04 M sodium phosphate, pH 6.5 containing 7500 KIU Trasylol per 100 µl and 5% (w/v) charcoal-extracted plasma. The composition of the incubation volume was:-

100 µl ^{125}I-GIP containing ~ 5000 cpm

100 µl GIP standard over the range 12.5-400 pg

or

100 µl assay control

or

50-200 µl unknown

100 µl antiserum at the appropriate initial dilution

Diluent buffer to a final volume of 1.0 ml.

Assays were set up in a cold tray at 4°C, in triplicate, in siliconized 10 x 75 mm glass tubes. Incubation was of the equilibrium type, at 4°C, for 48-72 hours.

The incubation volume has recently been altered to 300 µl, with the resultant decrease in the antiserum volume required. After the normal incubation period, the volume was corrected to 1.0 ml with diluent buffer prior to the separation of bound and free antigen.

In both cases, NSB tubes and total count tubes were included and the analysis of data was performed as described for motilin.

The standardization of the incubation milieu was examined by preparing standard curves with and without the addition of 100 µl charcoal-extracted plasma, to compensate for the protein added in the remainder of the assay when serum samples were being monitored. There was no significant difference in the

curves obtained (Fig.25) and the addition of plasma was deemed unnecessary.

(6) Preparation of standards

Working stock standards were prepared by dissolving 1 µg GIP in 0.04M sodium phosphate buffer, pH 6.5, containing 7500 KIU Trasylol per 100 ml and 0.5% BSA (w/v) to a final concentration of 8 ng/ml. These standards were stored in 1.5 ml polypropylene mictrotest tubes at -20°C and were used in the assay after being diluted 1:1 in diluent buffer, i.e to 400 pg/100 µl. The other standards were prepared by serial dilution. Working stock standards were discarded after being thawed.

(7) Preparation of controls

Artificial control sera were prepared by dissolving porcine GIP in 0.04M sodium phosphate buffer, pH 6.5, containing 2000 KIU Trasylol per 100 ml and 5% (v/v) charcoal-extracted plasma, to a concentration of 200 pg/100 µl. The controls were stored in 1.5 ml polypropylene microtest tubes at -20°C and assayed at the beginning and end of every assay, to provide an estimate of intra- and inter-assay variability. In 50 arbitrarily chosen assays, the initial control value was 254 $\pm$ 43 pg/100 µl (mean $\pm$ SD) and the final control was 258 $\pm$ 50 pg/100 µl. Any assay in which the controls varied more than 1 SD from these values was suspect and was discarded.

(8) Separation techniques

Separation of the free ^{125}I-GIP from the labelled peptide bound to the anti-serum was routinely performed using dextran-coated charcoal, exactly as

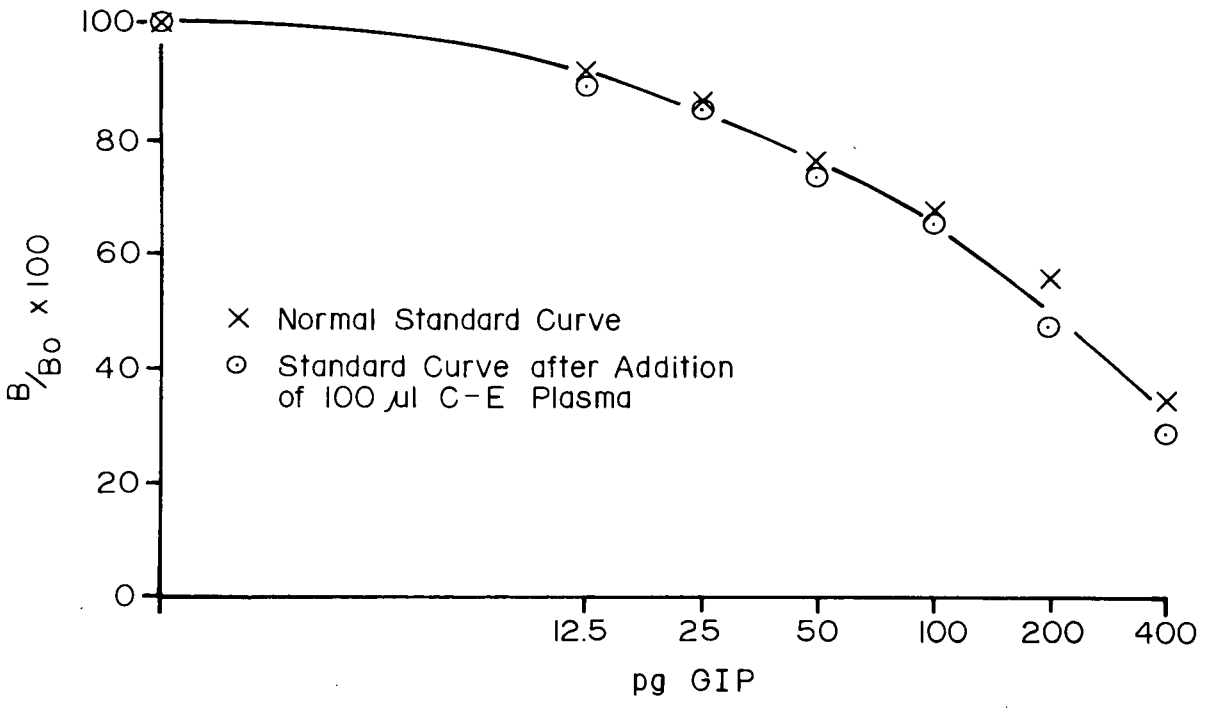


Fig. 25 Effect of the addition of charcoal-extracted plasma on the sensitivity of the routine standard curve for GIP.

described for motilin. An alternative method involves the precipitation of the antibody/antigen complex with the alcohol, dioxane. When standard curves were incubated under routine conditions and then separated by the addition of either 200 µl dextran-coated charcoal suspension or 1 ml dioxane, there was no significant difference in curves obtained (Fig. 26), but at this time the dioxane method has proved less reliable.

II. Insulin radioimmunoassay

(I) By commercially available kit

Measurement of immunoreactive insulin (IRI) was performed with the Amersham Searle Insulin Immunoassay Kit (IM-39) - developed from the method of Hales and Randle (1973). Insulin tracer, labelled with ^{125} - I at a minimum specific activity of 50 mCi/mg reacted with insulin antibody, provided as a dessicate already bound to a second antibody. The standards were human insulin, over the range 2.5 - 160 µU/ml. All dilutions were made in isotonic sodium phosphate buffer at pH 7.4, containing 0.5% BSA and trace thiomersal as a preservative. The assay was of the disequilibrium type with 6 hour incubation of antibody and unlabelled antigen, followed by label addition and incubation for a further 18 hours. After centrifugation, the supernatant was discarded and the pellet consisting of the Bound antibody/antigen complex was counted in an automatic gamma counter. Results were expressed as a percentage of the initially added counts (%B).

(2) By non-commercial radioimmunoassay

A laboratory radioimmunoassay was developed from the method of Dr. K.D. Buchanan (personal communication).

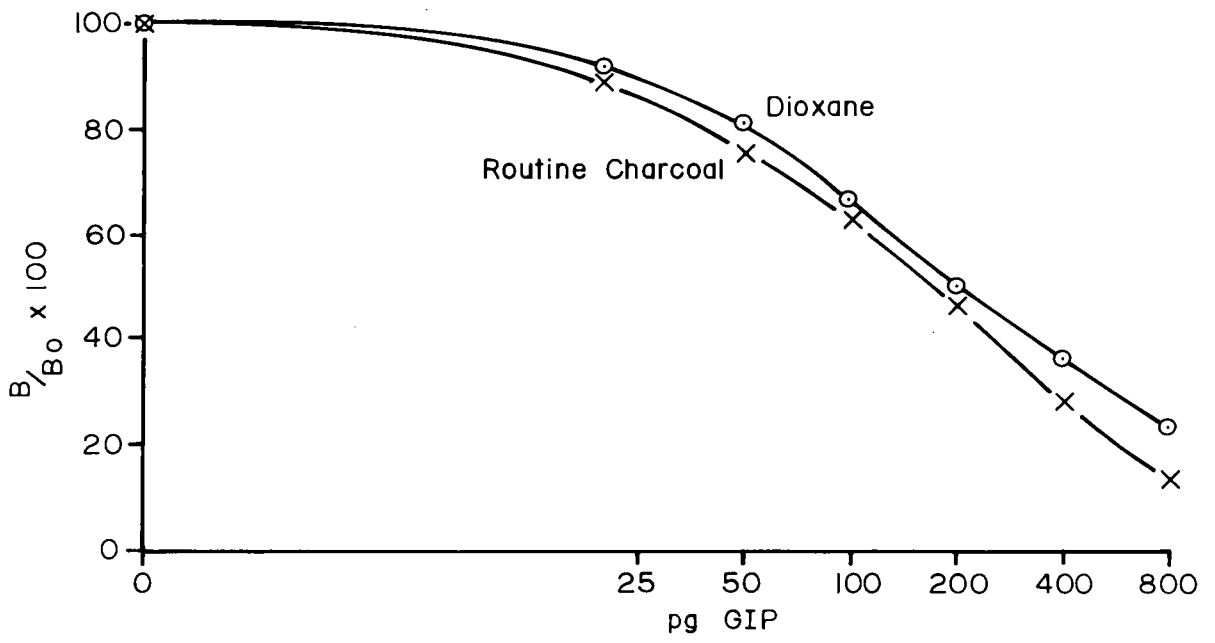


Fig. 26 Effect of separation of bound from free antigen by dextran-coated charcoal or dioxane on the sensitivity of the routine standard curve for GIP.

(a) Iodination of insulin

The following reagents were prepared just prior to use and mixed together in a siliconized glass 12 x 75 mm tube in the order given:-

^{125}-I	1 mCi	in	10 µl	
Insulin(porcine)	5 µg	in	20 µl	0.2M sodium phosphate pH 7.4
Chloramine-T	100 µg	in	20 µl	0.2M sodium phosphate pH 7.4
	10 sec exposure			
Sodium metabisulphite	240 µg	in	100 µl	0.2M sodium phosphate pH 7.4
	45 sec exposure			
Sodium iodide	1%	in	50 µl	0.2M sodium phosphate pH 7.4

The ^{125}I- insulin was purified by adsorption onto 10 mg microfine silica (QUSO) in a total volume of 2.0 mls sodium phosphate buffer. After vortexing and centrifugation, the free iodide was discarded with the supernatant. The silica complex was washed with 3.0 mls distilled water and the labelled insulin was eluted with 5.0 mls acid-ethanol (15 ml ethanol; 5.0 ml distilled water; 0.3 ml concentrated hydrochloric acid) and stored at -20°C.

The specific activity of the label was calculated from the percentage iodine incorporated into the insulin, by counting aliquots of the initial supernatant, the water wash, the final labelled product and the silica pellet, and correcting for the volume, i.e., a typical incorporation of 75% ^{125}-I into the polypeptide represented an approximate specific activity of 150 mCi/mg.

(b) The insulin antibody

The antiserum, obtained from a guinea pig, was kindly donated by Dr. K.D. Buchanan.

(c) The assay protocol

Sodium phosphate buffer (0.04M, pH 7.5, 5% charcoal-extracted plasma) was used in all dilutions and for correcting the final volume to 1.0 ml. The composition of the incubation volume was:-

100 µl standard human or porcine insulin, range 1.25-80 µU/ml

or

50-200 µl unknown

100 µl antiserum at the appropriate initial dilution

Diluent buffer to a final volume of 0.9 ml

24 hour incubation at 4°C

100 µl ^{125}-I insulin, containing ~12,000 cpm

24 hour incubation at 4°C

Non-specific binding and total count tubes were included (see motilin RIA).

Separation of counts bound to the antibody/antigen complex was achieved by adsorption of the free and damaged antigen onto dextran-coated charcoal (5% w/v charcoal, 0.5% w/v dextran in 0.04M sodium phosphate buffer pH 7.5 - 200 µl/tube) and centrifugation at 2800 rpm for 20 min. After the supernatant was discarded, the free cpm (the charcoal pellet) was counted in an automatic counter and the results expressed as % Bound.

B. Serum glucose

Serum glucose determinations were performed in a Beckman glucose analyzer. A precise volume of sample (10 µl) was pipetted into a standard amount of well-aerated enzyme reagent solution, containing glucose oxidase, ethanol, molybdate and iodide. The D-glucose in the sample reacted with the oxygen in solution to produce gluconic acid and hydrogen peroxide.

$$\text{Glucose} + O_2 \xrightarrow[H_2O]{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$$

The presence of ethanol, iodide and molybdate in the reagent solution prevented the destruction of the peroxide by a pathway resulting in further oxygen production.

The rate of oxygen consumption was directly proportional to the glucose concentration in the sample and the change of oxygen concentration in the solution was measured by an oxygen electrode and converted to produce a direct digital read out in mg% glucose.

RESULTS

ESTIMATION OF THE DEGREE OF CONJUGATION BETWEEN A POLYPEPTIDE AND BOVINE SERUM ALBUMIN

A. With motilin

A motilin/BSA conjugate was prepared as previously described with one difference i.e., the addition of an aliquot of ^{125}I-motilin, containing 9000 cpm, to the reaction mixture. The reaction was terminated by freezing the mixture.

A column of Sephadex G25 fine (0.9 x 100 cm) was calibrated with ^{125}I-motilin in 0.2M acetic acid, 1.5 ml fractions being collected. The conjugate reaction products were separated by chromatography on this same column, under identical conditions. The protein content of each fraction was determined spectrophotometrically at 280 nm in a 1 cm light path, and the radioactive content of each fraction was estimated by counting for 1 min in an automatic γ counter. The column profiles obtained are illustrated in Fig. 27.

The assumption was made that the labelled and unlabelled polypeptide would behave identically under the conditions of the conjugation procedure. Therefore, the percentage of the total reactivity eluting in the void volume of the column, with the major protein peak would give a measure of the percentage of polypeptide conjugated to the BSA in this reaction. From Fig.27 it was estimated that at least 60% of the motilin was conjugated.

B. With GIP

The method was as described for motilin except that ^{125}I-GIP (12,000 cpm) was added to the reaction mixture during the conjugation of GIP and BSA

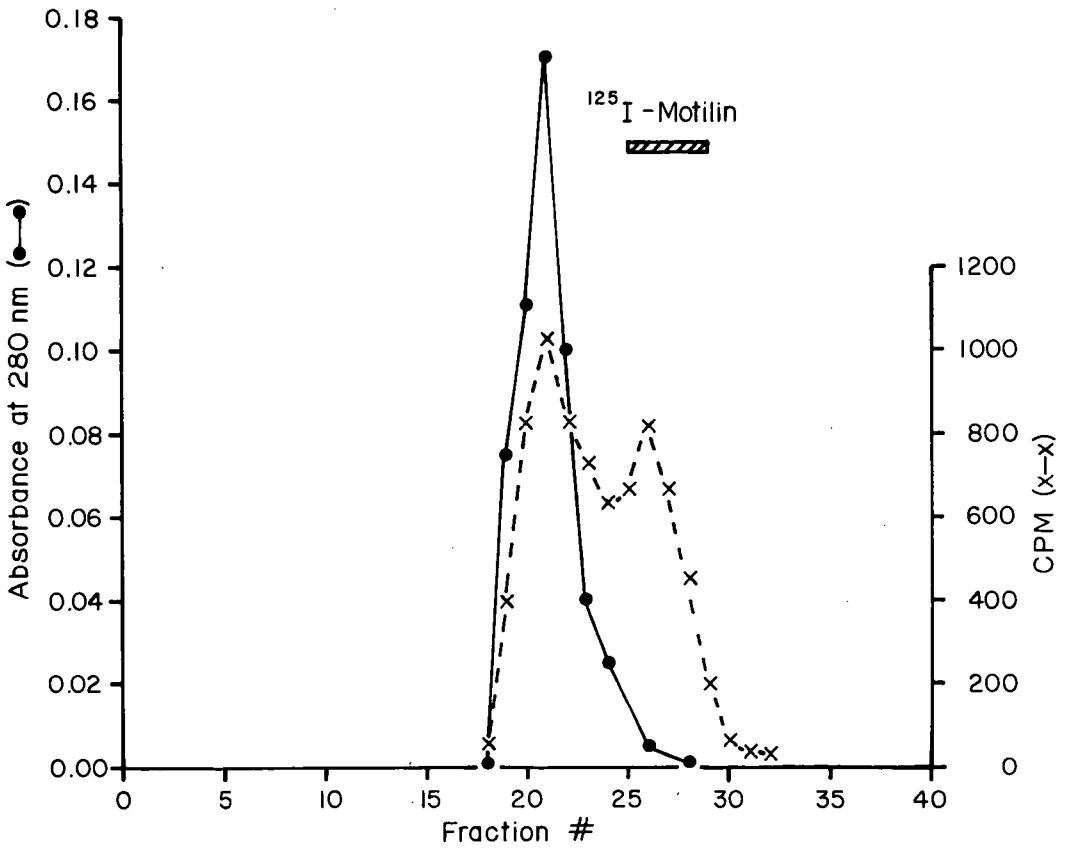


Fig. 27 Column profile obtained after elution of ^{125}I-motilin: motilin/BSA conjugate from Sephadex G25 in 0.2M acetic acid. The column had been previously calibrated with ^{125}I- motilin

by the carbodiimide method.

The column profile obtained indicates that 100% of the GIP was conjugated to the BSA as illustrated in Fig. 28.

REPRODUCIBILITY OF IR-MOTILIN DETERMINATIONS

Serum samples, containing motilin endogenously released by duodenal alkalinization, were obtained from 2 different studies in dogs. Sera from one experiment was immediately treated with the protease inhibitor, Trasylol, 100 µl per ml of serum. Both groups of sera were assayed for IR- motilin content, stored at -20°C and re-assayed 3 months later.

Table XII shows the results, in pg/ml IR- motilin, obtained in the two assays, after correction for the dilution by Trasylol in the appropriate experiment. In Fig. 29, the assay results have been plotted against each other. Protection against enzymatic degradation did not appear necessary for motilin. On re-assay only 1 sample deviated more than 25% from the line of identity.

COMPARISON OF RIA AND BIOASSAY OF MOTILIN

The motilin content of the commercially prepared duodenal extract "Pancreozymin" (PZN) estimated by RIA under routine conditions and by bioassay in the chronic dog preparation.

A. Immunological comparison

Serial dilutions of Boots "Pancreozymin" (PZN), ranging from 0.25 - 2.0 µg/

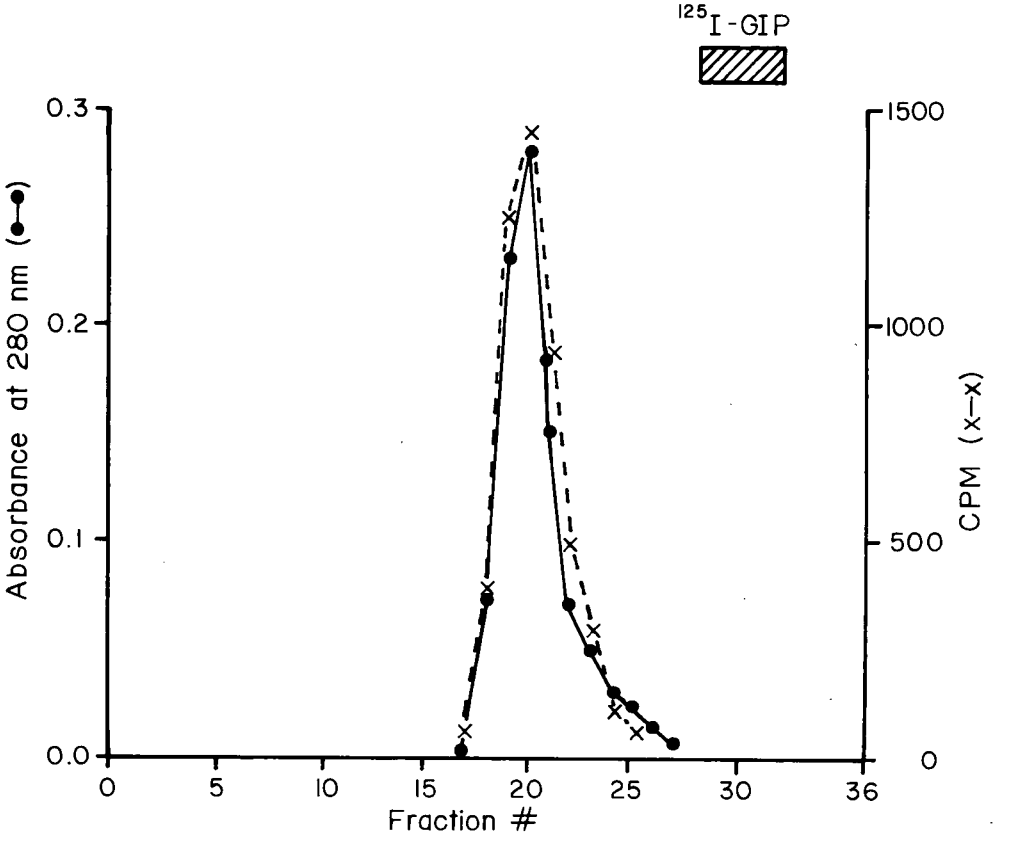


Fig. 28 Column profile obtained after elution of ^{125}I-GIP:GIP/BSA conjugate from Sephadex G25 in 0.2M acetic acid. The column had been previously calibrated with ^{125}I-GIP.

TABLE XII

Results, in pg/ml IR-motilin, demonstrating reproducibility of motilin determinations after storage for three months, with or without added Trasylol

Sample Time	Sample	With Trasylol		Without Trasylol	
		24/6/74	9/10/74	24/6/74	9/10/74
-30 min	1	530	580	680	670
-15 min	2	560	520	660	670
0 min	3	410	490	700	690
2 min	4	870	760	850	940
5 min	5	700	640	1100	1200
7 min	6	960	940	1100	1120
10 min	7	900	890	960	830
15 min	8	760	690	830	840
20 min	9	870	820	920	910
25 min	10	940	1100	840	730
30 min	11	830	940	840	720
45 min	12	720*	980*	600	590
60 min	13	520	600	550	580

* Deviates more than 25% from the line of indentity

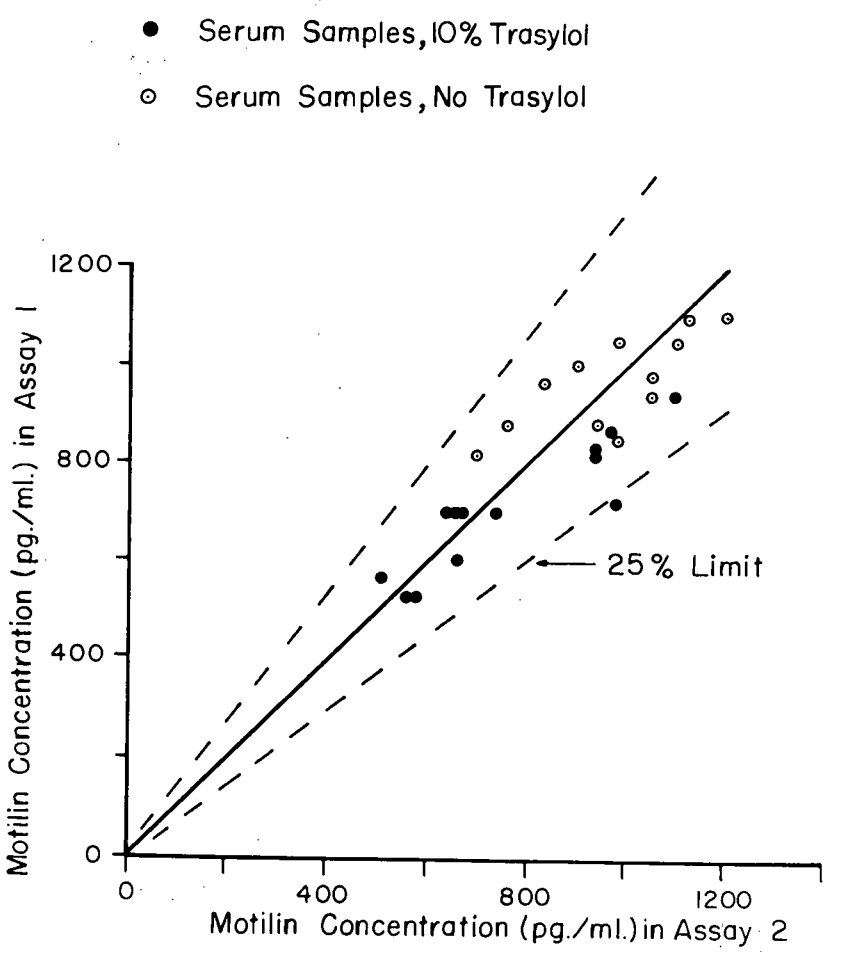


Fig. 29 Reproducibility of motilin determinations on serum samples stored between assays at -20°C, with (●) or without (o) Trasylol. Only 1 sample deviated more than 25% from the line of identity on reassay. (Dryburgh and Brown. Gastroenterology 68: 1169-1175, 1975).

100 µl were incubated with motilin antiserum in a routine RIA. The resultant displacement of ^{125}I - motilin obtained with 1 µg/100 µl PZN was fitted to the standard curve, the other dilutions being plotted accordingly. The immunoreactive material in the duodenal extract shared 100% immunological identity with the pure polypeptide and the motilin content in 4 separate estimates was 140 $\pm$ 40 pg/ µg Boots PZN (Fig. 30).

B. Biological comparison

In 2 experiments in each of 3 dogs, matched responses of increased fundic pouch motor activity were produced by bolus, intravenous injections of natural porcine motilin or Boots PZN given at least 40 mins. apart. The order of the injections was randomized. Fig. 31 and Table XIII illustrate the increase in motor activity, expressed as motility indices, in the 10 min. period immediately following the injection compared to the 10 min. period immediately prior to it, for 2 different doses of pure polypeptide and impure extract. Equivalent responses were observed after 1 and 2 µg pure motilin or 10 and 20 mg PZN (Table XIII, Fig. 31).

Therefore, by both immunological or biological estimations, the pure polypeptide represents a 10,000 fold purification over the impure extract.

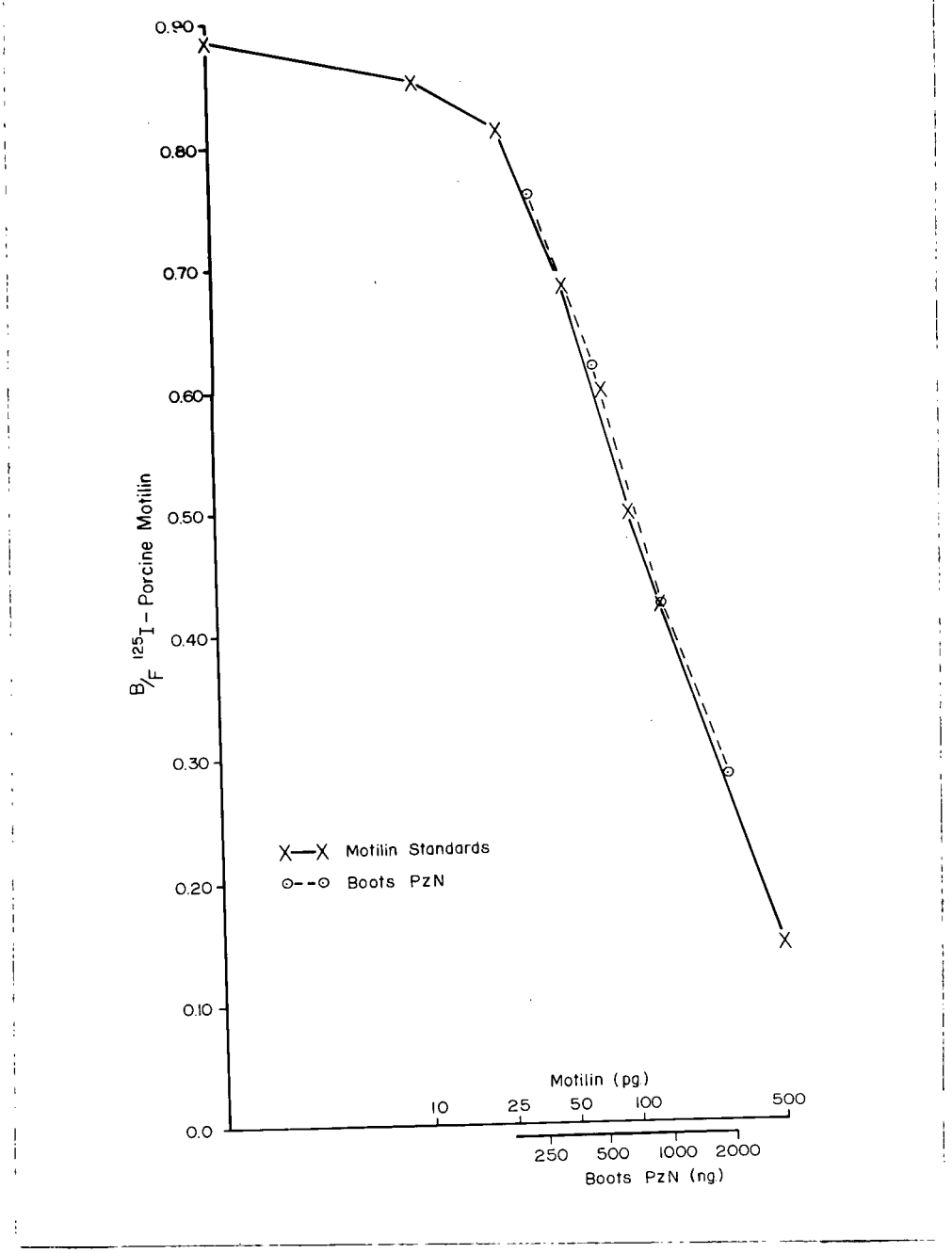


Fig. 30 Comparative immunoreactivities of porcine motilin and a crude duodenal extract (Boots PZN), the 1 µg dilution of the PZN being fitted to the standard curve and the other points fitted accordingly. (Dryburgh and Brown. Gastroenterology 68: 1169-1175, 1975).

TABLE XIII

Comparison in the increase in Motility Indices after single intravenous injections of motilin or Boots PZN +

A. 1 μg motilin / 10 mg Boots PZN

Dog Experiment	Motility Index*		
	Control	Post motilin	Post PZN
P 2/5/74	15.6	71.4	76.1
S 9/5/74	24.7	90.8	69.3
A 14/5/74	19.8	75.7	85.2
Mean ± SE	20.0 ± 2.6	79.3 ± 5.8	76.8 ± 4.6

B. 2 μg motilin / 20 mg Boots PZN

Dog Experiment	Motility Index*		
	Control	Post motilin	Post PZN
P 9/5/74	11.0	169.0	180.8
S 14/5/74	18.3	195.1	184.7
A 2/5/74	16.2	173.7	175.4
Mean ± SE	15.2 ± 2.2	179.3 ± 8.0	176.9 ± 5.9

* represents a 10 min period

+ PZN - Boots 'Pancreozymin'

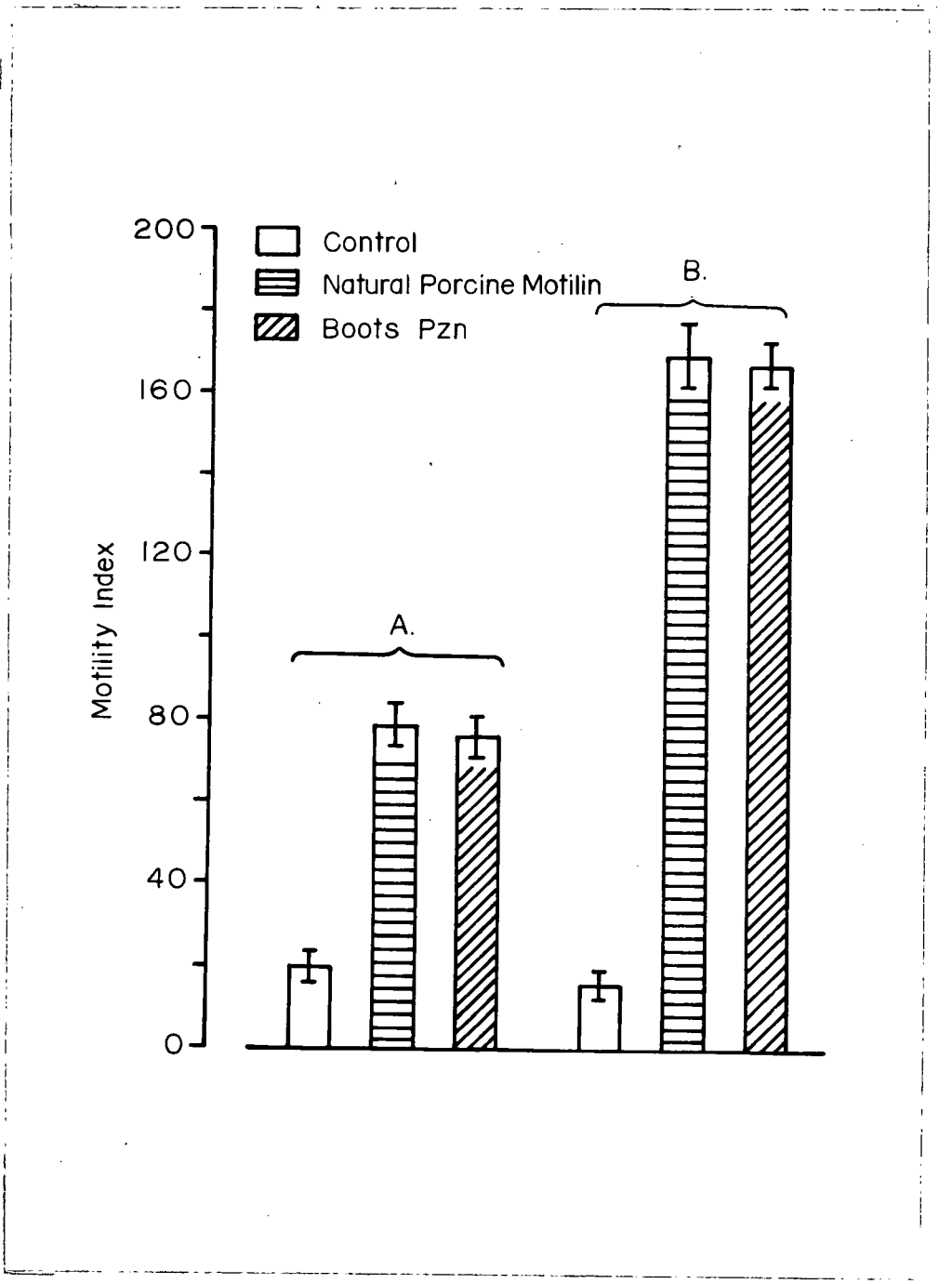


Fig. 31 Comparison of biological activity (expressed as motility indices) of pure motilin and a crude duodenal extract (Boots PZN) in the chronic dog bioassay, at 2 doses. (a) 1 ug/kg porcine motilin and 10 mg/kg PZN and (b) 2 ug/kg pure motilin and 20 mg/kg PZN. Each group of results represents the mean $\pm$ SE of 4 experiments in 2 dogs.

MOLECULAR HOMOGENEITY OF MOTILIN

A. In serum

To determine whether motilin existed in more than one detectable form sera containing endogenous motilin was subjected to gel filtration as follows:-

2 mls serum containing 900 pg/ml IR - motilin was applied to a colum of Sephadex G50 fine (0.9 x 100 cms), the column was developed with 0.2M acetic acid, and the eluant was collected in 1.0 ml fractions at a flow-rate of 10 mls/hour. Samples were stored at -20°C until 100 µl aliquots could be assayed. The column profile (Fig. 32) indicates that only one detectable form of motilin existed in this serum which was taken at the peak of a canine response to duodenal alkalinization.

B. In tissue extract

Presekretin, the starting material in the purification of motilin, was estimated, by RIA, to contain 13.5 ng/ µg IR - motilin. Three µg of this material in 2.0 mls 0.2M acetic acid was eluted from Sephadex G50 fine under the same conditions as in (A).

The column profile in Fig. 33 was obtained after monitoring each fraction at 1:1000 dilution for IR - motilin content.

The chromatography system was calibrated routinely with ^{125} 1- albumin and ^{125} 1- motilin, 50,000 cpm of each in 0.2M acetic acid.

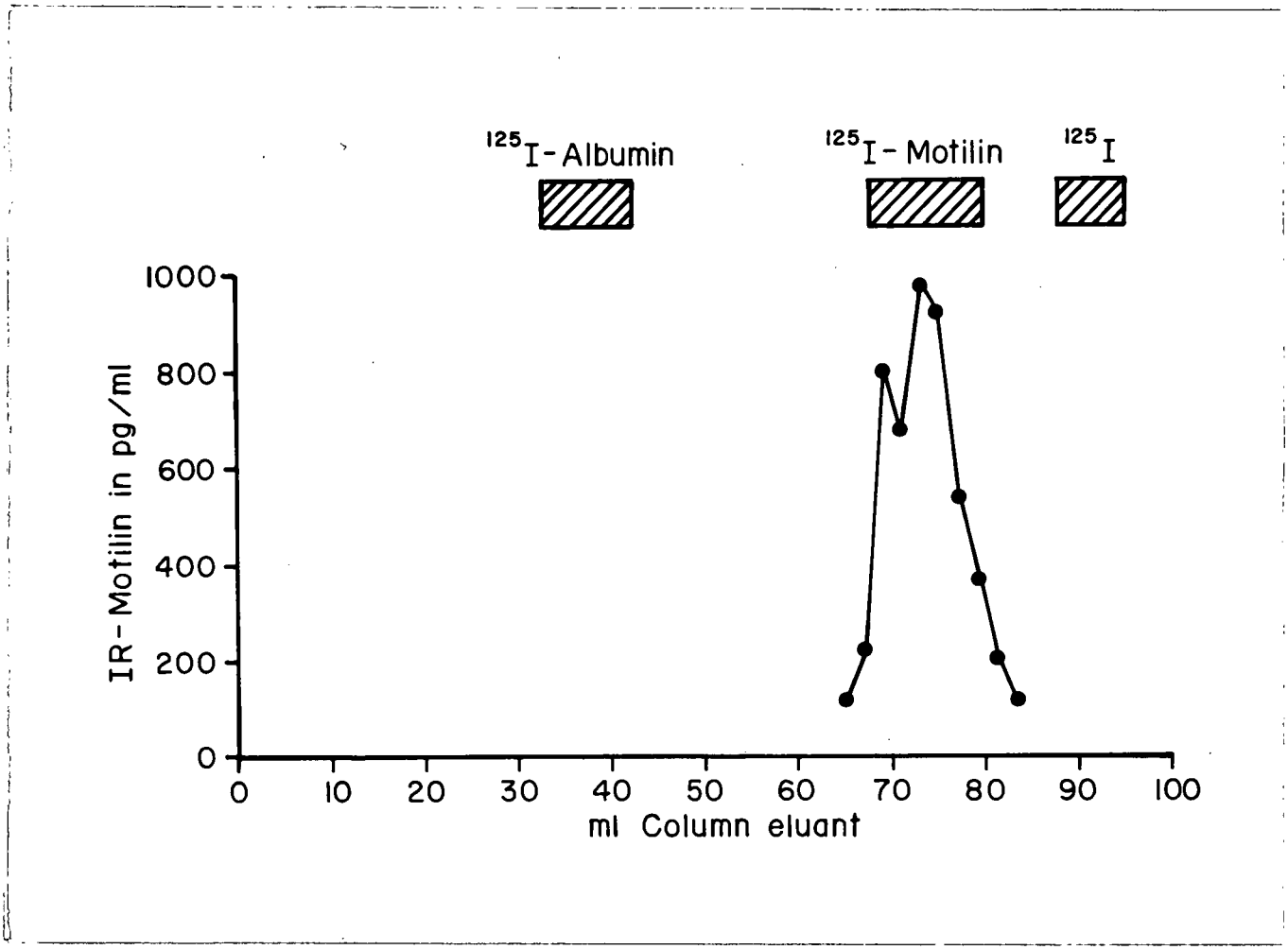


Fig. 32 Column profile obtained after chromatography of 2 ml serum containing 900 pg/ml IR- motilin on Sephadex G-50 (0.9 x 100 cm) in 0.2M acetic acid. The column was calibrated with ^{125}I-motilin and ^{125} Iodine

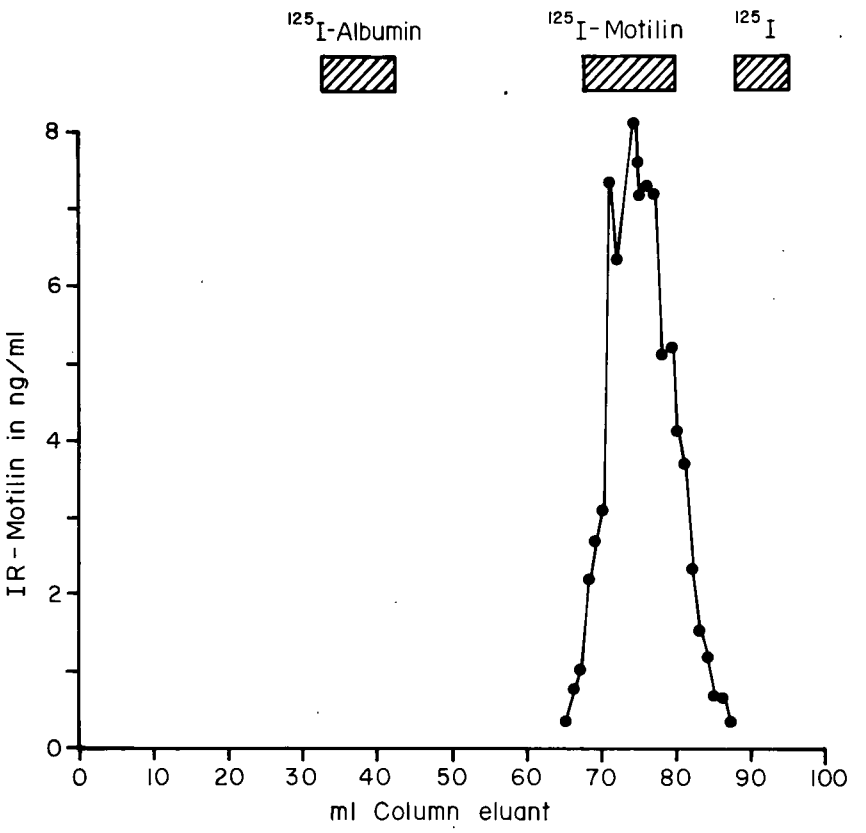


Fig. 33 Column profile obtained after chromatography of 3 ug Presekretin in 2 ml eluting buffer on Sephadex G-50 in 0.2M acetic acid. The column was calibrated with ^{125}I- albumin, ^{125}I- motilin and ^{125}Iodine.

DISTRIBUTION OF IR- MOTILIN THROUGHOUT THE HOG GASTROINTESTINAL TRACT

The tissue was collected fresh and divided into appropriate sections. It was boiled briefly, cleared of connective tissue, frozen, coarsely minced and extracted into acid-ethanol.

Composition of acid-ethanol:-

3.75 litres	95% ethanol
75 ml	glacial acetic acid
1.25 litres	distilled water

The filtrate was cleared by successive passage through several layers of cheese cloth and nylon gauze, and the pH lowered to 2.5 by addition of 2.0M hydrochloric acid. Adsorption onto alginic acid was allowed to proceed for 1 hour at 4°C and the alginic sediment was then removed by gentle filtration under vacuum and washed well with 0.2M acetic acid. The combined washings were cooled to 4°C and the protein was precipitated by saturation with sodium chloride (35 g/litre), redissolved in distilled water and lyophilized.

After lyophilization, each extract was desalted and partially purified by chromatography on Sephadex G25 coarse in 0.2M acetic acid. Fig. 34 shows a typical column profile, obtained in the purification of a duodenal extract. The appropriate fractions were pooled as indicated, lyophilized and monitored for IR- motilin content at several dilutions. The results are presented in Table XIV.

TABLE XIV

Distribution of IR-motilin throughout the hog gastrointestinal tract

Region	IR-motilin in pg/ug dry weight extract	Ratio
Duodenum (Fr 1)	94	7
Jejunum (Fr 1)	1300	100
Ileum (Fr 1)	0.6	0.04
Fundus	*ND	0
Antrum	*ND	0
Oesophagus	*ND	0

* ND - non-detectable

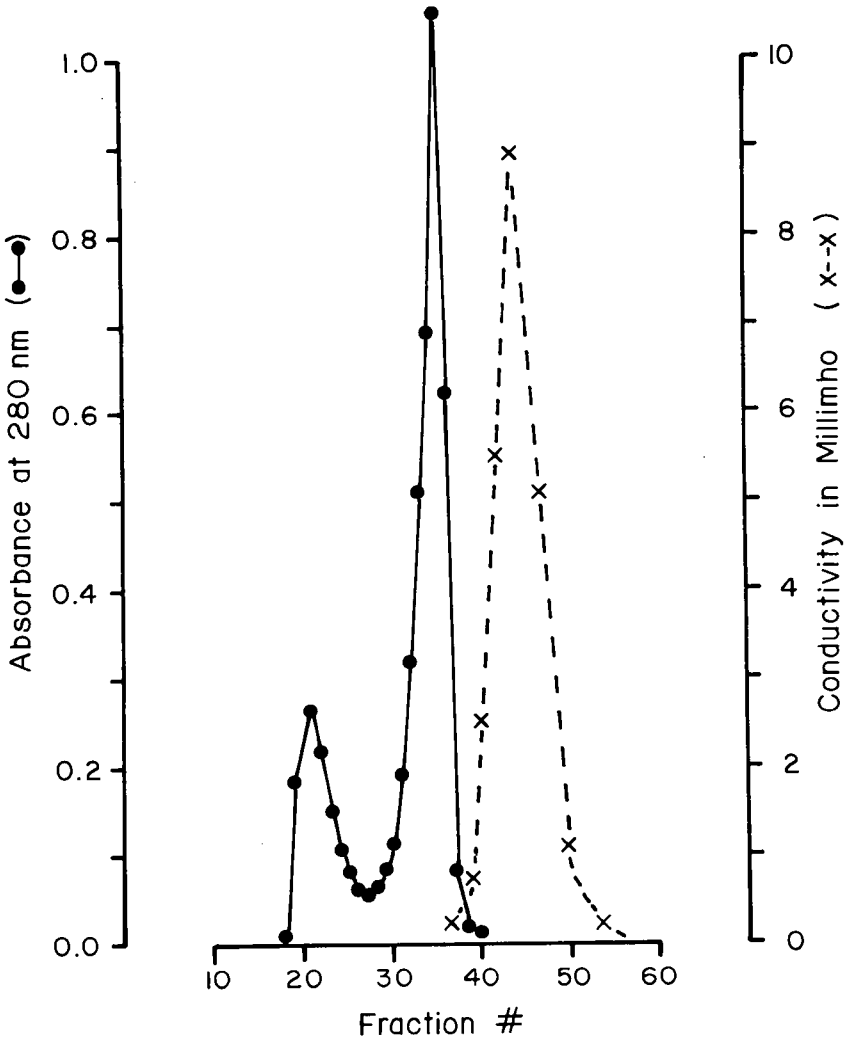


Fig. 34 Desalting of a crude extract of hog duodenal mucosa on Sephadex G-25 coarse (2.5 x 100 cm) in 0.2M acetic acid. Absorbance at 280 nm in a 1 cm light path and conductivity in millimho was determined for each fraction. Fractions 28-38 were pooled and lyophilized and subsequently referred to as Fr I.

The jejunal extract content was assigned an arbitrary value of 100 and in descending order anatomically, the following ratios were obtained:-

Oesophagus	0
Fundus	0
Antrum	0
Duodenum	7
Jejunum	100
Ileum	0.04

RELATIONSHIP BETWEEN GASTRIC (FUNDIC) MOTOR ACTIVITY AND ENDOGENOUS MOTILIN RELEASE

Six dogs prepared for the chronic bioassay study were used in this study. Blood samples were obtained whilst the dogs exhibited low spontaneous activity in the fundic pouch, during infusion of 50 mls 0.3M Tris buffer, pH 9.5, and whilst the motor activity was returning to (or below) basal levels. In 3 experiments, 50 mls 0.1M hydrochloric acid was substituted for the alkali and control experiments were performed in 4 dogs, during duodenal infusion with 50 mls 0.9% saline. Blood sampling and gastric motor activity recording were performed as in the alkali experiments.

On 2 different occasions dogs exhibited a spontaneous and significant increase in fundic motor activity. The experiment was continued and blood samples taken to monitor any changes in IR- motilin levels.

The fasting serum level of IR- motilin in the dogs was 412 $\pm$ 44 pg per ml (mean $\pm$ SE) and the duodenal pH was 7.5. Passage of the infusion into the duodenum was complete in 3 mins. After alkali, the circulating IR-

motilin had increased to 498 ± 100 pg per ml within 2 mins, the duodenal pH having reached 8.2. At the end of 5 mins the IR- motilin levels were at their peak value of 916 ± 96 pg/ml. The gastric motor activity also reached its maximum within the first 5 min post-infusion and the duodenal pH had returned to its pre-infusion level (Fig 35 and Tables XV, XVI). There was no increase in either IR- motilin or fundic pouch motor activity when the duodenum was perfusedn with saline, (Fig 36 and Tables XVII, XVIII). In the 2 instances, when the fundic pouch motor activity increased spontaneously, a concomitant and spontaneous elevation in IR- motilin was also noted, (Fig 37 and Tables XIX, XX).

The results obtained after duodenal perfusion with 0.1M hydrochloric acid were equivocal. The increase in circulating IR- motilin was not as great, was slower in reaching its peak value and was also slower in returning to the pre-infusion levels. There was no associated increase in fundic motor activity. The comparison between the incremental IR-motilin responses to acid, alkali and saline is summarized in Fig 38 and Table XXI.

EFFECT OF INGESTION OF GLUCOSE OR A MIXED MEAL ON THE CIRCULATING LEVELS OF IR- MOTILIN

These studies were performed in 6 fasted, human volunteers (aged 20 - 36 years) with no history of gastrointestinal disorder. On the first occasion the subjects were given, orally, 1g/kg glucose in a 20% solution. Blood samples were taken during the control period and at 5, 10, 15, 30, er, 60, 75 and 90 min after the ingestion of the glucose. On a second occasion, these same subjects were given a normal meal, consisting of

TABLE XV

Fundic motor activity response to duodenal infusion with alkali.

DOG	CONTROL				Minutes after onset of duodenal infusion											
I.D.	-20 -15	-15 -10	-10 -5	-5 -0	0 5	5 10	10 15	15 20	20 25	25 30	30 35	35 40	40 45	45 50	50 55	55 60
P_1	31.4	0	10.7	4.8	94.9	41.2	0	13.2	0	8.1						
P_2	69.5	73.0	42.2	44.0	97.2	23.3	34.8	23.2	45.8	35.0	16.0	17.2	26.8			
R_1	19.0	55.3	6.4	7.6	124.8	154.0	66.2	56.3	24.6	30.4	7.8	11.0	5.0	9.8	4.8	0
S_1	14.0	35.7	36.1	42.7	127.8	65.0	40.6	27.1	67.6	46.6	41.1	38.4	35.0	0	16.0	14.6
S_2	17.1	51.4	3.3	0	226.6	107.5	65.5	55.1	33.2	72.6	53.7	54.0	28.4	33.2		
R_2	64.7	62.5	46.3	45.4	179.8	84.2	48.6	30.6	42.8	33.4	18.8	35.3	8.4	0	0	5.1
$\overline{X}$	35.9	46.3	24.1	24.0	141.8	79.2	42.6	34.2	35.6	37.6	27.4	31.2	20.7	10.7	6.9	6.5
SE	10.1	10.5	7.9	8.9	21.0	19.3	9.9	7.1	9.2	8.6	8.5	7.7	5.9	7.8	4.7	4.2

TABLE XVI

IR-motilin (pg/ml) response to duodenal infusion with alkali.

DOG	CONTROL			Minutes after start of duodenal infusion								
I.D.	-20	-10	0	2	4	6	8	10	15	30	45	60
P_1	450	550	200	200	800			600	500	400		300
P_2	450	450	600	1800	1300	800	550			400	350	
R_1	300	280	280	280	450	960	540	560	620	380	300	280
S_1	310	270	460	500	650	1100		600	550	520	700	
S_2	377	311	277	622	544	477		577	440	300		280
R_2	890	570	560	970	1100	940	980	940	740	560	430	400
$\overline{X}$	412	405	396	728	807	855	690	655	430	426	445	315
SE	44	55	67	241	135	106	145	71	101	39	89	28

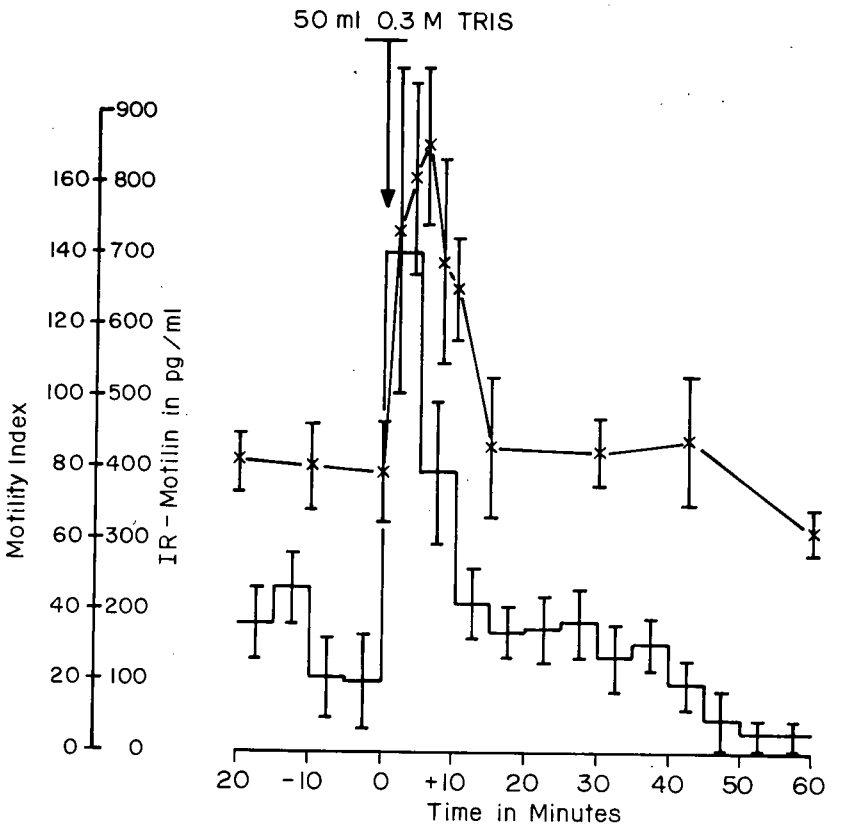


Fig. 35 Fundic pouch motor activity, expressed as an index of motility and circulating levels of IR- motilin in pg/ml after intraduodenal infusion of 0.3M tris buffer. Each motility index represents the mean ($\pm$ SE) for a 5 min period, whilst the serum levels of motilin (mean $\pm$ SE) are measured at a specific time. (Dryburgh and Brown (1975), Gastroenterology 68: 1169-1176) n= 6

TABLE XVII

Fundic motor activity response to duodenal infusion with saline.

DOG	CONTROL				Minutes after start of duodenal infusion of saline					
I.D.	20 15	-15 10	-10 5	-5 0	0 5	5 10	10 15	15 20	20 25	25 30
R_3	54.0	28.4	33.2	58.3	56.7	47.0	27.5	26.0	14.8	12.8
P_3	23.6	28.4	14.6	11.2	4.7	4.7	0	0	11.6	7.8
R_4	62.1	54.7	30.6	0	0	0	0	11.4	6.5	11.7
P_4	20.4	20.9	11.3	12.6	7.8	13.4	14.8	9.6	18.4	20.6
$\bar{X}$	40.2	33.1	22.4	20.5	17.3	16.3	10.5	11.7	12.8	13.2
SE	10.5	7.4	5.5	12.9	13.2	10.6	6.6	5.3	2.5	2.6

TABLE XVIII

IR-motilin (pg/ml) response to duodenal infusion with saline.

DOG	CONTROL			Minutes after start of duodenal infusion								
I.D.	-20	-10	0	2	4	6	8	10	15	30	45	60
R_3	320	470	510	410	390	240	350	360	400	280	280	280
P_3	450	390	380	125	310	350	125	300	280	280	350	280
R_4	380	280	240	280	320	310	260	200	125	200	280	300
P_4	410	440	450	380	350	380	270	310	300	400	350	300
$\overline{X}$	390	395	395	298	342	320	251	292	276	290	315	290
SE	27	41	58	64	17	30	46	33	56	41	20	5

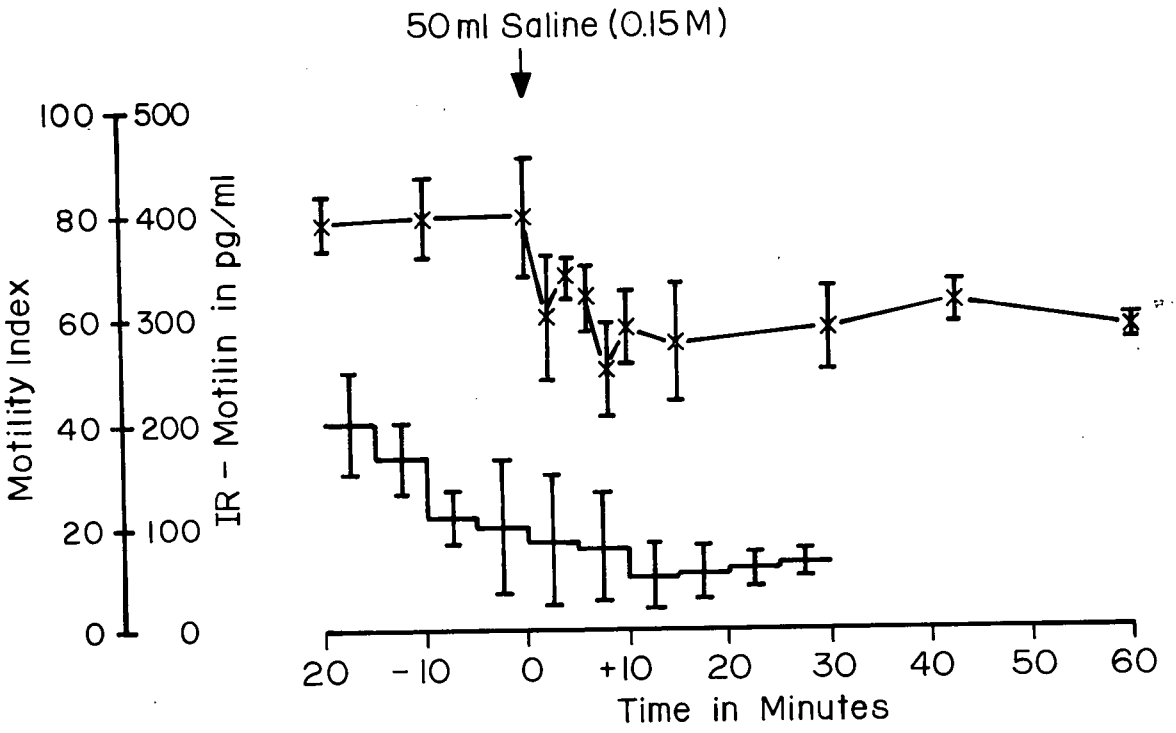


Fig. 36 Fundic pouch motor activity, expressed as an index of motility, and circulating levels of IR- motilin in pg/ml after intraduodenal infusion of 0.15M saline. Each motility index represents the mean ($\pm$ SE) for a 5 min period, whilst the serum levels of motilin are measured at a specific time, as mean $\pm$ SE (n = 4)

TABLE XIX

Spontaneously induced fundic motor activity.

DOG	CONTROL				Spontaneously induced fundic motor activity					
I.D.	-20 -15	-15 -10	-10 5	-5 0	0 5	5 10	10 15	15 20	20 25	25 30
R_5			82.2	63.1	143.8	127.7	25.7	11.6	0	0
P_7			38.4	14.2	67.8	65.8	16.0	14.6	39.2	40.6
$\bar{X}$			60.3	38.6	105.8	96.7	20.8	8.1	19.6	20.3
SE			21.9	24.4	38.0	30.9	4.8	6.5	19.6	20.3

TABLE XX

IR-motilin (pg/ml) response to spontaneously increased motor activity.

DOG	CONTROL			Time after onset of spontaneous activity							
I.D.	-20	-10	0	2	4	6	8	10	15	30	45
R_5	460	520	800	720	1000	430	400	400	270	350	300
P_7	390	520	700	1100	2600	640	520	680	500	300	300
$\bar{X}$	425	520	750	910	1800	535	460	540	385	325	300
SE	35	0	50	190	800	105	60	140	115	25	0

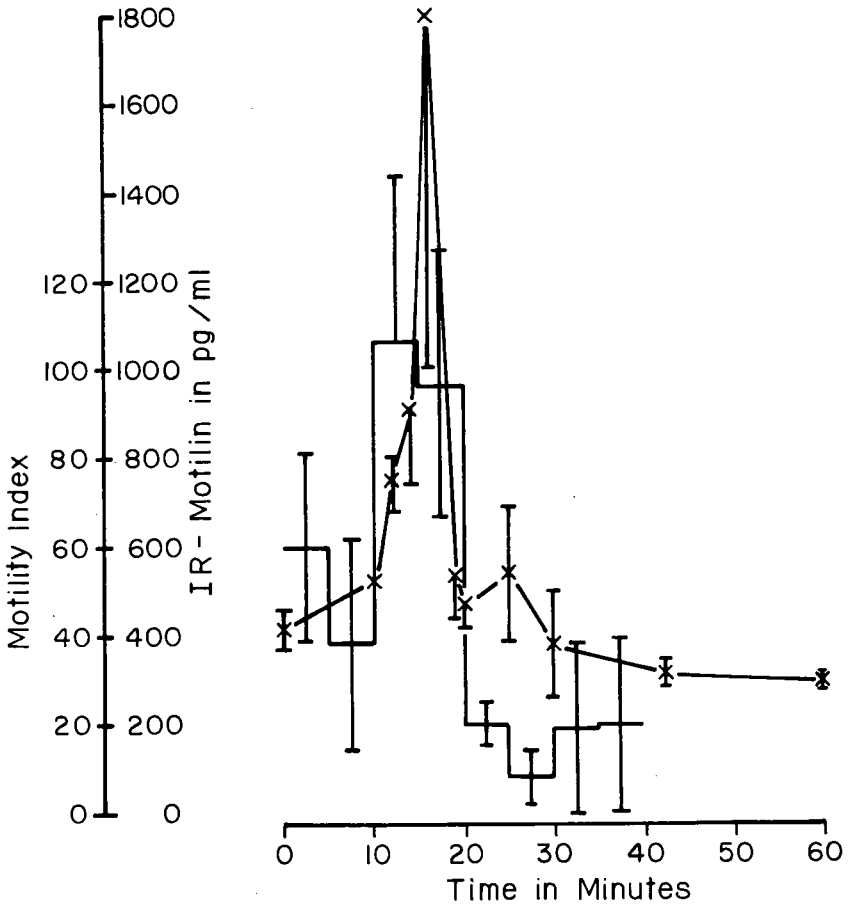


Fig. 37 Fundic pouch motor activity, expressed as an index of motility, and circulating levels of IR- motilin in pg/ml, during a spontaneous burst of fundic motor activity. Each motility index represents the mean $\pm$ SE for a 5 min period, whilst the serum levels of motilin (mean $\pm$ SE) are measured at a specific time. (n = 2)

TABLE XXI

Effect of duodenal infusion of alkali, acid or saline on the incremental IR-motilin (ΔIR-motilin) response.

	Time in min. after duodenal infusion of alkali.							
CONTROL	2	5	7	10	15	30	45	60
400	-200	400	-	200	100	0	-	-100
500	1300	800	300	-	-100	-	-150	50
280	0	170	680	280	340	100	20	0
350	150	300	750	250	200	170	350	-50
320	302	224	157	250	120	-20	20	-40
570	400	530	33-	370	170	-10	-130	-170
Mean	325	404	451	270	138	48	22	-77
±SE	214	95	115	28	59	37	89	31

TABLE XXI (Cont.)

	Time in min. after duodenal infusion of acid.							
CONTROL	2	5	7	10	15	30	45	60
165	155	355	395	365	285	185	35	165
160	100	200	270	180	200	5	130	70
330	40	-80	220	0	80	160	30	20
266	34	144	164	234	159	69	-86	-26
175	105	25	165	115	105	105	25	115
Mean	86	129	243	179	166	105	28	69
±SE	22	74	43	60	36	32	34	34
	Time in min. after duodenal infusion of saline.							
CONTROL	2	5	7	10	15	30	45	60
430	-20	-40	-190	-70	-30	-150	-150	-150
400	-275	-90	-40	-100	-120	-120	-50	-120
300	-20	20	10	-100	-175	-100	-20	0
430	-50	-80	-50	-120	-130	-30	-80	-130
Mean	-91	-48	-67	-97	-113	-100	-75	-100
±SE	61	24	42	10	30	25	27	33

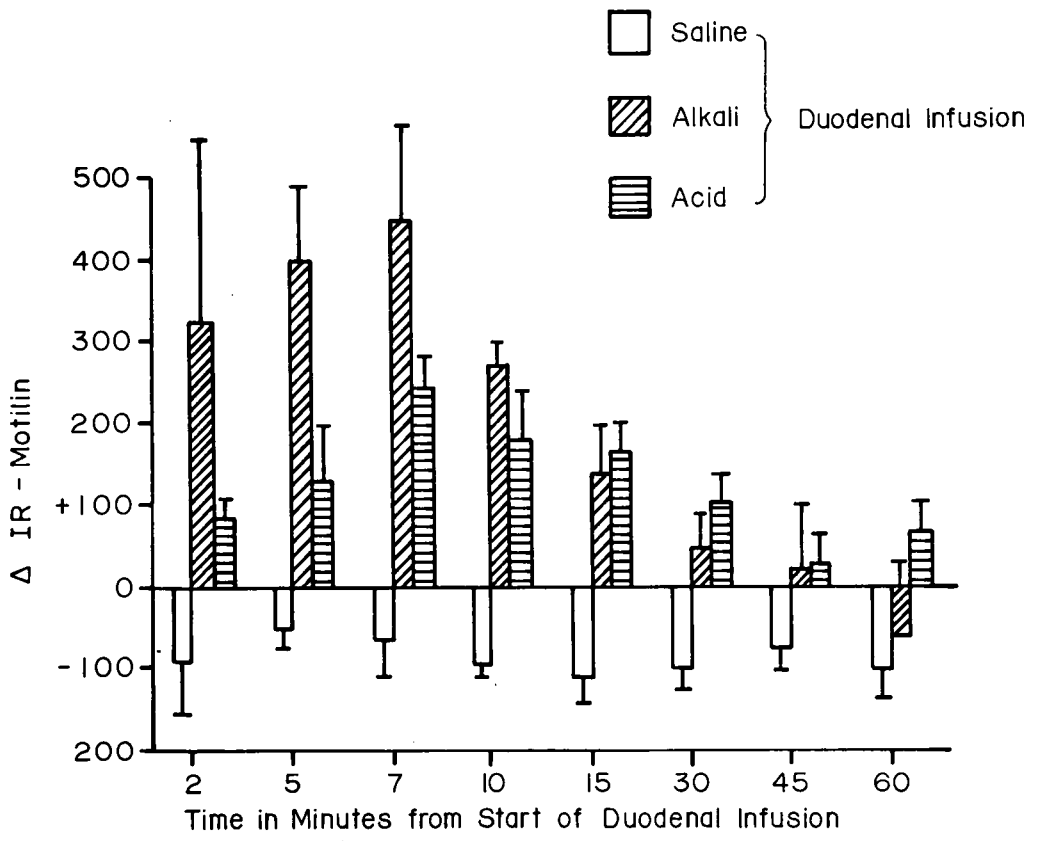


Fig. 38 A comparison of the incremental IR- motilin responses (IR- motilin in pg/ml to intraduodenal infusion of alkali, acid or saline, each point representing the mean ± SE for 5 experiments of each type.

orange juice, bacon and eggs with fried potatoes, toast with conserves and coffee. Blood samples were taken before breaking the fast and at 15 min. intervals thereafter for 2 hours.

No increase in systemic serum IR- motilin levels was observed. A significant ($P > 0.025$) decrease in IR- motilin (from the control values) was noted at 30 and 45 min. after ingestion of either glucose or the meal. The IR- motilin levels then tended to return to the control values (Tables XXII and XXIII; Fig. 39).

COMPARISON OF THE IMMUNOLOGICAL AND BIOLOGICAL ACTIVITIES OF SYNTHETIC AND NATURAL MOTILIN FRAGMENTS AND ANALOGUES

A. Immunological comparison.

(I) Synthetic motilin and fragments.

The initial purification products, MoB_1 and MoB_2, were found to contain 50% and 10% of the immunological activity of natural motilin respectively. The polypeptide MoC_1 shared complete immunological identity with the natural polypeptide whereas MoC_2, although chemically identical, was immunologically inactive, and may rêpresent an isomer of the active form.

Synthetic fragments, motilin 9-22 and 13-22 passed no immunological activity. The results are summarized in Fig. 40.

(II) Fragments of natural motilin

(a) Cyanogen bromide cleavage

The immunological cross-reactivity was measured on the mixture of products

TABLE XXII

Effect of ingestion of oral glucose

on serum levels of IR-motilin in pg/ml

	CONTROL			Post glucose ingestion							
	-30	-15	0	5	10	15	30	45	60	75	90
RAP	280	280	260	260	230	240	235	190	160	200	160
JRD	200	210	180	200	190	170	180	180	200	210	200
JCB	300	310	260	240	255	260	200	170	125	100	125
KM	190	200	160	140	125	100	100	100	100	140	160
HS	160	100	140	125	125	160	125	100	140	135	140
TM	200	200	230	215	190	160	170	135	140	200	210
Mean	221	216	205	196	185	181	168	145	144	164	165
±SE	23	30	21	17	22	24	20	16	14	18	13

TABLE XXIII

Effect of ingestion of a normal mixed meal

on serum levels of IR-motilin in pg/ml.

	CONTROL			Post meal ingestion							
	-30	-15	0	15	30	45	60	75	90	105	120
HS	230	280	200	300	190	160	200	220	200	250	225
KM	160	280	210	210	200	100	200	210	240	190	200
JRD	140	260	180	260	160	140	230	205	235	255	260
RAP	100	160	200	240	140	125	215	190	210	235	190
JCB	330	230	310	230	225	210	200	180	190	160	210
TM	280	235	240	180	100	125	170	160	140	200	205
Mean	206	240	223	236	169	143	202	194	202	215	215
±SE	36	18	19	17	18	15	8	9	15	15	10

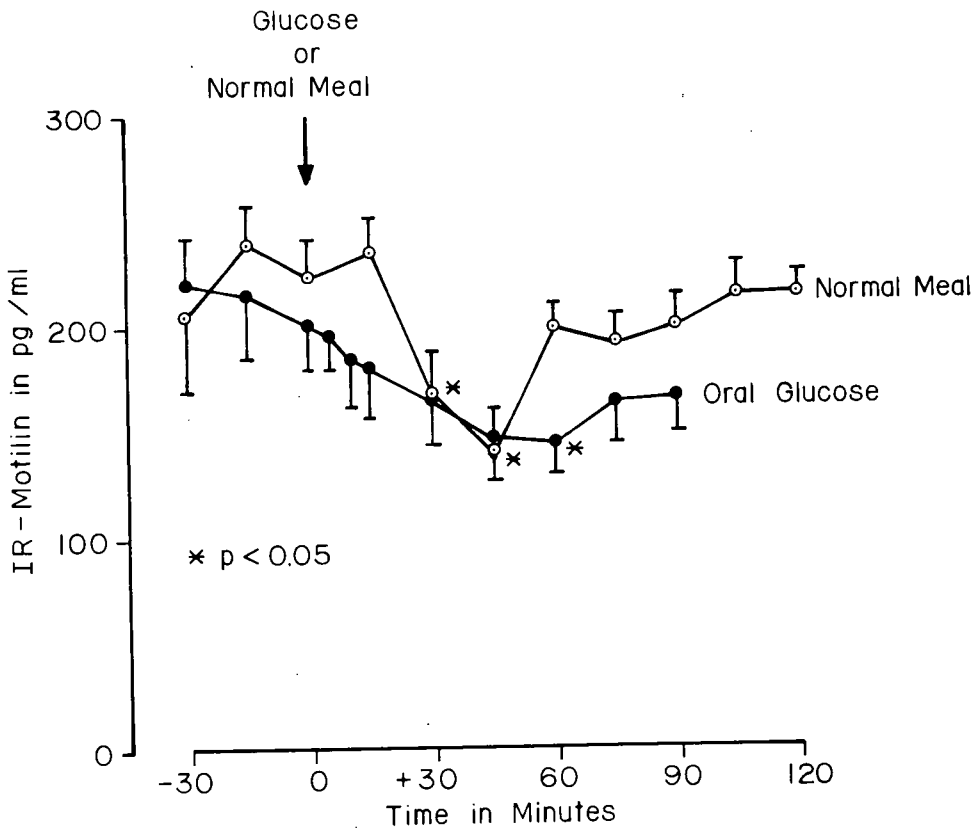


Fig. 39 The serum IR- motilin levels (mean $\pm$ SE) in pg/ml measured after ingestion of either 1 g/kg 20% dextrose or a normal, mixed meal in 6 normal subjects. Points marked * indicate a significance of >0.05 by the Mann-Whitney U test.

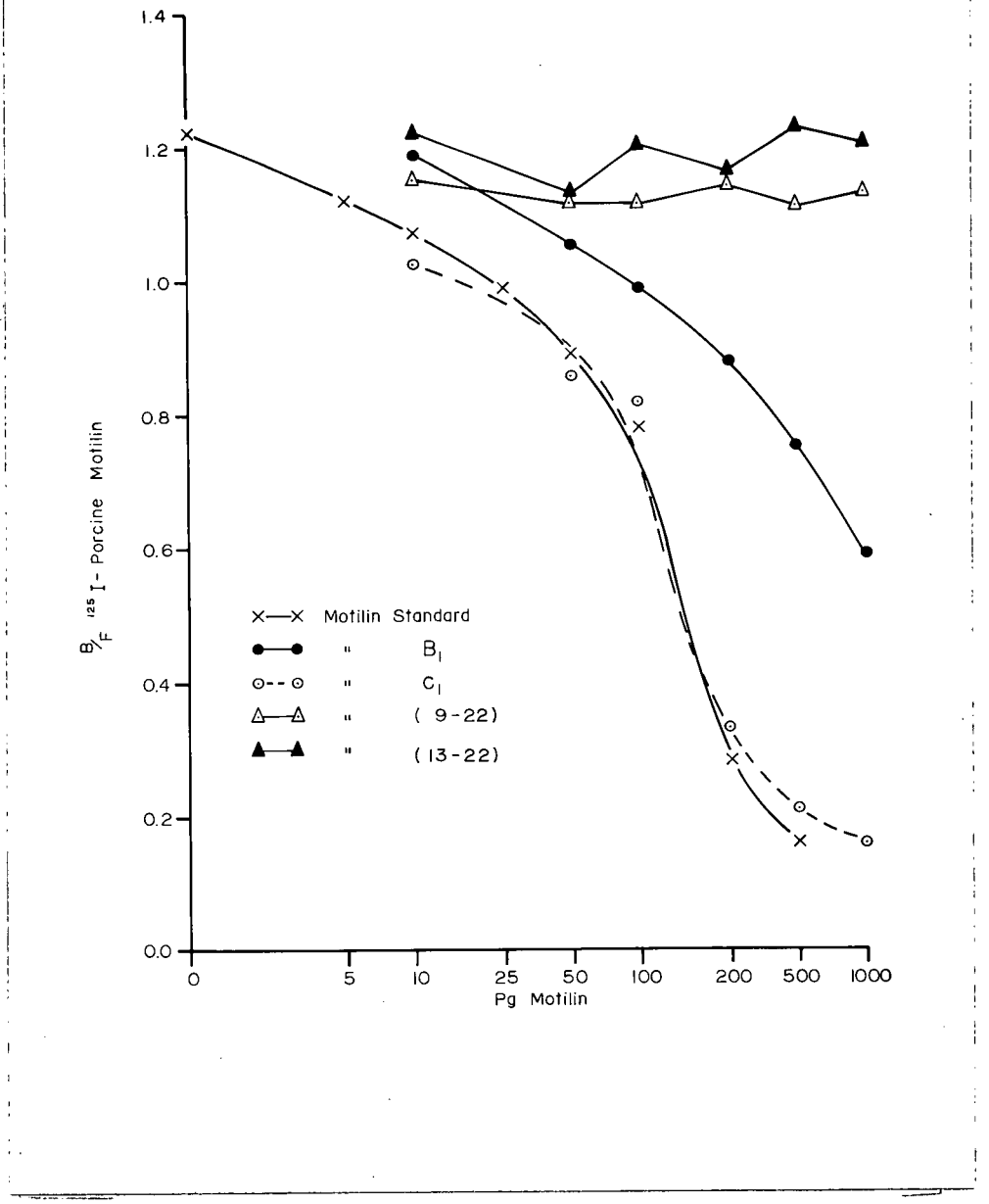


Fig. 40 Standard curve to motilin showing comparative immunoreactivities with synthetic 13-norleu-motilins B_1 and C_1 and synthetic fragments 9-22 and 13-22. Synthetic motilin C_1 showed 100% crossreactivity with natural porcine motilin. (Dryburgh and Brown (1975) Gastroenterology 68: 1169-1176)

produced by cyanogen bromide cleavage of motilin. The degree of cross-reactivity observed was approximately 30% of that seen with the intact molecule (Fig. 41). This can be explained by the presence in the mixture of uncleaved material, the cleavage being incomplete due to the presence of a small percentage of methionine sulphoxide residues which are resistant to the action of cyanogen bromide (Gross, 1967).

(b) Tryptic and chymotryptic digestion

After enzymatic degradation of the molecule by trypsin and chymotrypsin, acting specifically at the carboxyl terminals of the basic and aromatic amino acids respectively, tryptic peptides showed no cross-reactivity with motilin antiserum (Fig. 41).

(III) Modifications of natural motilin

(a) Removal of the C-terminal residue(s)

A 6 hour digestion of motilin with carboxypeptidase A resulted in release of 80% of the C-terminal glutamine and 30% of the penultimate C-terminal glycine. The degree of immunological activity remaining, approximately 18% that of the intact molecule, may be accounted for by the remaining undigested polypeptide.

(b) After removal of the N- terminal residue

After one cycle of the Edman's degradation reaction and the removal of the N- terminal phenylalanine only 18% of the original immunological activity remained. This decrease in immunoreactivity may be due to the loss of the aromatic N- terminal.

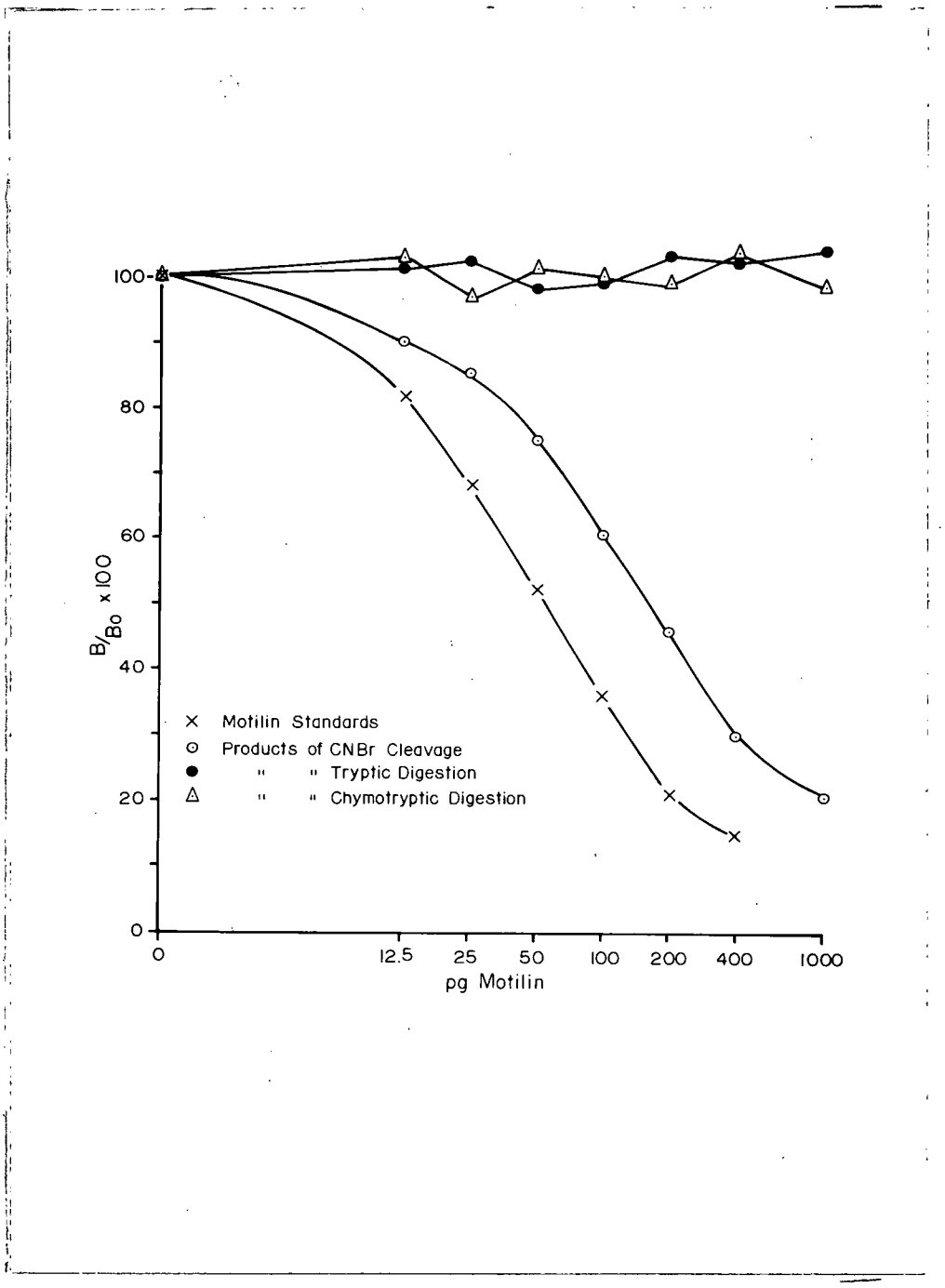


Fig. 41 Standard curve to motilin showing comparative immunoreactivities with the products of tryptic and chymotryptic digestion of motilin, or cleavage with cyanogen bromide.

(c) Acylation - acetylation

Treatment of the molecule with acetic anhydride resulted in acetylation of the lysine residues, the acyl derivatives having no charge, and loss of 50% of the immunoreactivity.

(d) Acylation - succinylation

Acyl derivatives obtained after treatment of motilin with succinic anhydride bear unit negative charge and have only 50% of the immunoreactivity of the original molecule.

B. Biological comparison

(I) Synthetic motilin

Comparison of the biological activity of the synthetic and natural polypeptides was achieved by matching the increase in fundic pouch motor activity in the chronic dog preparation produced by single intravenous injections of 1 µg or 2 µg of natural motilin with injections of the test material. No significant difference in the biological activity of MoC_1 (13- norlencine - motilin) or natural motilin could be detected (Fig. 42 and Table XXIV). No biological activity was observed after injections of the motilin fragments 9-22 or 13-22, even in doses equivalent to 10 times that of the natural peptide, on an equimolar basis.

(II) Fragments of natural motilin

The mixture of peptides produced by tryptic digestion were biologically inactive in doses equimolar with 10 µg natural motilin.

TABLE XXIV

Comparison of biological activities of natural and synthetic motilin

Dog ID.	Motility Index*			
	2µg dose		1µg dose	
	Natural motilin	Synthetic motilin	Natural motilin	Synthetic motilin
Polly	161.4	147.3	50.8	47.3
Abraham	178.2	168.7	59.1	76.5
$\bar{X}$	169.8	158.0	59.1	61.9
$\pm$SE	8.4	10.7	8.3	14.6

* represents a 10 min period

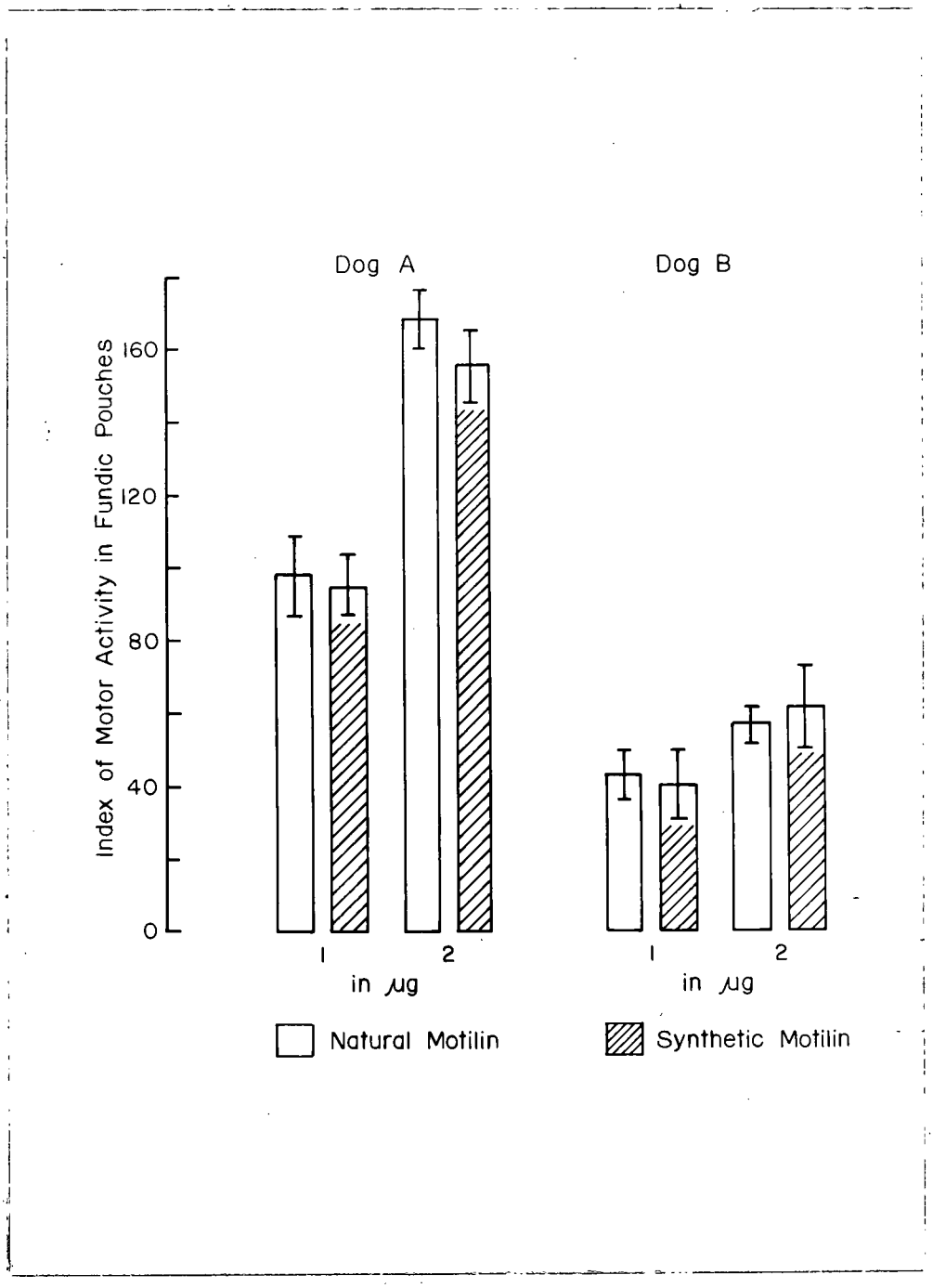


Fig. 42 Comparison of biological activities of synthetic 12-norleu-motilin and natural porcine motilin, assayed by measuring the increase in fundic pouch motor activity after bolus injections of 1 or 2 µg of each peptide. Each pair of results represents the mean $\pm$ SE of 4 experiments in 2 dogs. (Dryburgh and Brown (1975). Gastroenterology 68: 1169-1176).

(III) Modifications of natural motilin

(a) Removal of the C- terminal residue(s)

There was no loss in biological activity observed after the release of 80% of the glutamine residues and 30% of the glycine residues.

(b) Removal of the N- terminal residue

Comparable increases in fundic pouch motor activity were produced by 1 µg intact motilin and 10 µg motilin 2 - 22, i.e., removal of phenylalanine or the net loss of positive charges on the lysine residues reduced the activity by 90%.

(c) Acetylation

The biological activity was reduced by 90% after the net loss of positive charge in the acyl derivative.

(d) Succinylation

The biological activity of motilin was almost completely destroyed, i.e., to less than 5%, in the acyl derivative bearing negatively charged succinyl groups.

The comparative immunological and biological activities of the natural and synthetic peptides are summarized in Table XXV.

TABLE XXV

Comparison of the immunological and biological activities of synthetic and natural motilin fragments and analogues

Material	% Immunological activity	% Biological activity
MoB_1	50	90 - 100
MoB_2	10	Not tested
MoC_1	100	100
MoC_2	0	Not tested
Motilin 9 - 22	0	0
Motilin 13 - 22	0	0
CNBr motilin	30	Not tested
Tryptic digest	0	0
Chymotryptic digest	0	0
After C-terminal removal	18	100
After N-terminal removal	18	10
Acetylated derivative	50	10
Succinylated derivative	50	5

AFFINITY CHROMATOGRAPHY

A. Application to RIA

(I) Motilin

(a) Antiserum dilution and change in activity

Serial dilutions of the coupled ligand were incubated with ^{125} I- motilin in a total volume of 1.0 ml, for 48 hours at 4°C. All other variables were identical to those of the routine RIA. The tubes were centrifuged at 2800 rpm for 30 min., the supernatants discarded and the pellets counted. The antiserum dilution curve (coupled ligand) is shown in Fig. 43 in comparison with an antiserum dilution curve (uncoupled ligand) obtained under identical conditions in a routine RIA. There was no significant loss in activity produced by coupling the antibody to a solid matrix.

(b) RIA standard curves and change in sensitivity

Motilin standards over the range 12.5 - 400 pg were incubated with ^{125} I- motilin and equivalent final dilutions of coupled or uncoupled antisera as follows:-

(i) routine RIA conditions, including uncoupled antiserum and usual charcoal separation;

(ii) at 1.0 ml incubation volume, with coupled antiserum;

(iii) at 0.3 ml incubation volume, with coupled antiserum;

(iv) at 0.3 ml incubation volume, with coupled antiserum, in siliconized tubes.

The standard curves obtained (Fig.44) were judged by the slope at zero dose (calculated as shown in Fig. 45 and Table XXVII) and L.D.D. The greatest sensitivity was established when coupled antiserum was incubated with radioactive tracer in a total volume of 0.3 ml, in non-siliconized tubes (Table XXVI).

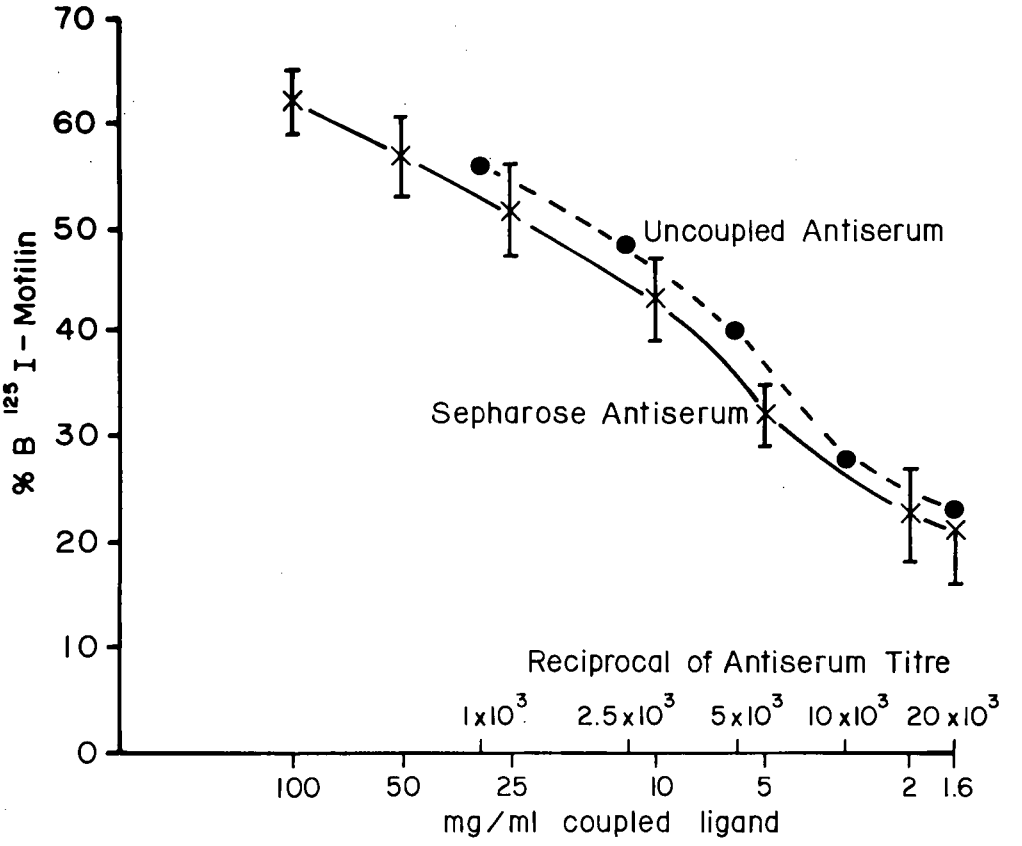


Fig. 43 Comparison of dilution curves obtained with antiserum to motilin, performed on aliquots taken before and after coupling of the antiserum to CNBr-activated Sepharose 4B.

TABLE XXVI

Coupled versus uncoupled antisera to motilin at varying incubation volumes

Standard curve	Criteria for Evaluation		
	Slope at Zero Dose	Midrange	L.D.D.
a.	4.5×10^{-13} 1/mole	88 pg	25 pg
b.	2.5×10^{-13} 1/mole	320 pg	25 pg
c.	7.5×10^{-13} 1/mole	120 pg	12.5 pg
d.	5.5×10^{-13} 1/mole	150 pg	25 pg

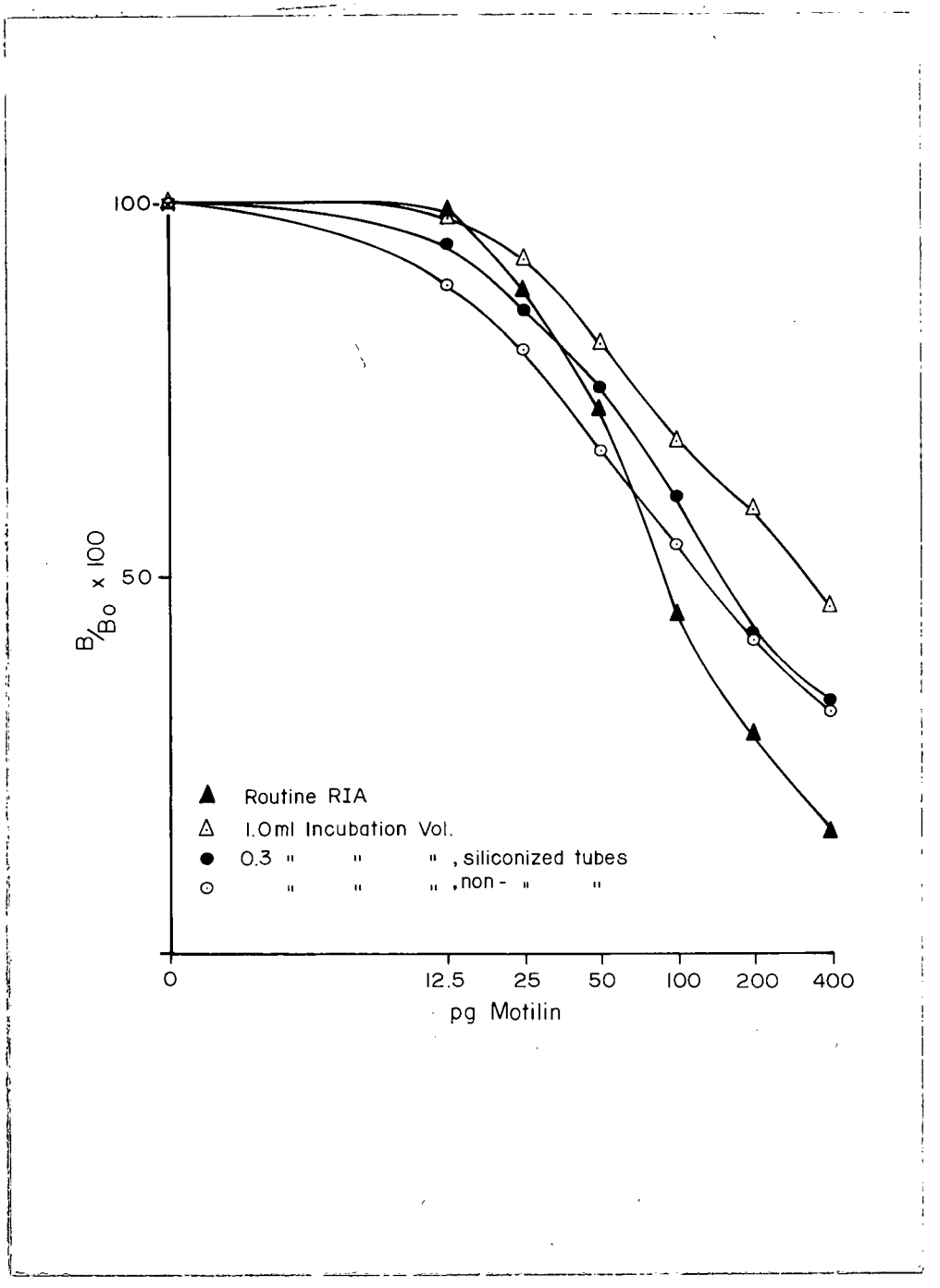


Fig. 44 Comparison of the standard curves obtained with antiserum to motilin under routine assay conditions or after coupling to Sepharose 4B, at various incubation volumes, in siliconized or non-siliconized tubes.

TABLE XXVII

Calculation of the slope at zero dose.

CURVE	STD in pg	CONC in moles ($\times 10^{-15}$)	BOUND (%)	B/F	B x CONC ($\times 10^{-15}$)	SLOPE AT (L/mole)
	0	1.3	0.315	0.46	0.42	
a	12.5	5.9	0.309	0.40	1.8	
	25.0	10.5	0.267	0.36	2.6	
	50.0	19.8	0.223	0.28	4.4	4.5×10^{-13}
	0	1.3	0.309	0.44	0.40	
b	12.5	5.9	0.305	0.43	1.8	
	25.0	10.5	0.284	0.39	2.9	
	50.0	19.8	0.251	0.33	4.9	2.5×10^{-13}
	0	1.3	0.448	0.81	0.58	
c	12.5	5.9	0.398	0.66	1.3	
	25.0	10.5	0.359	0.56	3.7	
	50.0	19.8	0.298	0.42	5.9	7.5×10^{-13}
	0	1.3	0.428	0.74	0.55	
d	12.5	5.9	0.402	0.67	2.3	
	25.0	10.5	0.367	0.57	3.8	
	50.0	19.8	0.323	0.44	6.4	5.5×10^{-13}

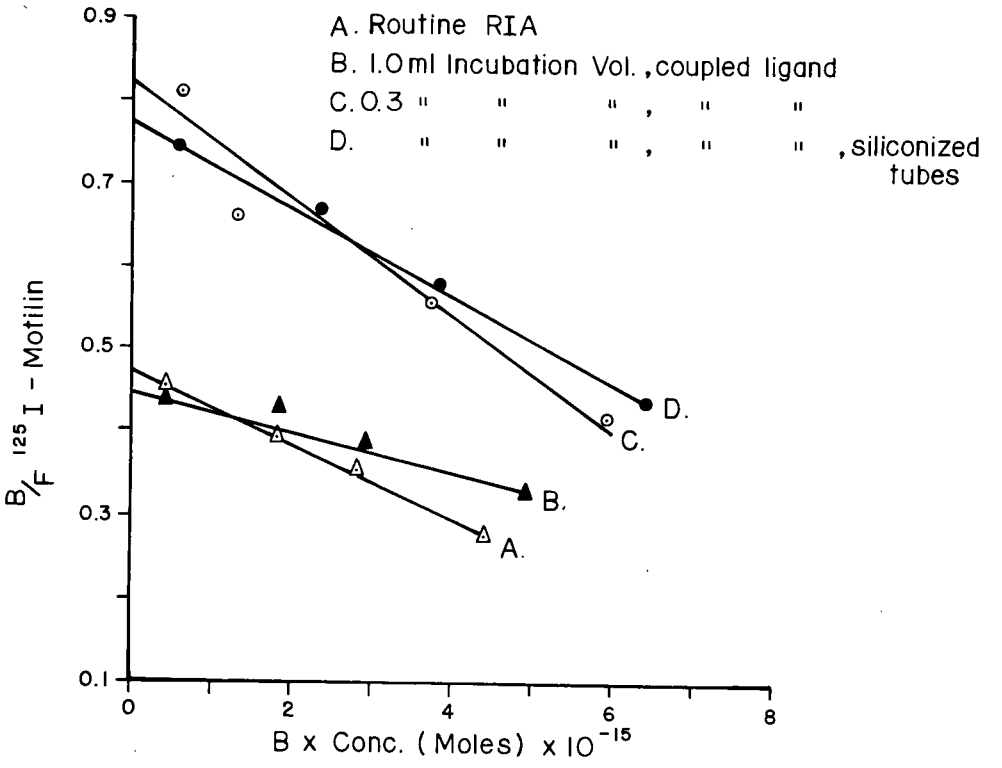


Fig. 45 Standard curves, from Fig. 44, presented as Scatchard plots, demonstrating the slope at zero dose.

II Gastric inhibitory polypeptide

a. Antiserum dilution and change in activity

Comparison of the antiserum dilution curves obtained with coupled and uncoupled antisera to GIP after incubation with ^{125}I- GIP under routine RIA conditions is shown in Fig. 46. Coupling of GIP antiserum to a solid matrix resulted in a recovery of only 10% (approximately) of the antibody activity.

b. RIA standard curves and change in sensitivity

Standard GIP in the range 12.5 - 400 pg was incubated with different final dilutions of coupled and uncoupled antisera, to give approximately the same maximum binding. Not only did the coupled antiserum have to be used at a much lesser dilution but it also produced a less sensitive assay curve, with a midrange value of 340 pg and LDD of 50 pg compared to values of 170 pg and 25 pg respectively obtained with the same antiserum when used uncoupled, (Fig. 47).

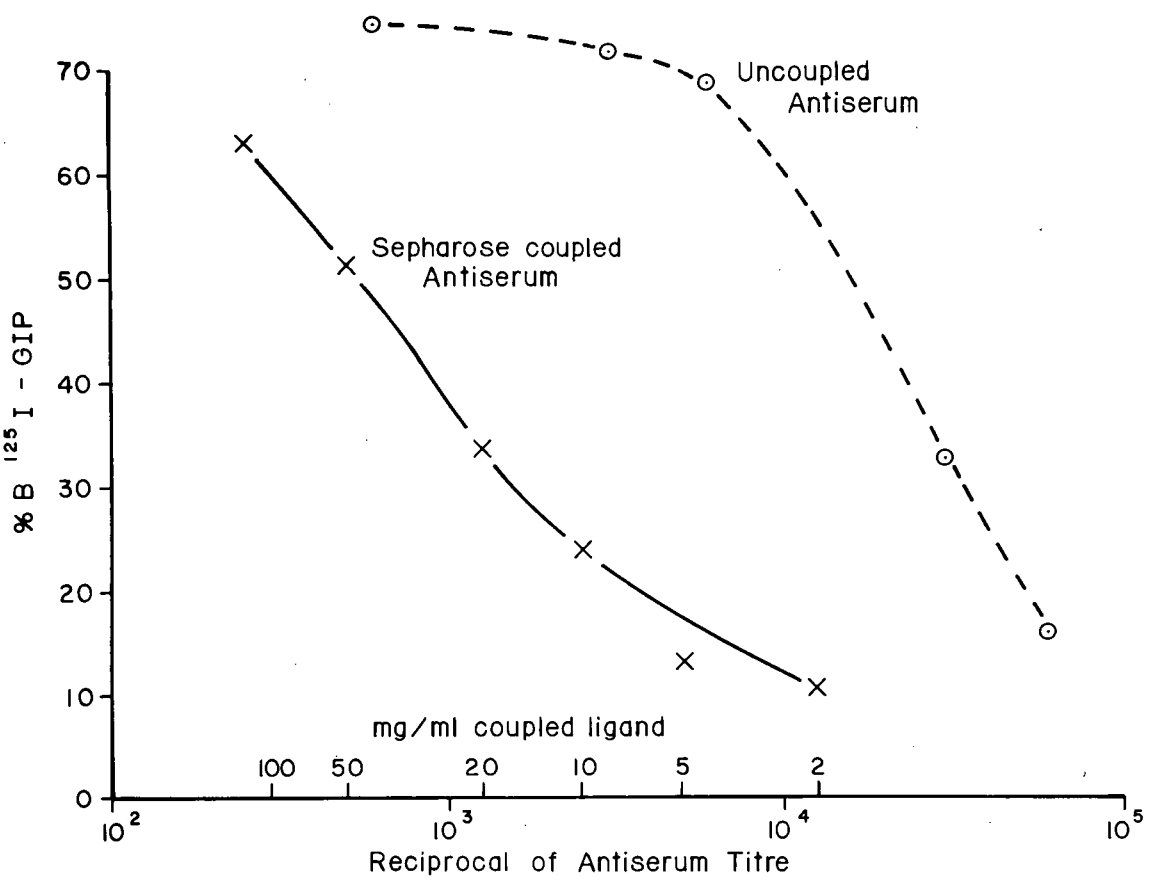


Fig. 46 Comparison of dilution curves obtained with antiserum to GIP, performed on aliquots taken before and after coupling of the antiserum to CNBr-activated Sepharose 4B.

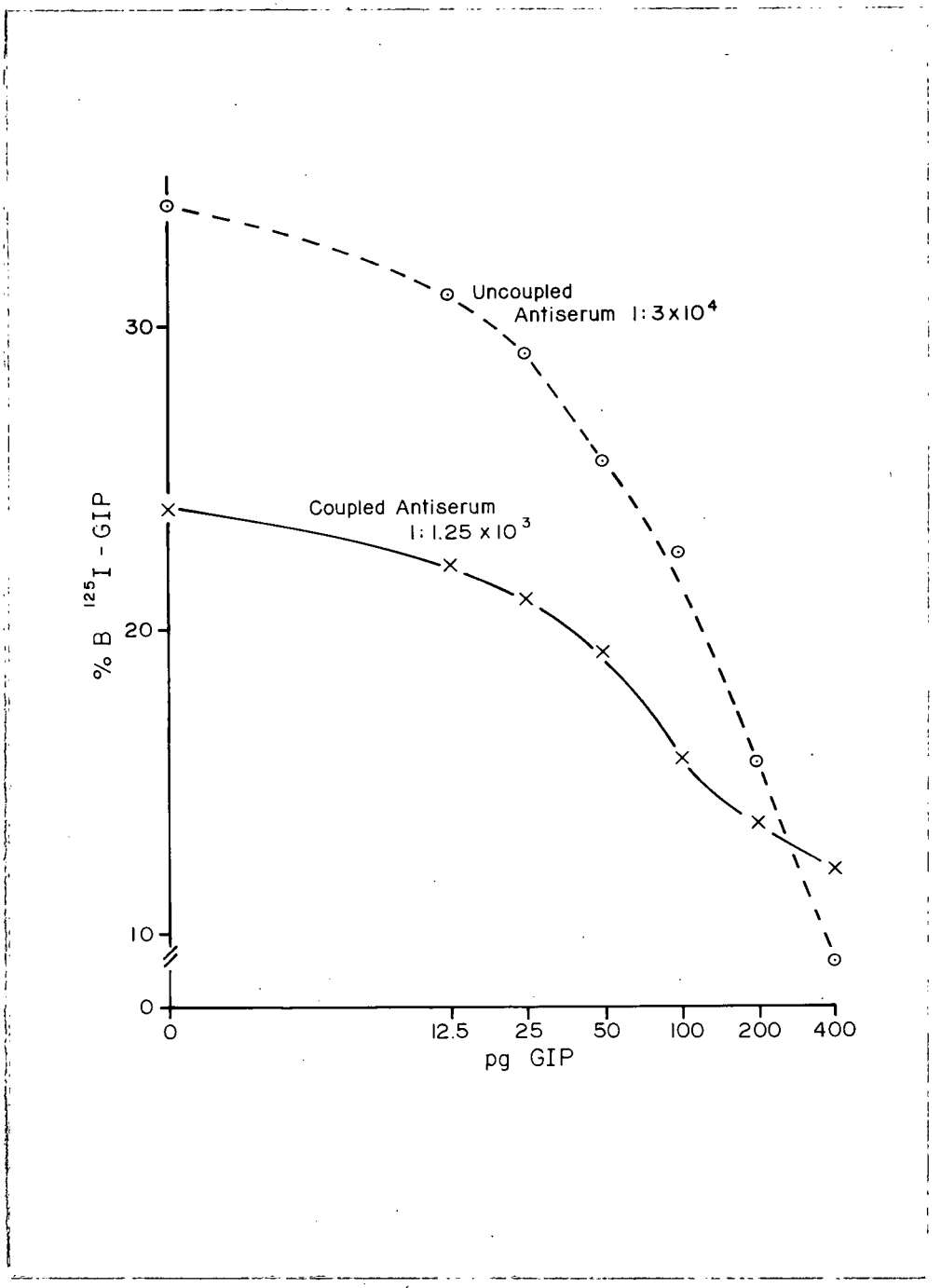


Fig. 47 Comparison of the standard curves obtained with antiserum to GIP, under routine assay conditions or after coupling to Sepharose 4B, demonstrating the loss of both antiserum activity and sensitivity potential after the coupling procedure.

B. Application to purification of motilin

(I) Purification of ^{125}I- Motilin

A column of activated Sepharose 4B, coupled to motilin antiserum, was prepared in a Pasteur pipette, the bed volume being 1.0 ml. The gel was well equilibrated in 0.04M sodium phosphate buffer, pH 6.5. A lyophilized sample of labelled motilin, containing 2×10^6 cpm (estimated to contain 4 ng IR- motilin from the label specific activity) was applied in 0.5 ml of the equilibrating buffer and the column developed in this buffer until no further counts were eluted. That portion of the ^{125}I- motilin which had been bound to the gel was eluted with 0.2M acetic acid. One ml fractions were collected throughout and the column profile in Fig. 48 obtained by plotting cpm/fraction against ml of eluant.

Eighty percent of the initially applied cpm remained bound to the gel until the pH of the eluting buffer was lowered to 2.4, when 78% of that bound material was eluted. The remaining 22% was distributed between the gel and the glass wool at the bottom of the column.

An aliquot of this same radioactive tracer was treated with coupled Sepharose 4B in a batchwise manner, as follows:- 2×10^6 cpm ^{125}I- motilin was diluted in 5.0 mls 0.04M phosphate buffer, pH 6.5 and mixed with 1.0 ml Sepharose slurry, equilibrated in the phosphate buffer, for 18 hours at 4°C. The supernate was discarded and the gel washed with 5x5 ml phosphate buffer, i.e., until the counts in the wash were negligible. The gel was resuspended in 5 mls 0.2M acetic acid and mixed, by rotation, for 1 hour. The supernate was diluted in assay diluent buffer to give ~ 5000 cpm/100 µl. An aliquot of

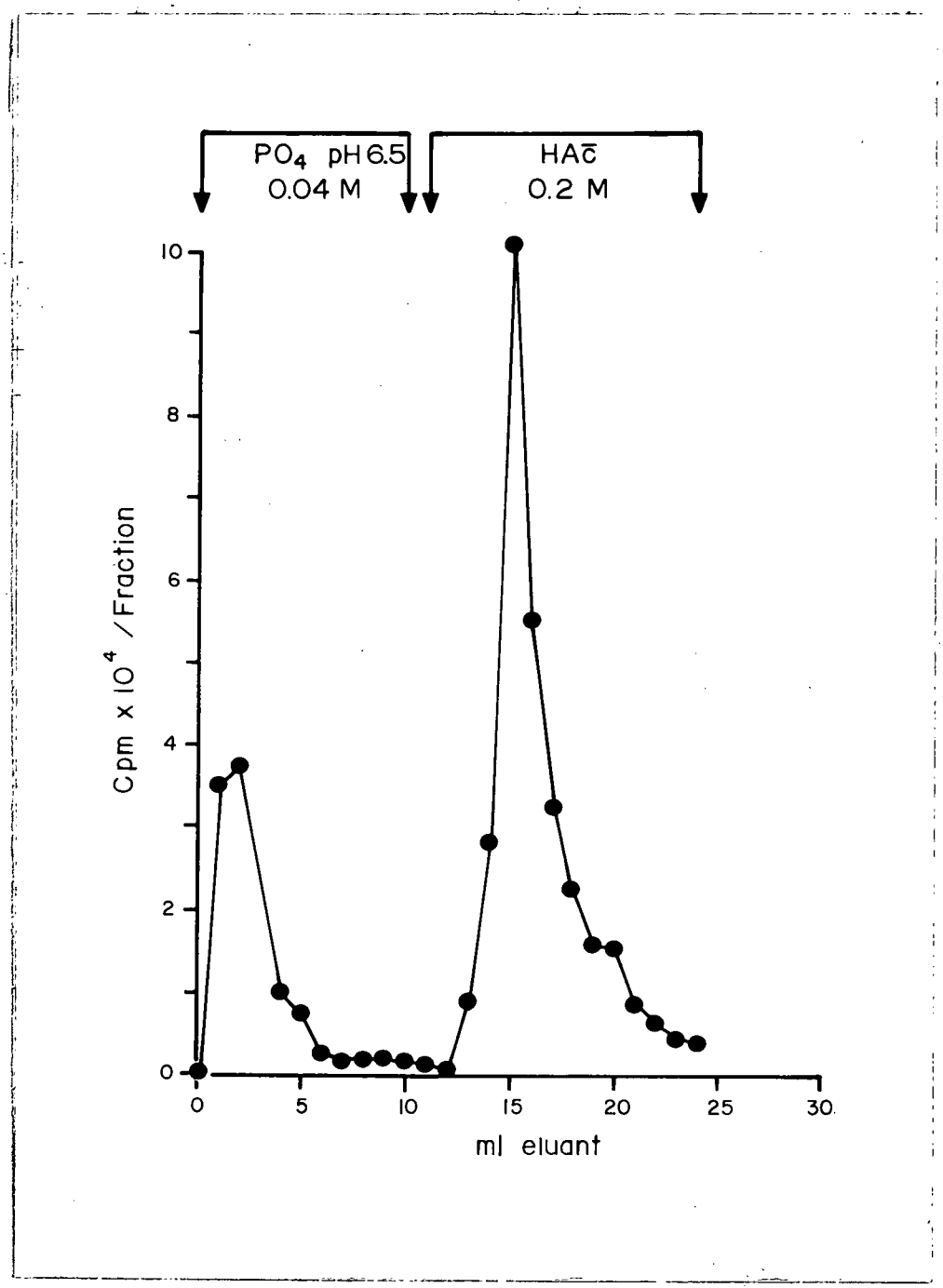


Fig. 48 Column profile obtained after elution of ^{125}I- motilin, adsorbed to Sepharose 4B, with 0.04M phosphate, pH 6.5 and 0.2M acetic acid, pH 2.4. One ml samples were collected and counted for 1 min in an automatic γ counter.

untreated ^{125}I- motilin was similarily diluted to produce the same concentration and standard curves were prepared with both labels.

The non-specific binding of the untreated label was 18.9%B, that of the treated portion was 10.6%. This improvement was due to the removal of free iodine and damaged, iodinated but non-immunoreactive fragments. There was no significant difference in the curves produced (see Fig. 49), the iodinated motilin appeared unaffected by its passage through the gel.

(II) Extraction of motilin added to plasma

Natural porcine motilin was diluted in charcoal-extracted plasma to a concentration of 4 ng/100 µl. When 4 ng was applied to a column of Sepharose 4B and the column developed as previously described, RIA was used to monitor the recovery of motilin. The column profile, in Fig. 50, is a plot of pg/ml IR- motilin against ml eluant. Initially 83% of the motilin was bound to the gel and 100% of that amount was recovered.

(III) Extraction of endogenous motilin from serum

Two mls serum (subject R.K.), which contained 680 pg IR- motilin/ml was subjected to affinity chromatography as in II.

Virtually 100% of the applied motilin was bound to the gel and then recovered by elution with a lower pH buffer (Fig. 51).

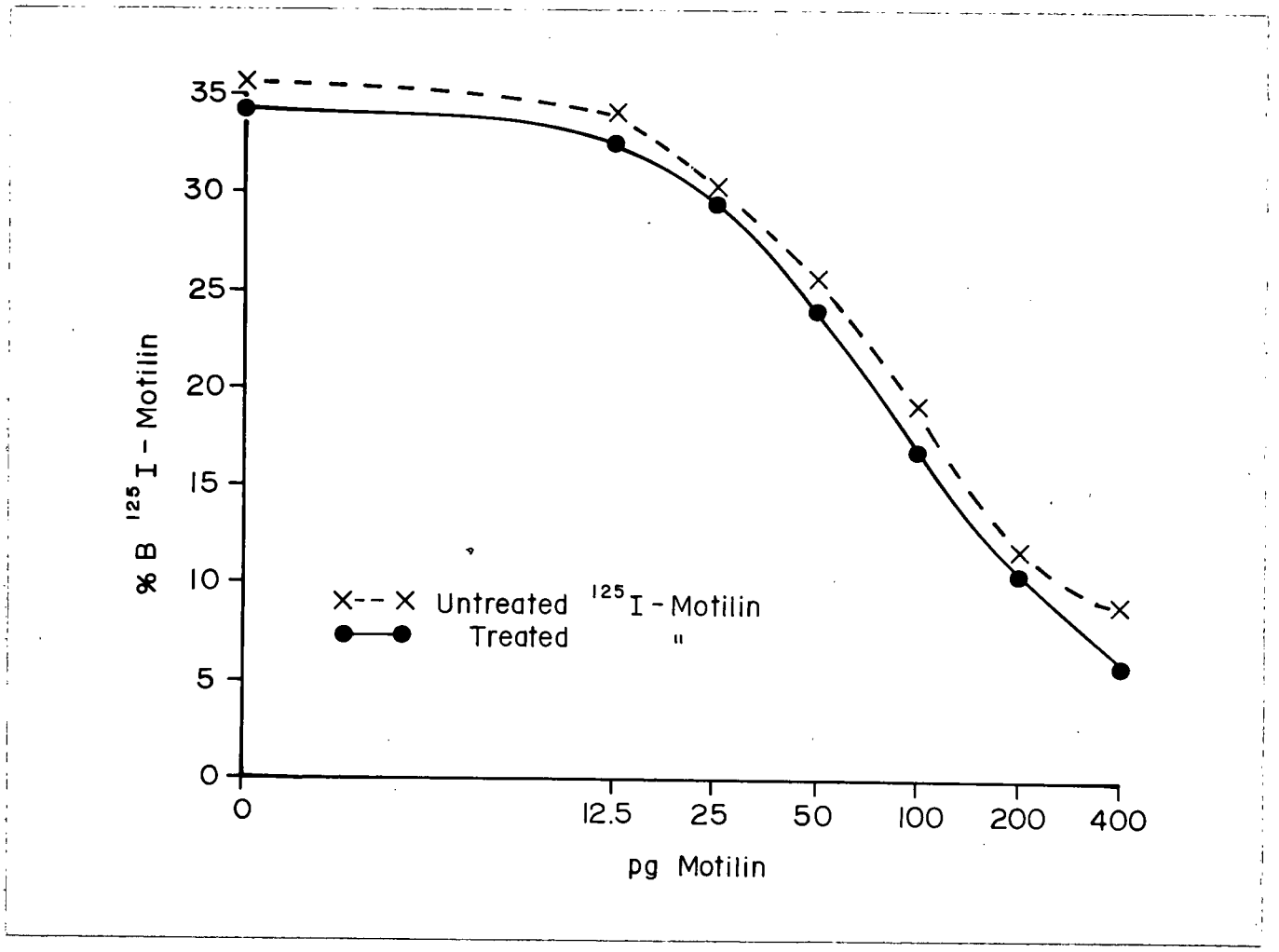


Fig. 49 Comparison of standard curves obtained with ^{125}I- motilin, before and after treatment by chromatography on a column of Sepharose 4B.

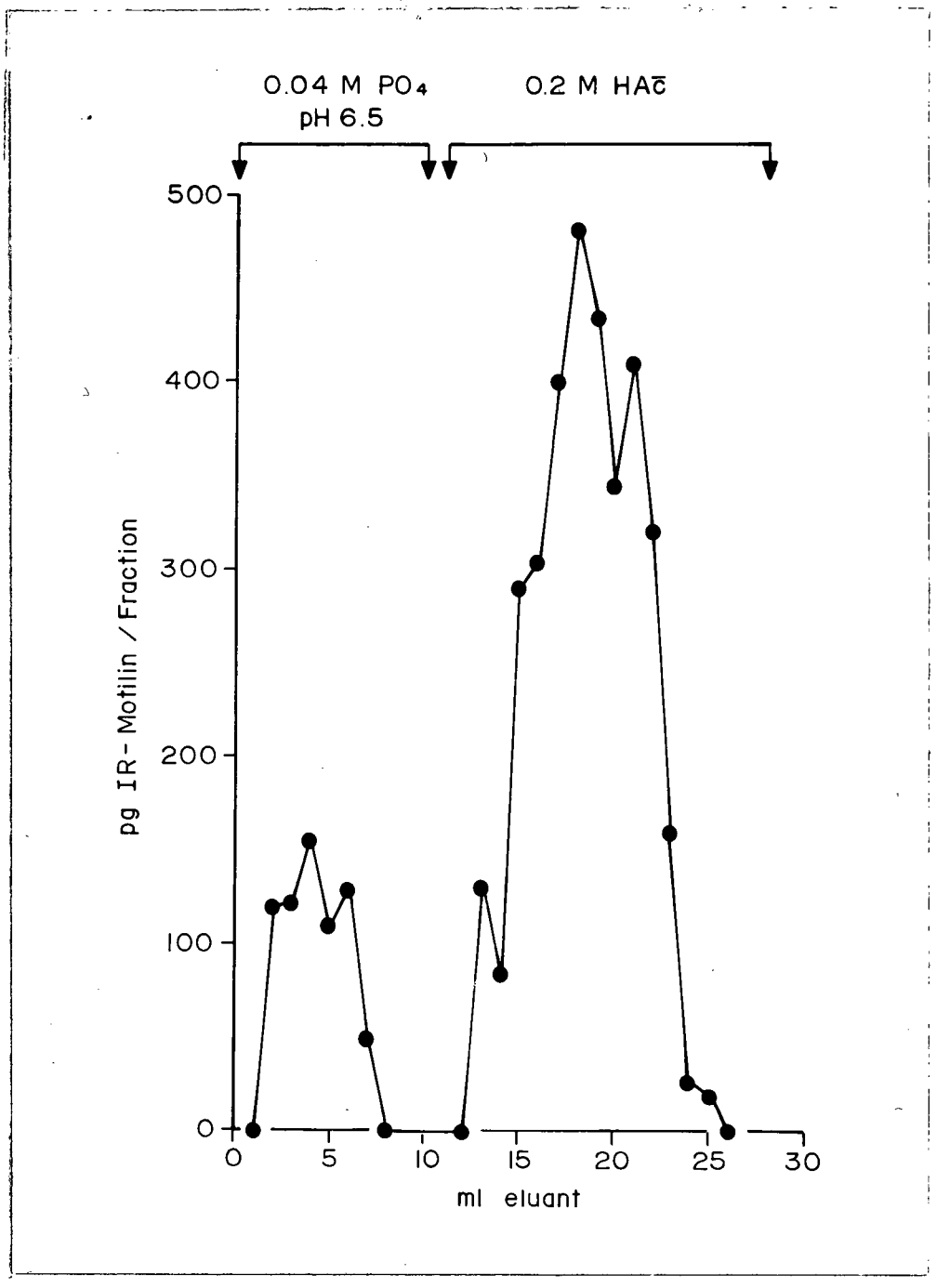


Fig. 50 Column profile obtained after elution of a motilin-containing plasma, adsorbed onto Sepharose 4B, with 0.04M phosphate, pH 6.5 and 0.2M acetic acid, pH 2.4. One ml samples were collected and monitored for IR- motilin by radioimmunoassay.

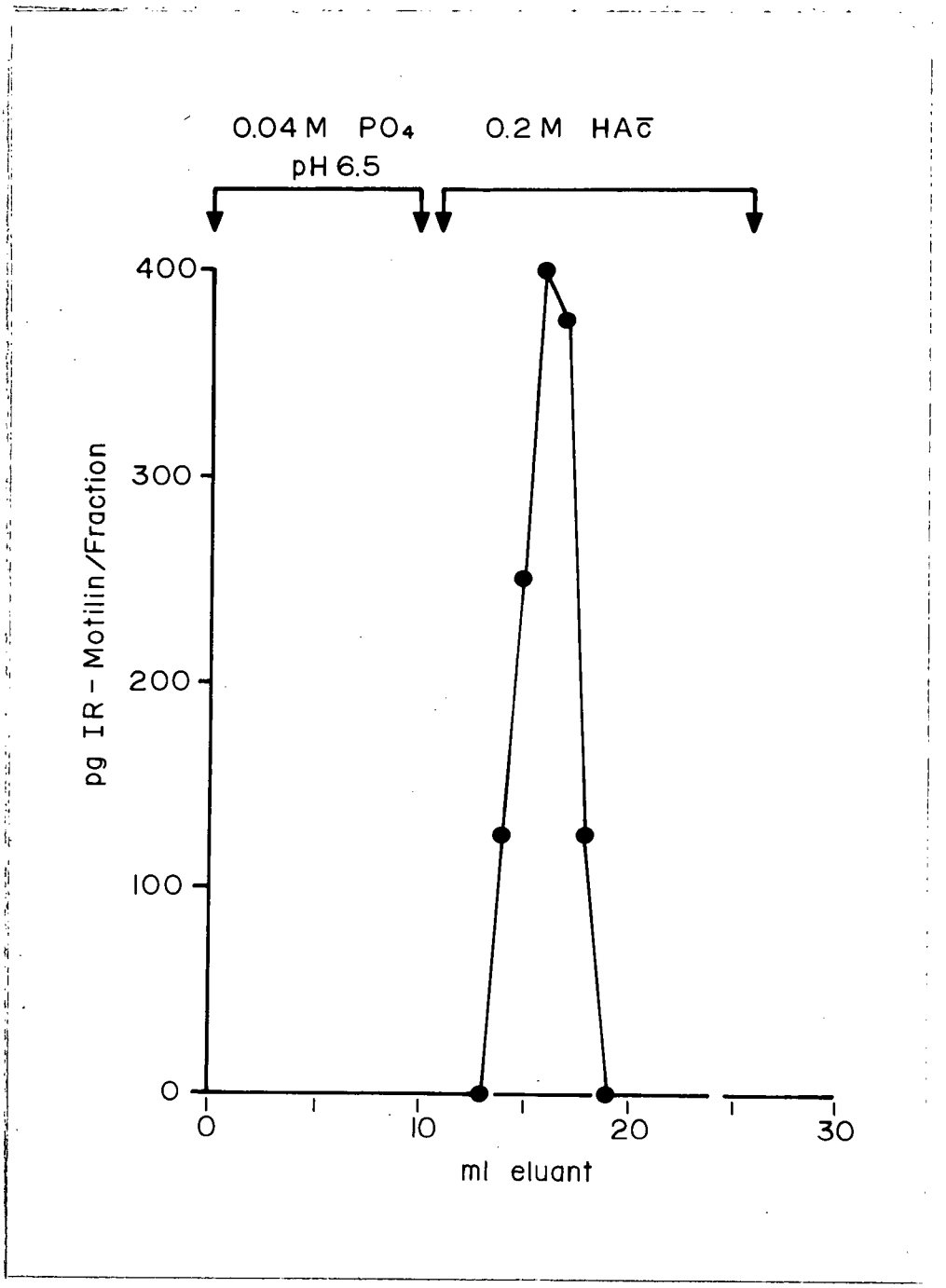


Fig. 51 Column profile obtained after elution of a motilin-containing serum, adsorbed onto Sepharose 4B, with 0.04M phosphate, pH 6.5 and 0.2M acetic acid, pH 2.4. One ml samples were collected and monitored for IR- motilin by radioimmunoassay.

(IV) Isolation of motilin from presekretin

Presekretin, a side fraction produced during the purification of secretin, is the starting material in the isolation of motilin (Brown et al, 1971). The original purification procedure involved 5 stages of column chromatography, was monitored in the chronic dog bioassay and resulted in production of an active moiety representing 0.5-1.0% of the starting material by weight.

Two µg Presekretin, estimated to contain 4 ng IR- motilin, was dissolved in 0.04M phosphate buffer, pH 6.5 and treated as previously described. The IR- motilin content was 90% recovered and the yield represented a 5% recovery of the starting material by weight, (Fig. 52).

EFFECTS OF MOTILIN ON THE RATE OF GASTRIC EMPTYING

It was desirable to perform the studies on the rate of gastric emptying during a relatively constant background of circulating motilin. Pilot studies indicated that IR- motilin levels reached a plateau within 20 mins. of the start of the infusion and therefore measurements of the rate of gastric emptying were performed in the last 10 mins. of a 30 mins. infusion (Fig. 53).

A. Control studies in the gastric fistula dog

(I) Effect of motilin on the rate of gastric emptying of liquids

Motilin infusions in the range 0.125 - 2.0 µg/kg/hour accelerated the rate of emptying of the test liquid in a dose-related manner. The basal rate (C)

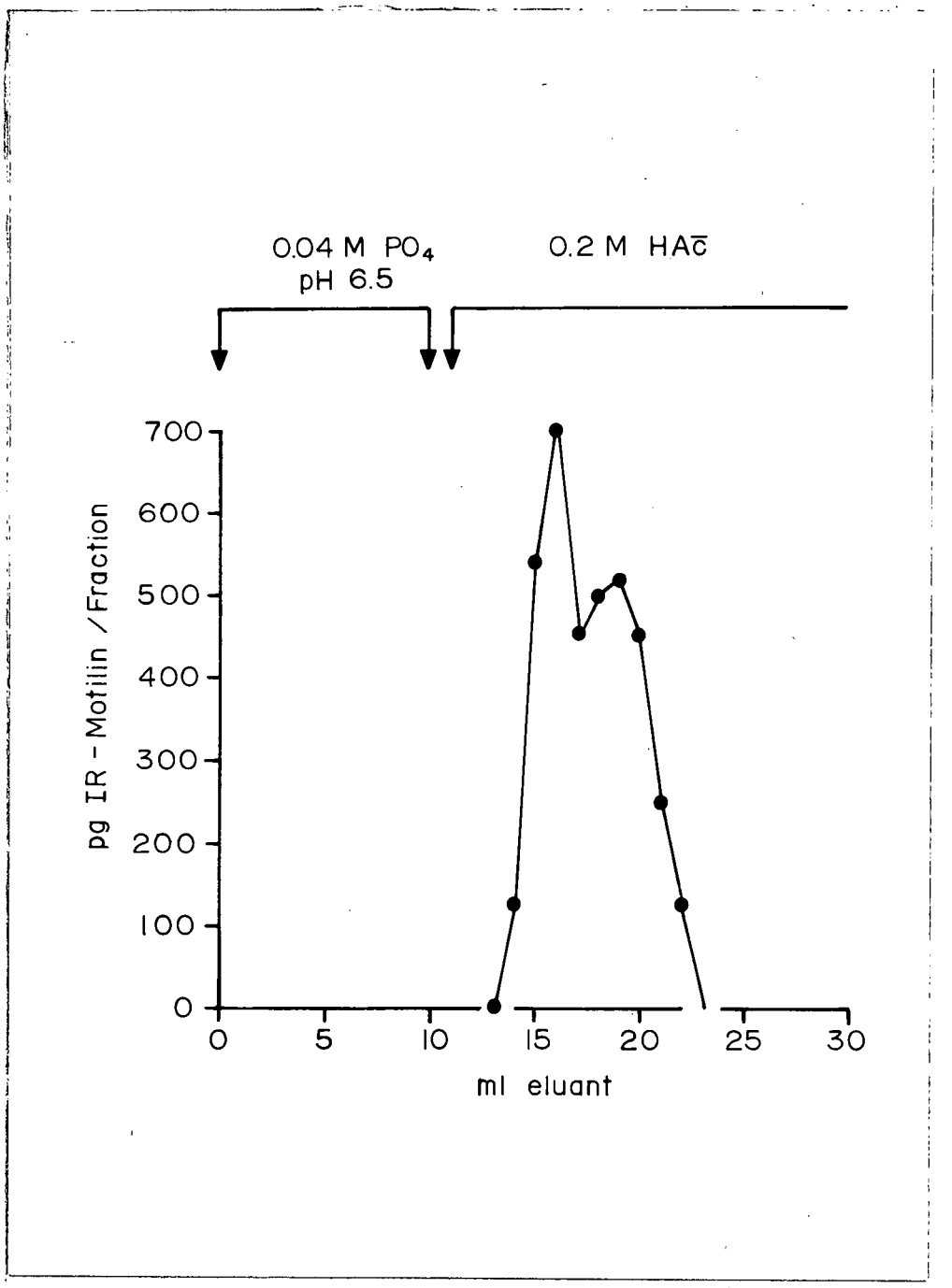


Fig. 52 Column profile obtained after elution of a motilin-containing duodenal extract (Presekretin), adsorbed onto Sepharose 4B, with 0.04M phosphate pH 6.5 and 0.2M acetic acid, pH 2.4. One ml samples were collected and monitored for IR- motilin by radioimmunoassay.

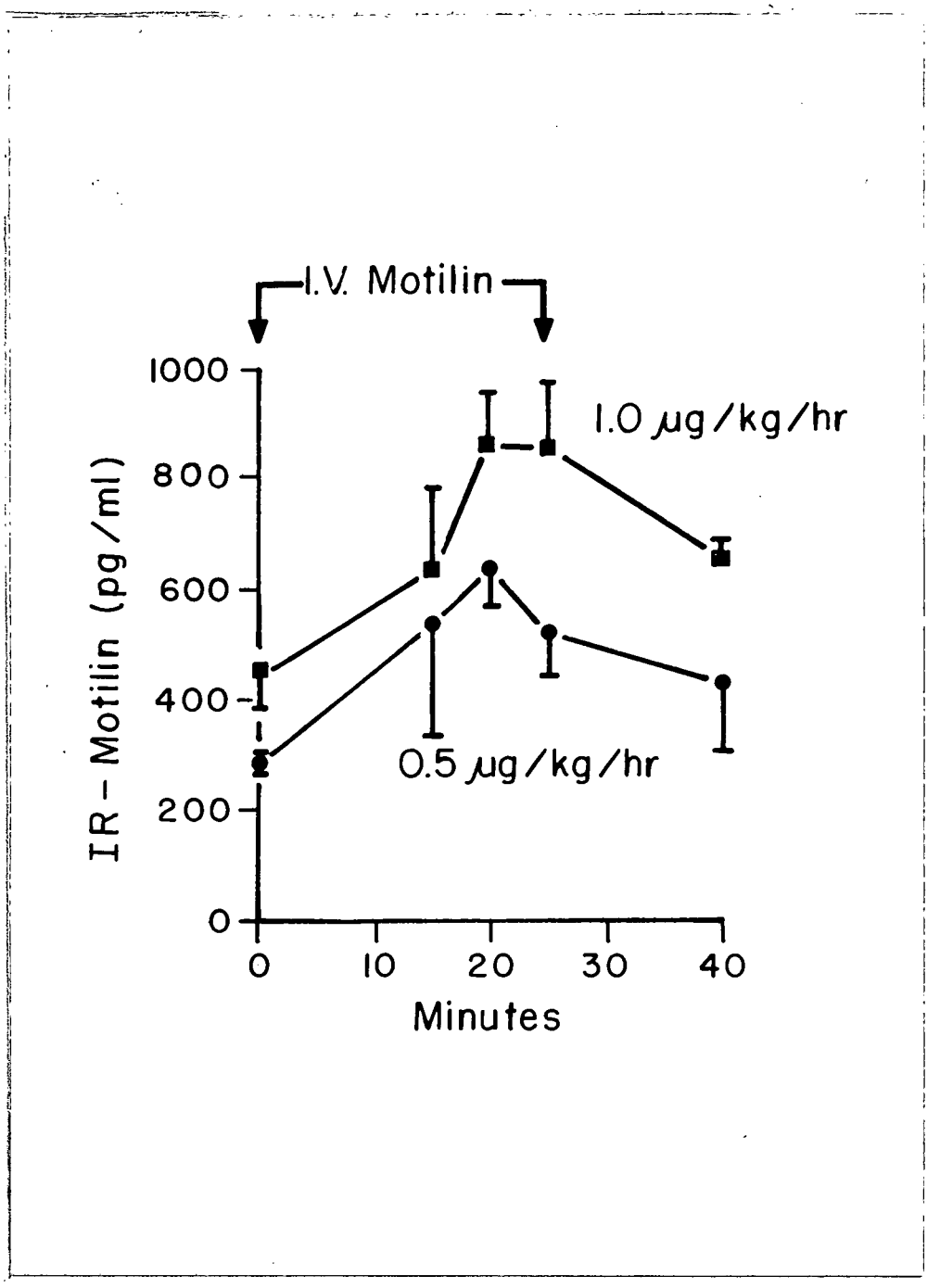


Fig. 53 Mean ± SE serum concentrations of IR- motilin (pg/ml) following infusion of pure natural motilin 0.5 and 1.0 µg/kg/hour. Each point is the mean of two experiments on each of six dogs (Debas et al, Gastroenterology in press, 1977).

was measured during the infusion of 0.15M saline, (Fig. 54).

The lowest effective dose was 0.25 µg/kg/hour motilin ($p < 0.001$) and the maximum effect was achieved at 0.5 µg/kg/hour motilin.

(II) Effect of motilin on the rate of gastric emptying of solids

The fraction of the solid meal remaining in the stomach at 30, 60, 90 and 120 mins after its ingestion was virtually the same whether the infusion was 1.0 µg/kg/hour motilin or 0.15M saline (Fig. 55).

B. Effects of motilin on the rate of gastric emptying of liquids after truncal vagotomy and/or antrectomy

(I) Effect of motilin after truncal vagotomy

The dose of motilin producing the maximum effect in the control dogs, 0.5 µg/kg/hour, was ineffective after vagotomy. The doses of 1.0 and 2.0 µg/kg/hour, however, did produce a significant increase in the fraction of the liquid meal emptied from the stomach. The effect of vagotomy, therefore, was to decrease the sensitivity of the response to motilin.

The basal rate of gastric emptying, measured during saline infusion, was significantly lower in the vagotomized animal - as shown in Fig. 56.

(II) Effect of motilin after antrectomy

There was no difference in the response to 0.25, 1.0 and 2.0 µg/kg/hour motilin in the control and post-antrectomy dogs. There was an, as yet, in-

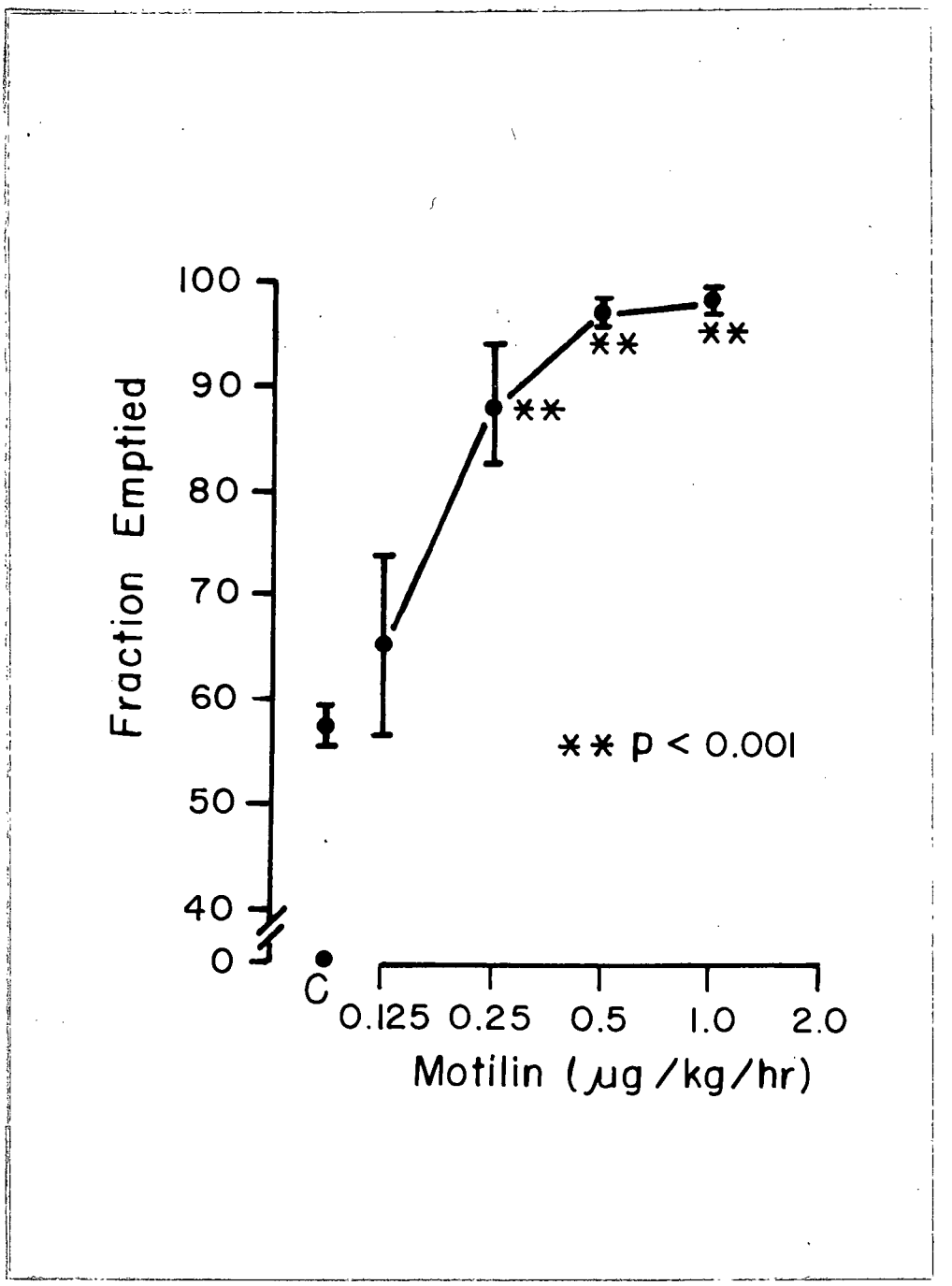


Fig. 54 Mean ± SE fraction of liquid meal emptied while different doses of motilin were infused intravenously. Each point is the mean of two experiments on each of six dogs. (Debas et al, Gastroenterology, in press, 1977).

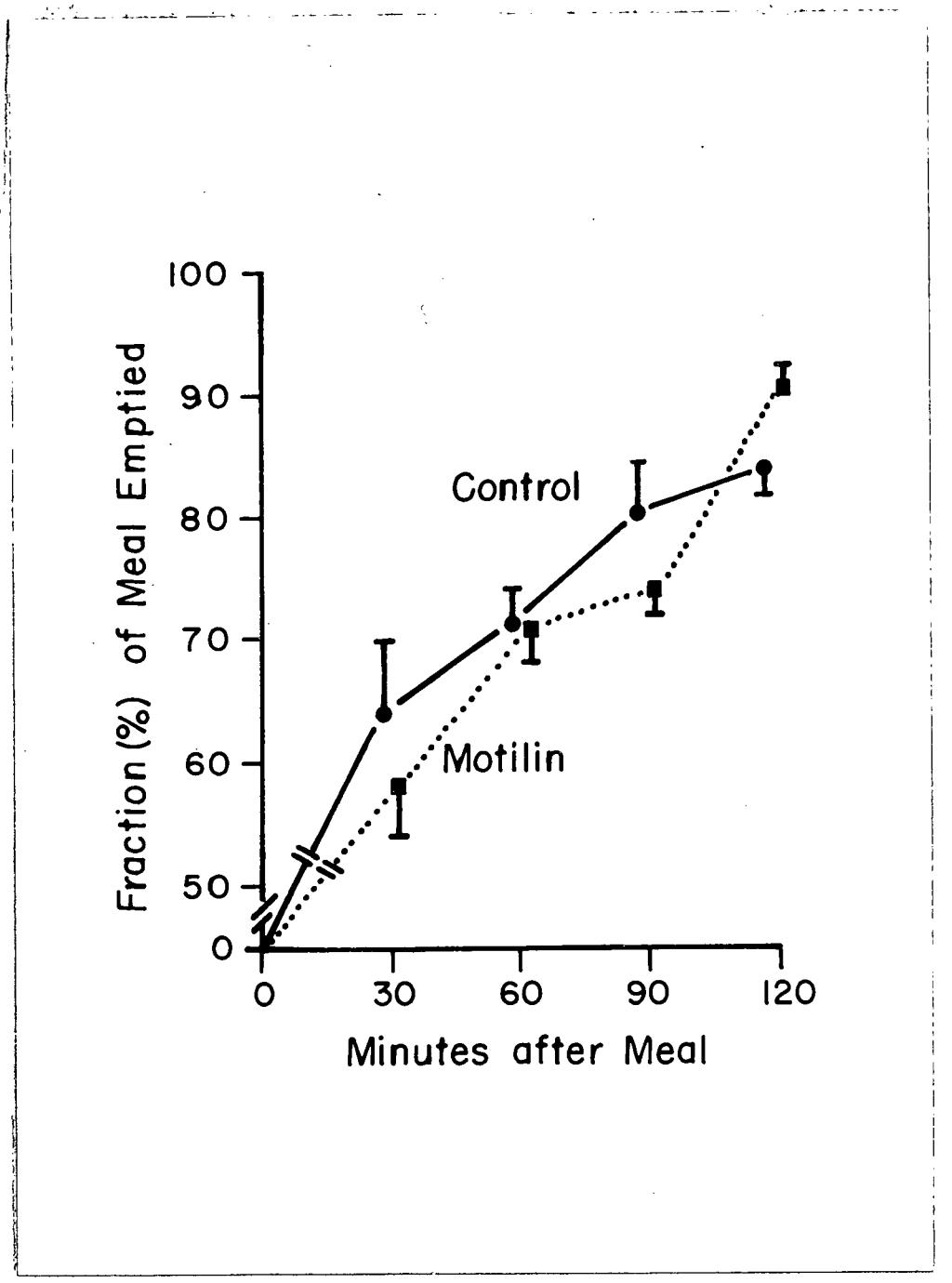


Fig. 55 Mean $\pm$ SE fraction of solid meal emptied at different time periods following ingestion. Each point is the mean of two experiments on each of six dogs (Debas et al, Gastroenterology, in press, 1977).

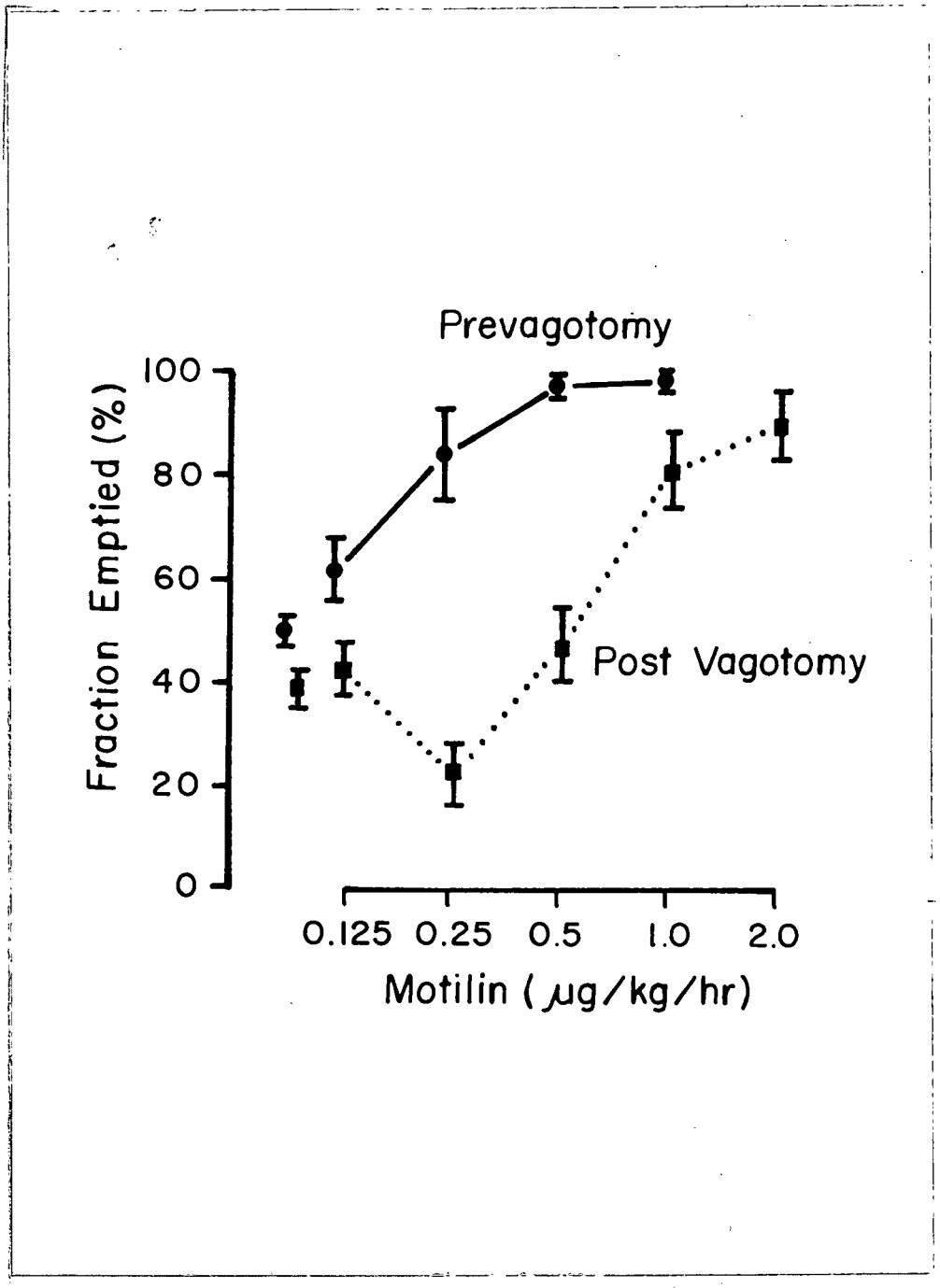


Fig. 56 Mean $\pm$ SE fraction of liquid meal emptied while different doses of motilin were infused I.V before and after truncal vagotomy. Each point is the mean of two experiments on each of three dogs (Debas et al, Gastroenterology, in press, 1977).

explicable, but significant decrease in the rate of gastric emptying at 0.5 µg/kg/hour motilin in the antrectomized animal, as shown in Fig. 57.

The basal rates were not altered by removal of the antrum.

(III) Effect of motilin after truncal vagotomy and antrectomy

The basal rate of gastric emptying was significantly increased in the vagotomized, antrectomized animal, compared to that in the control animal.

There was a significant increase in the rate of gastric emptying of the liquid meal after 0.5 and 1.0 µg/kg/hour motilin ($P < 0.01$). This was less significant, however, than the increase in the control animals ($P < 0.001$) - (Fig. 58)

MODIFICATIONS TO GIP RADIOIMMUNOASSAY

A. Antisera to GIP

All animals received at least 3 immunizations with 50 µg porcine GIP, conjugated to BSA and emulsified with FCA. After 6 months, 10% of the animals were producing antisera usable at titres of $1:20 \times 10^3$. The affinity constants (K) of the best guinea pig (Van 8) and best rabbit (Gö 5) were 7.5×10^{14} and 1.0×10^{15} l./mole respectively.

Rabbit antiserum Ro7 demonstrated a maximum binding of >30% at a titre of $1:30 \times 10^3$ but the displacement of label by standard antigen was unsatisfactory after an incubation of the equilibrium type. When this antiserum was incubated

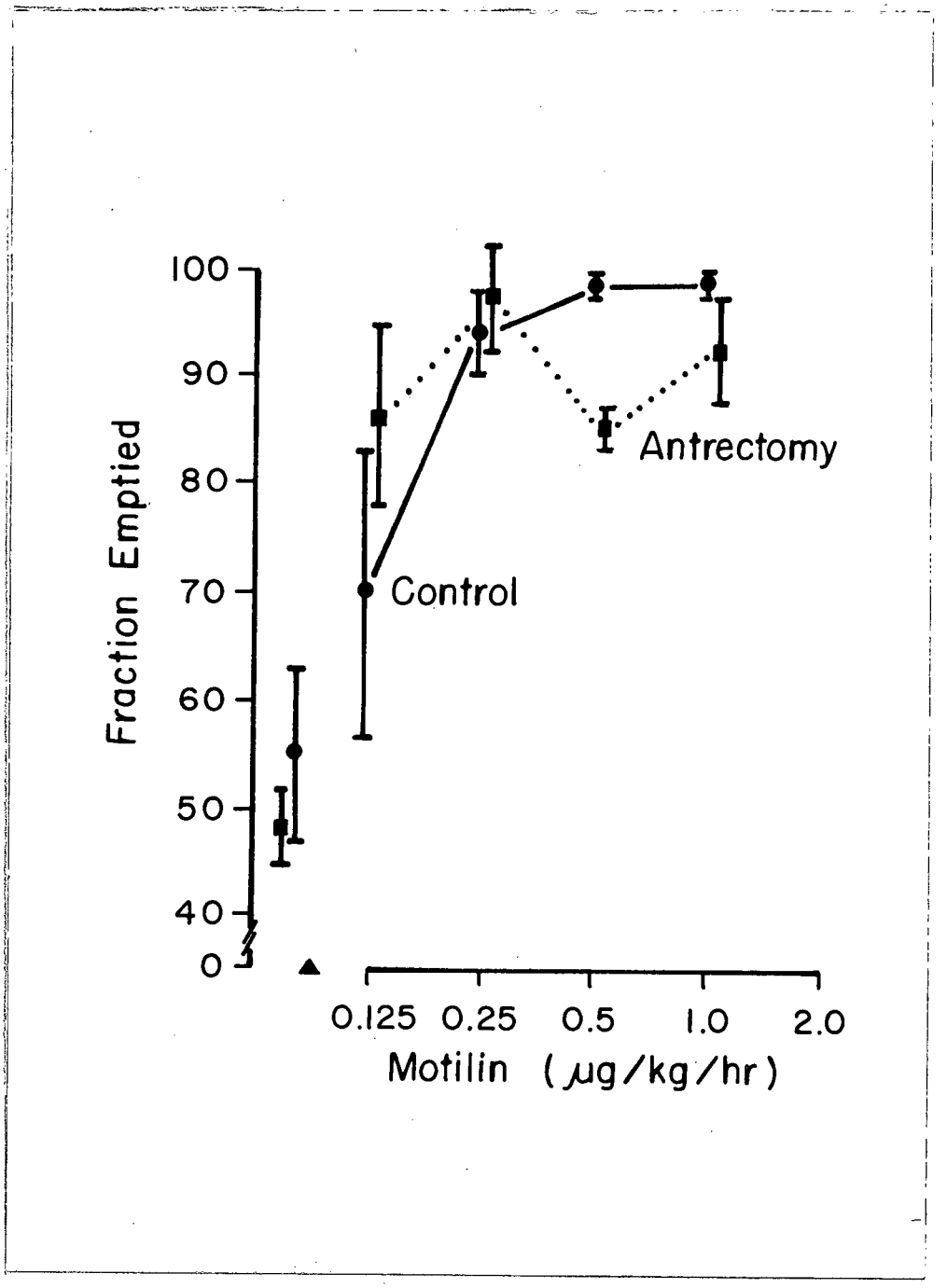


Fig. 57 Mean ± SE fraction of liquid meal emptied while different doses of motilin were infused I.V. before and after antrectomy. Each point is the mean of two experiments on each of three dogs, (Debas et al, Gastroenterology, in press, 1977).

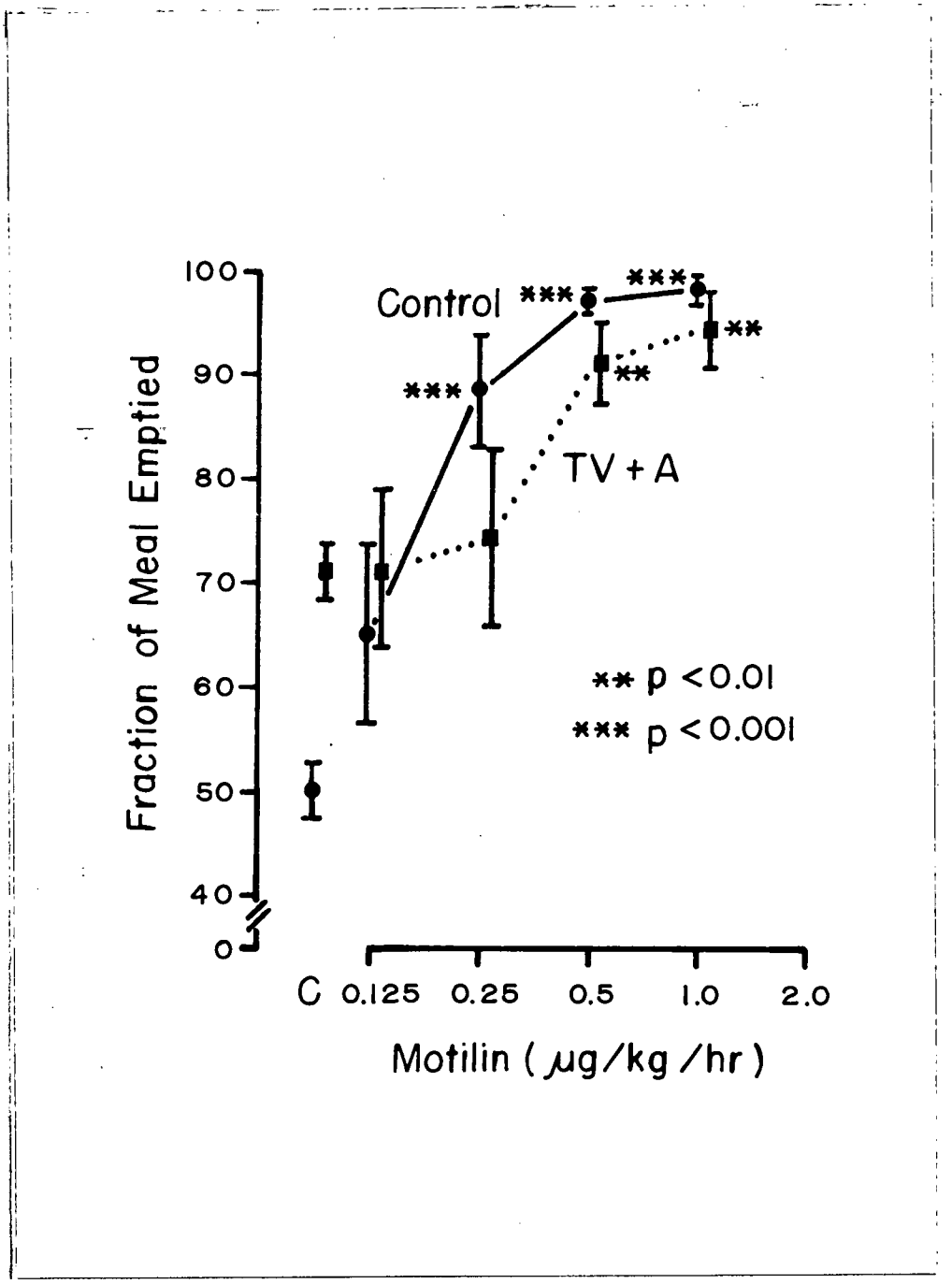


Fig. 58 Mean ± SE fraction of a liquid meal emptied while different doses of motilin were infused I.V before and after truncal vagotomy and antrectomy. Each point is the mean of two experiments on each of 6 dogs, (Debas et al, Gastroenterology, in press, 1977).

with cold antigen for 24 hours prior to the addition of ^{125}I- GIP, and for another 48 hours after the label addition a satisfactory standard curve was obtained, the antiserum affinity constant (K) being 2.5×10^{14} 1/mole. Fig. 59. illustrates the improvement obtained when Ro7 was incubated in a disequilibrium assay system. In contrast, assays containing Van 8 showed no significant difference when incubated under either equilibrium or disequilibrium conditions.

B. Iodination of GIP

The specific activity of the ^{125}I- GIP, purified by gel filtration was only 70 mCi/mg. After further purification by ion exchange chromatography on QAE Sephadex A-25 the specific activity was greatly improved, the value being ~ 250 mCi/mg. A comparison of the standard curves obtained with the label at each stage is shown in Fig. 60.

EFFECT OF SOMATOSTATIN ON THE CANINE RESPONSE TO GIP

A. Effect of somatostatin on the release of endogenous GIP

(I) On the insulinotropic action of GIP released by oral glucose

These studies were performed in the intact, conscious dog. Somatostatin was administered intravenously either as a single rapid injection (3 µg/kg) immediately prior to the oral administration of glucose, or as an infusion of 6 µg/kg over 1 hour, the glucose load being given after 30 mins. The glucose load in either case was 1g/kg of 20% dextrose in distilled water and was administered alone in control studies.

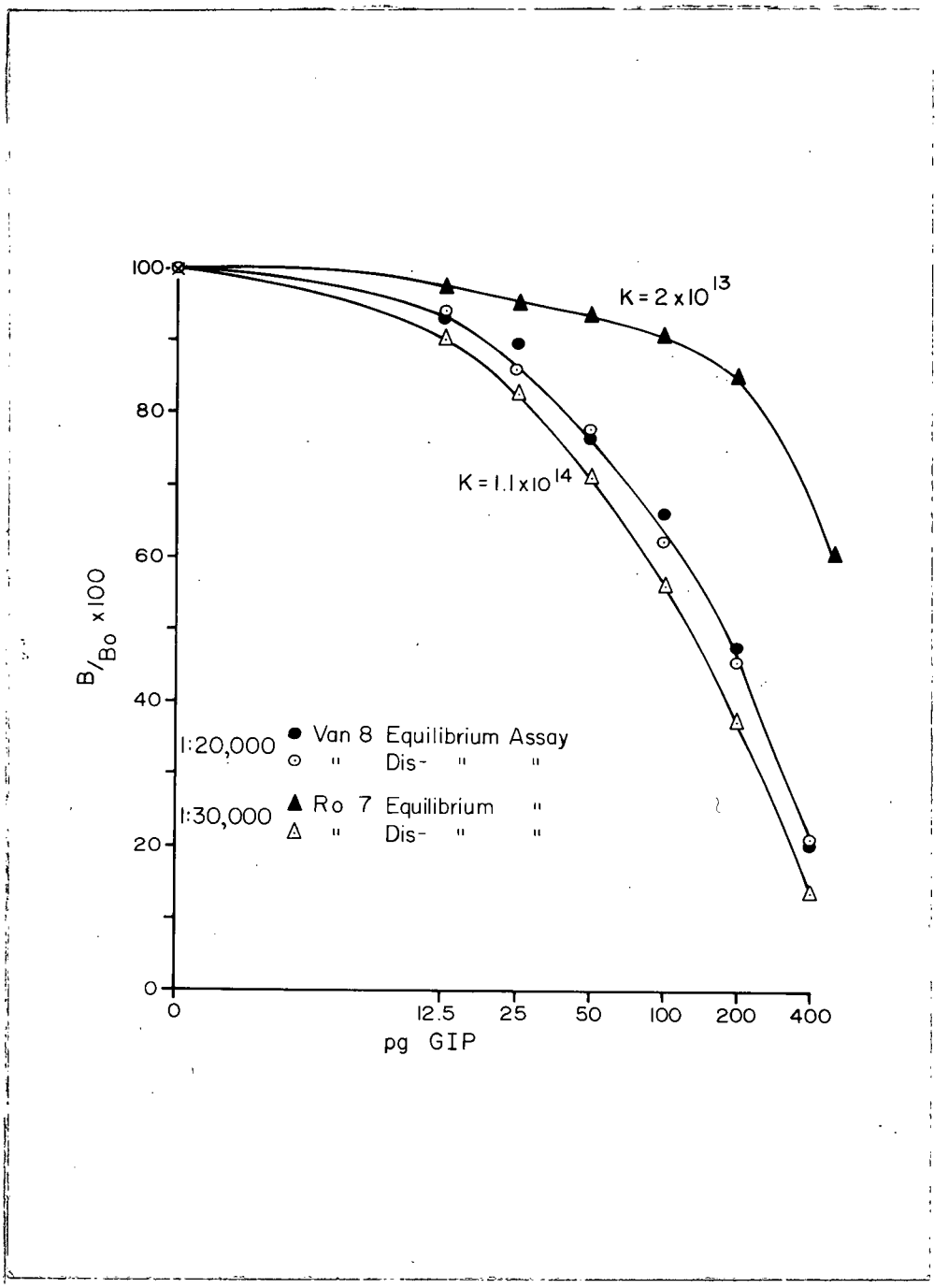


Fig. 59 The effect of incubations of the equilibrium and disequilibrium type on the sensitivity of the standard curves obtained with GIP antisera Van 8 and Ro7.

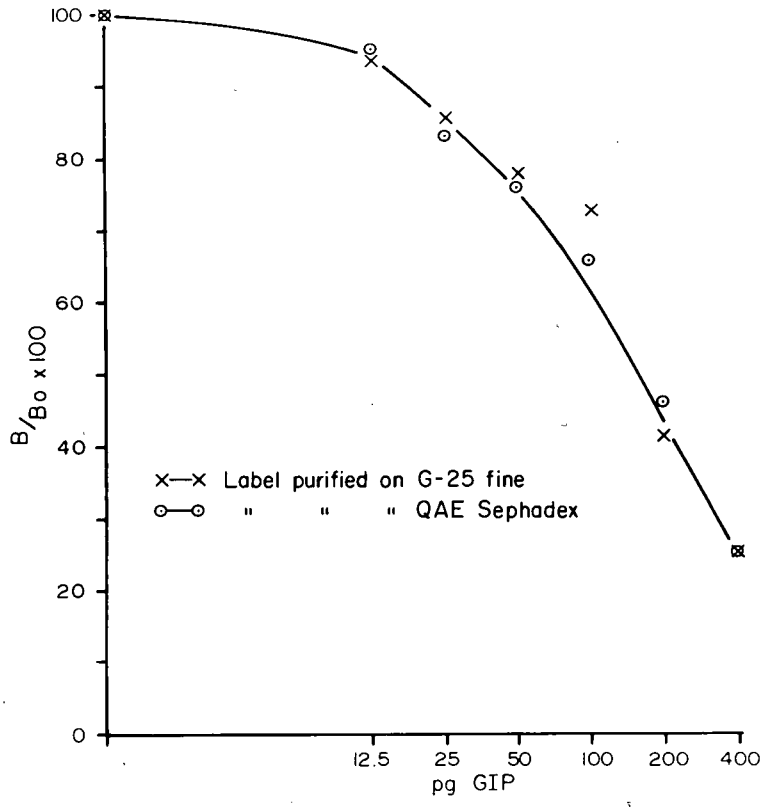


Fig. 60 Comparison of the standard curves for GIP obtained with radioactive tracer isolated by gel filtration on Sephadex G25 only and after subsequent elution from Sephadex QAE A25.

After the single bolus injection of somatostatin, the fasting levels of serum glucose, IR- GIP and IR- insulin were inhibited and the response to the oral glucose was delayed. There was a significant reduction from the control values of all parameters at 15 mins. (IR-GIP, $P < 0.0025$; IR-insulin $P < 0.0005$, glucose, $p < 0.0005$), (Fig. 61, Table XXVIII).

When somatostatin was administered as an infusion, the IR-GIP and IR-insulin responses to oral glucose were suppressed until the infusion had ended at 60 mins. when the IR insulin response rebounded to values significantly above the control. The delayed elevation of the serum glucose levels was similar to that seen after the bolus injection of of somatostatin and by 60 mins. there was no significant difference from the control values (Fig. 62, Table XXIX).

(II) On the release of endogenous GIP by fat

The effect of a single, rapid intravenous injection of 3 µg/kg somatostatin in the IR-GIP response to ingestion of 100 mls Lipomul was compared with control studies when fat alone was administered. The IR-GIP response after somatostatin was found to be delayed and reduced ($P < 0.05$) after somatostatin and then rebounded above the control values (as may be observed in Fig. 63 and Table XXXI).

B. Effect of somatostatin on the response to exogenous GIP

A comparison was drawn between the responses of IR insulin and serum glucose to an intravenous infusion of 1.5 µg/kg porcine GIP over 5 mins, given with and without a prior single, rapid, intravenous injection of 3 µg/kg somatostatin. There was an 80% reduction in the peak IR insulin response to GIP

TABLE XXVIII

The effect of a single rapid i.v. injection of somatostatin on the incremental IR-GIP, IRI and serum glucose response to oral glucose.

n = 7			Incremental Response (Δ)												
Time (mins)			5	10	15	20	25	30	45	60	75	90	105	120	135	150
Serum Glucose mg%	Control	$\bar{X}$	10.2	19.6	32.0	33.7	46.6	45.7	43.9	31.1	24.6	14.1	11.3	10.2	8.7	5.7
		±SE	4.5	5.4	4.8	4.2	8.7	5.8	4.6	4.3	4.0	3.9	3.3	2.8	3.7	3.3
	Test	$\bar{X}$	7.6	3.8	5.9	11.9	24.4	24.8	36	39	36.1	25	22	14	10.3	9.1
		±SE	2.9	4.9	6.4	5.6	5.8	6.2	11	8.2	5.7	5.2	7.1	5.3	3	3
IR-GIP pg/ml	Control	$\bar{X}$	205	520	551	980	1063	909	1348	1188	910	673	518	336	221	60
		±SE	95	149	156	202	132	239	134	182	117	97	123	124	85	75
	Test	$\bar{X}$	-192	-128	87	136	316	357	842	1314	1007	750	668	614	578	378
		±SE	120	101	148	134	210	172	367	295	298	241	240	261	332	117
IRI μU/ml	Control	$\bar{X}$	22.8	54.6	68.7	63.0	72.8	62.6	42.5	36.4	20.4	8.2	1.3	-2.9	-2.7	-5.4
		±SE	11.3	14.2	9.1	5.9	9.4	8.0	7.0	7.6	4.7	7.7	4.1	1.7	4.8	4.2
	Test	$\bar{X}$	-6.5	-4.4	-0.5	7.5	39	58	59	49	37.4	25.2	5.5	2.3	1.4	-1.8
		±SE	2.4	2.8	6.8	5.2	12	9.2	13	16	12	10.5	4.9	5.3	2.4	1.8

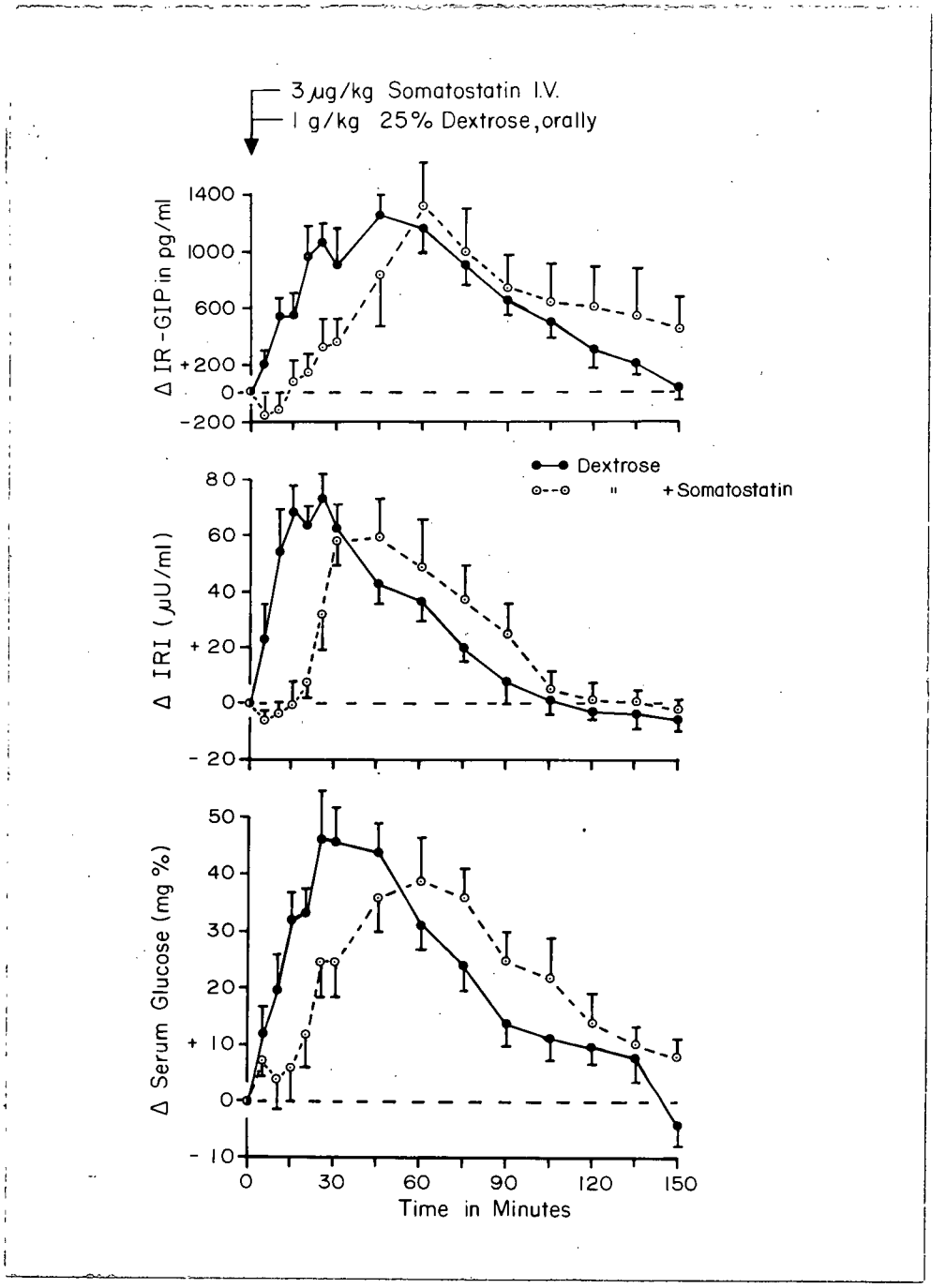


Fig. 61 The effect of an intravenous injection of 3 µg/kg somatostatin on the incremental IR-GIP, IR- insulin and serum glucose responses to the ingestion of glucose. (1 g/kg) (Pederson et al, Can J. Physiol. Pharmacol. 53: 1200-1205, 1975)

TABLE XXIX

The effect of a 1 hr infusion of somatostatin on the incremental IR-GIP, IRI and serum glucose response to oral glucose.

n = 6			Incremental response (Δ)																		
Time (mins)			5	10	15	20	25	30	35	40	45	50	55	60	75	90	105	120	135	150	165	180
Serum glucose mg%	Control	$\bar{X}$	10.2	19.6	32.0	33.7	46.0	45.7			43.9			31.1	24.7	14.1	11.3	10.2	8.7	5.7	6.4	6.3
		±SE	4.5	5.4	4.8	4.2	8.7	5.8			4.6			4.3	4.0	3.9	3.3	2.8	3.7	3.3	3.5	4.1
	Test	$\bar{X}$	2.4	4.7	2.2	0.5	4.6	3.0	5.0	0.2	6.0	10	18.2	25	43	27	29	35.4	32.3	21.5	20	11.4
		±SE	0.7	3.1	2.1	2.6	5.0	3.4	3.9	1.7	3.7	4.2	5.6	6	6.8	5.2	5.5	5.4	4.7	7.9	6.3	2.1
IR-GIP pg/ml	Control	$\bar{X}$	208	520	551	980	1063	909			1348			1188	910	673	518	336	221	60	143	115
		±SE	95	149	156	202	132	238			134			182	117	97	123	124	85	75	121	74
	Test	$\bar{X}$	72	115	150								843	176	802	752	684	763	886	750	500	324
		±SE	89	339	339								128	58	200	183	156	210	212	143	200	136
IRI μU/ml	Control	$\bar{X}$	22.8	54.6	68.7	63.0	72.8	62.6			42.5			36.4	20.4	8.2	1.3	-2.9	-2.7	-5.4	-5.0	2.7
		±SE	11.3	14.2	9.1	5.9	9.4	8.0			7.0			7.6	4.7	7.7	4.1	1.7	4.8	4.2	4.8	5.1
	Test	$\bar{X}$	0	-3	-1.6	-9.6	-10	-6.5	-6.8	-6.6	-5.6	-7.6		-1.8	119.8	43.2	3.5	34.6	17	10.2	7.1	8.3
		±SE	2.4	1.8	2.9	3.7	3.3	3.6	3.6	3.1	2.6	2.2		4.7	13	8.4	9.2	7.6	5.6	4.7	5	6

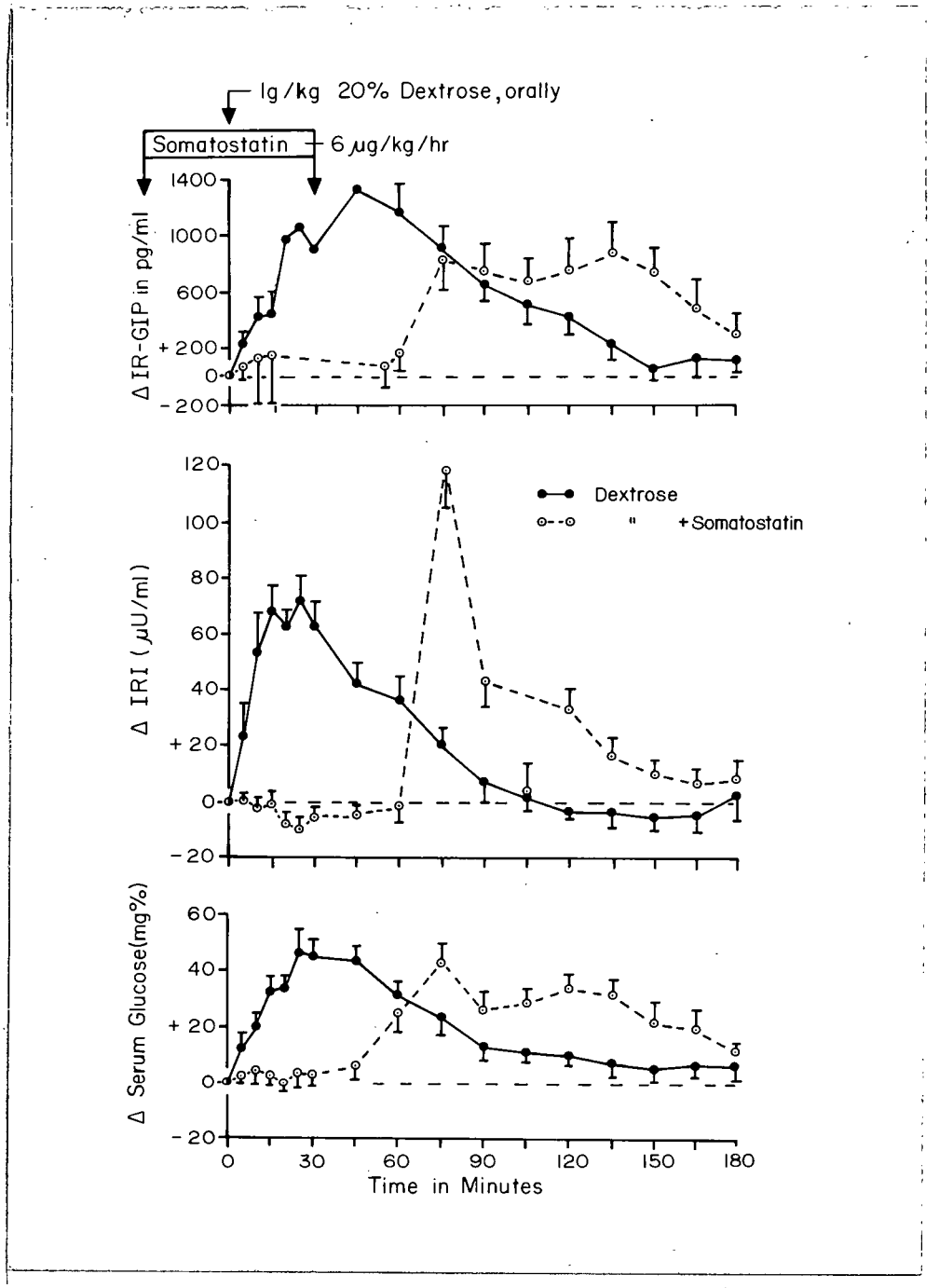


Fig. 62 The effect of an infusion of somatostatin (6 ug/kg/hour) on the incremental IR-GIP, IR- insulin and serum glucose responses to the ingestion of 1 g/kg glucose. (Pederson et al, Can. J.Physiol. Pharmacol. 53: 1200-1205, 1975)

TABLE XXX

Effect of a single rapid i.v. injection of somatostatin on the incremental IR-GIP, IRI and serum glucose response to oral fat.

			Incremental Response (Δ)															
Time (mins) -			5	10	15	20	25	30	35	45	60	75	90	105	120	135	150	165	180
Serum glucose mg%	Control	$\bar{X}$			3.8			3.6		5.8	3.7	3.7	7.5	8.3	6.5	7.5	4.3	5.5	7.5
		±SE			1.3			3.1		3.3	2.0	2.6	3.6	2.5	4.0	4.4	4.6	3.6	3.4
	Test	$\bar{X}$	0	0.4	5.0	3.3	4.2	3.9	4.0	3.0	1.9	1.8	-0.3	2.0	0	0.2	3.4	3.0	4.8
		±SE	1.2	1.9	1.9	1.3	1.4	4.2	1.3	1.8	1.5	0.7	1.4	2.0	1.5	1.1	1.2	2.2	3.7
IR-GIP pg/ml	Control	$\bar{X}$			674			1210		1712	2641	2696	3083	3035	3325	3270	3548	3526	2704
		±SE			174			274		441	497	560	614	612	605	635	782	707	565
	Test	$\bar{X}$	29	12	30	-5	-10	242	210	394	946	1235	1585	1492	1642	1435	1903	1721	1964
		±SE	43	30	35	29	46	154	140	164	257	323	122	103	215	221	414	416	322
IRI μU/ml	Control	$\bar{X}$			-1.0			0.83		-0.3	4.1	5.6	6.5	9.5	3.1	3.9	3.6	5.0	4.5
		±SE			0.45			1.6		1.6	2.9	1.8	3.1	3.8	0.9	1.9	1.2	2.2	0.8
	Test	$\bar{X}$	-6	-9	-5	-3	-3	-2	-1.3	4.2	9.3	17.6	12.8	5.8	8.3	7.8	5.6	1.6	8.8
		±SE	2.8	1.8	2.0	3.0	3.0	2.9	2.8	4.3	4.6	3.4	5.4	2.7	2.6	2.3	2.5	1.9	7.0

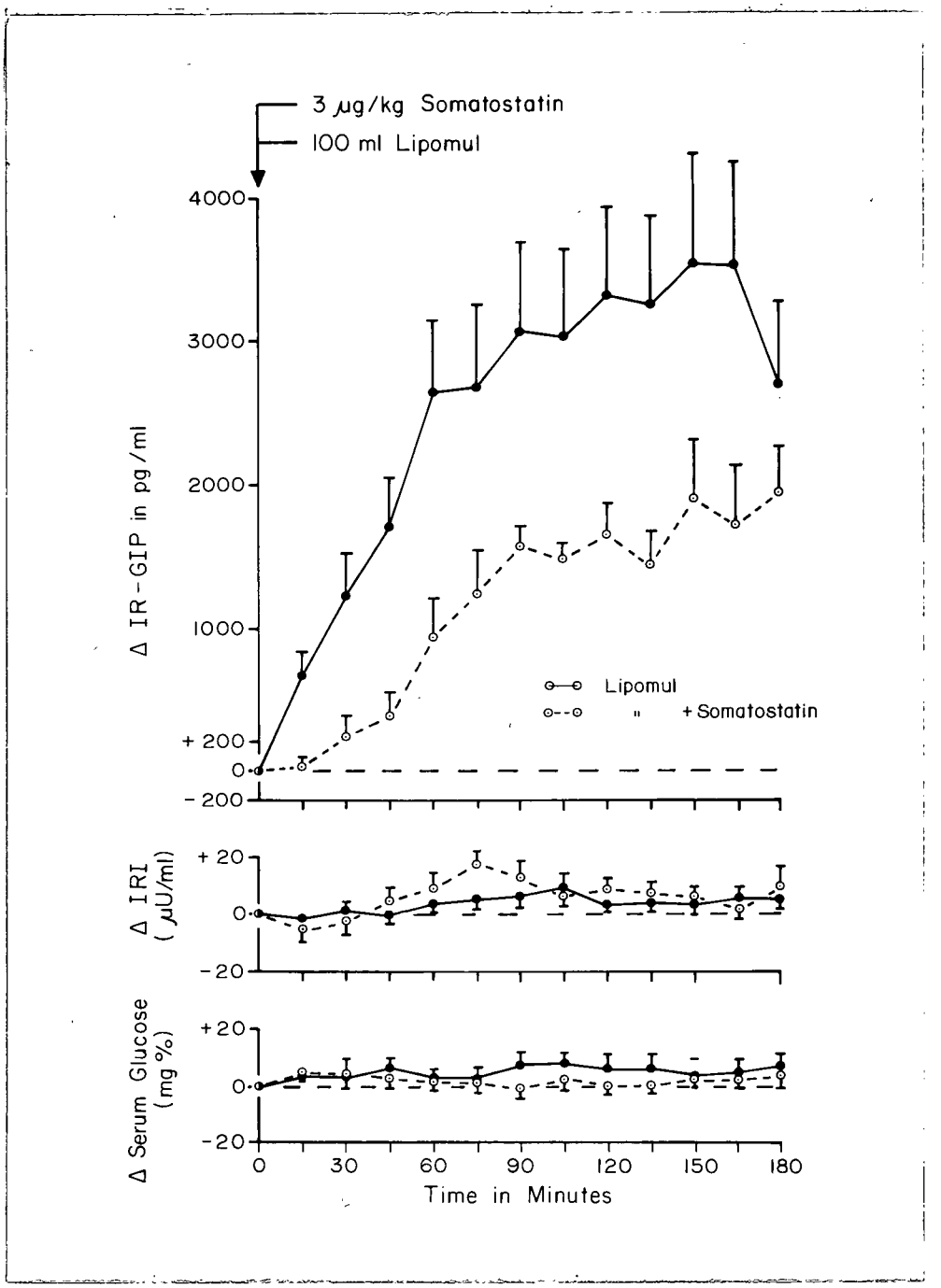


Fig. 63 The effect of an intravenous injection of 3 µg/kg somatostatin on the incremental IR-GIP, IR- insulin and serum glucose responses to the ingestion of 100 mls Lipomul. (Pederson et al, Can. J. Physiol. Pharmacol. 53: 1200-1205, 1975).

after somatostatin, with a concomitant and significant ($P < 0.01$) reduction in the depression of the serum glucose values (Fig. 64, Table XXXI).

RELATIONSHIP BETWEEN GIP AND GASTRIC ACID SECRETION

A. Effect of exogenous GIP on gastric acid secretion

The effect of a 60 min. intravenous infusion of 1.0 µg/kg/hour porcine GIP on a gastric acid plateau stimulated by pentagastrin (2.0 - 4.0 µg/kg/hour) in 9 experiments in 3 dogs is illustrated in Fig. 65 and Table XXXIII. A 60% inhibition of gastric acid secretion was observed in the second half of the GIP infusion, associated with IR-GIP levels in the range 1200-1400 pg/ml above the control value. During the post-infusion period, the IR-GIP gradually declined back toward the pre-infusion values and the H^+ output returned toward the control plateau levels. Control experiments were performed in 3 dogs which received pentagastrin only (Table XXXII).

B. Effect of endogenous GIP on gastric acid secretion

(I) After an intraduodenal infusion of fat

A triglyceride emulsion (Lipomul) was infused intraduodenally at 1.91 ml/min. over 30 mins. after a plateau of gastric acid secretion had been achieved by intravenous infusion of pentagastrin in 3 experiments in each of 3 dogs. The results (Fig. 66 and Table XXXIV) showed that a marked increase in IR-GIP occurred to levels of 800 - 1000 pg/ml above the pre-infusion levels, co-incident with a 68% inhibition of gastric acid secretion. During the post-infusion period both IR-GIP and H^+ values returned toward the control levels.

TABLE XXXI

Effect of a single rapid i.v. injection of somatostatin on the incremental IRI and serum glucose response to i.v. porcine GIP.

n = 4			Incremental Response (Δ)									
Time (mins)			3	4	5	7	10	15	20	25	30	45
Serum glucose mg%	Control	$\bar{X}$	0.6	-3.6	-1.6	-5.0	-8.4	-9.8	-6.8	-4.2	-0.2	2.6
		±SE	1.5	3.3	1.4	2.4	3.1	7.3	7.7	2.5	3.7	1.3
	Test	$\bar{X}$	3.7	4.7	2.2	1.5	0.25	-1.7	-1.25	-1.25	3.0	4.7
		±SE	1.8	1.3	1.7	1.8	1.7	1.4	2.4	5.3	1.5	3.9
IRI μU/ml	Control	$\bar{X}$	25.4	25.4	30.0	16.4	9.4	-1.4	-3.8	-2.6	-2.2	-1.4
		±SE	11.9	6.4	6.9	3.3	2.1	1.3	1.4	1.1	1.2	2.1
	Test	$\bar{X}$	-1.25	-1.75	5	15	5.8	1.7	-2.0	1.0	1.25	1.25
		±SE	1.3	1.0	3.7	2.2	1.7	4.4	0.8	3.1	1.0	1.0

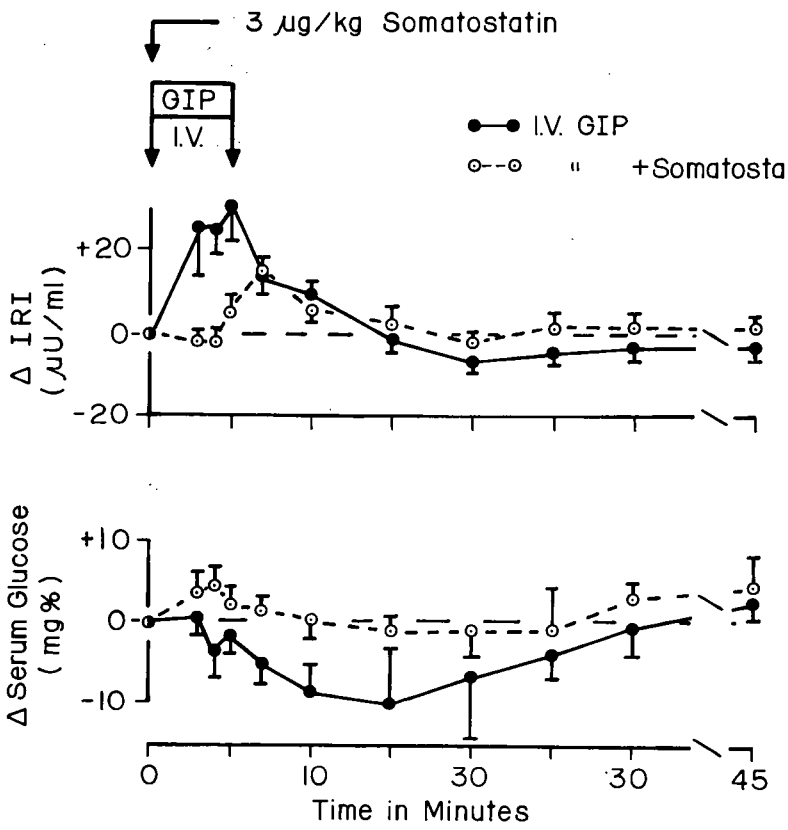


Fig. 64 The effect of an intravenous injection of 3 µg/kg somatostatin on the incremental IR- insulin and serum glucose responses to an intravenous infusion of 1 µg/kg porcine GIP over 5 min. (Pederson et al, Can. J. Physiol. Pharmacol. 53: 1200-1205, 1975).

TABLE XXXII

Effect of a continuous infusion of pentagastrin on H^+[1] output of an extrinsically denervated fundic pouch.

Expt. #	INTRAVENOUS PENTAGASTRIN INFUSION										
	0_1	0_2	0_3	15	30	45	60	75	90	105	120
Ma	161	149	140	220	198	208	261	202	213	198	201
Di	330	326	310	264	238	235	280	274	264	251	283
Be	1720	1705	1669	1647	1324	1307	1120	1205	1409	1460	1103

1. μEq H^+/15 min.

TABLE XXXIII

Effect of an intravenous infusion of porcine GIP[1] on pentagastrin-stimulated H^+ output[2].

Experiment #		CONTROL			Intravenous Porcine GIP Infusion								Post Infusion Period			
		0_1	0_2	0_3	5	10	15	20	25	30	45	60	75	90	105	120
Ma 1	H^+	329	374	314			137			106	97	120	103	145	193	143
	IR-GIP[3]	340	400	320		800	1475	1500	1650	1675	1750	1325	1225	1425	1050	920
Ma 2	H^+	378	389	297			217			89	124	216	235	233	228	
	IR-GIP	380	320	420	210	620	780	950	1300	1050	1750	450	700	230	220	
Ma 3	H^+	269	288	245			195			75	60	48	40	84	128	171
	IR-GIP	125	125	125	370	440	310	1500	1450	1300	1500	1050	1600	140	130	130
Be 1	H^+	292	351	313			248			102	158	79	162	149	283	
	IR-GIP	280	230	430	320	500	540	1200	1150	1200	1700	1150	430	310	400	
Be 2	H^+	235	205	205			124			85	123	90	61	117	159	165
	IR-GIP	125	125	125	440	1350	1500	1850	1800	1750	2600	2400	1550	570	550	580
Be 3	H^+	275	237	259			202			130	151	99	102	156	186	197
	IR-GIP	125	125	125	125	460	840	1450	1900	1400	1900	2400	700	300	350	480
Di 1	H^+	252	224	225			197			153	146	109	84	155	166	199
	IR-GIP	200	150	125	140	700	830	1200	1700	1450	1900	1700	1800	1050	620	320
Di 2	H^+	300	329	292			236			184	134	124	163	171	234	200
	IR-GIP	125	125	125	125	470	720	650	1000	1620	2000	1450	1750	1200	650	270

1. 1 µg/kg/hr GIP over 60 min.
2. µEq H^+/15 min.
3. IR-GIP in pg/ml.

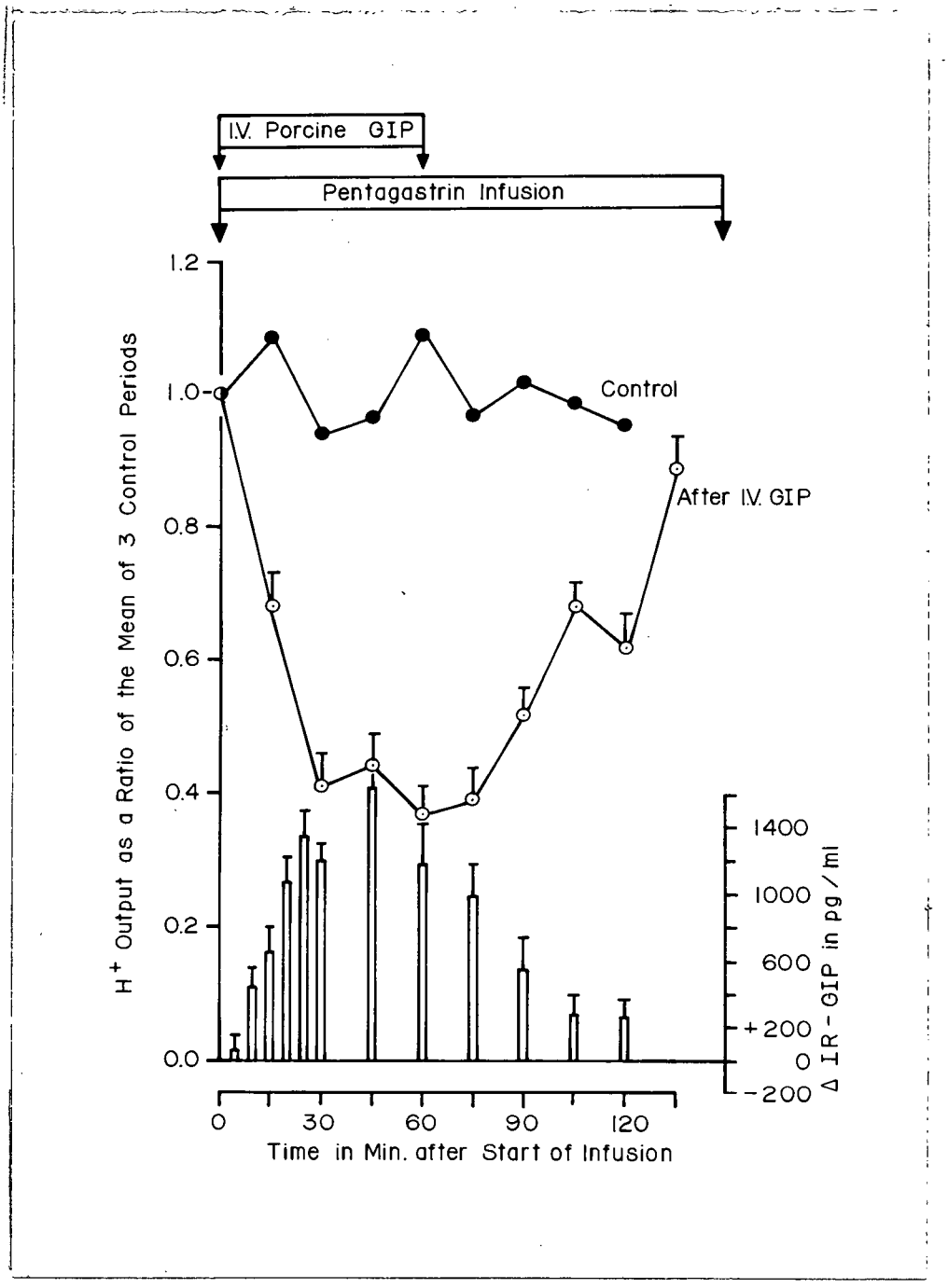


Fig. 65 The incremental IR-GIP response and inhibition of pentagastrin-induced gastric acid secretion caused by an intravenous infusion of 1 µg/kg/hour porcine GIP. The results represent the mean ± SE of 8 experiments in 3 dogs.

TABLE XXXIV

Effect of duodenal infusion of fat on pentagastrin-stimulated H^+ output[2] and IR-GIP[3] release.

Experiment #		CONTROL			Duodenal Fat Infusion						Post Infusion Period					
		0_1	0_2	0_3	5	10	15	20	25	30	45	60	75	90	105	120
Ma 1	H^+	499	528	533			358			342	167	162	420	486	416	440
	IR-GIP	125	600	300			820			720	1650	1175	1050	440	660	385
Ma 2	H^+	270	234	218			50			82	25	116	125	168	245	282
	IR-GIP	520	420	500	520	920	730	690	1025							
Ma 3	H^+	555	508	518			78			157	40	25	80	155	178	185
	IR-GIP	130	220	330	200	460	470	610	690	840	420	1125	1875	1425	1200	900
Be 1	H^+	176	162	128			220			150	98	68	145	86	230	209
	IR-GIP	150	190	120			500			800	1550	1550	1650	1550	700	500
Be 2	H^+	220	230	250			275			162	70	117	146	160	192	335
	IR-GIP	910	1000	800			360			840	1700	1450	1250	1150	1000	860
Be 3	H^+	298	273	297			185			186	166	216	249	201	278	286
	IR-GIP	650	710	600	620	860	1250	1450	1500	1600	1750	1900	1350	930	700	360
Di 1	H^+	127	110	156			168			-	71	21	70	172	177	210
	IR-GIP	180	125	500	700	590	1600	1500	1750	1800	1400	1350	1250	1350	1350	1330
Di 2	H^+	262	250	299			340			65	46	74	68	210	245	198
	IR-GIP	125	125	125	125	125	550	970	1550	1350	680	580	400	340	170	125

1. 100 mls Lipomul over 30 min.
2. μEq H^+/15 min.
3. IR-GIP in pg/ml.

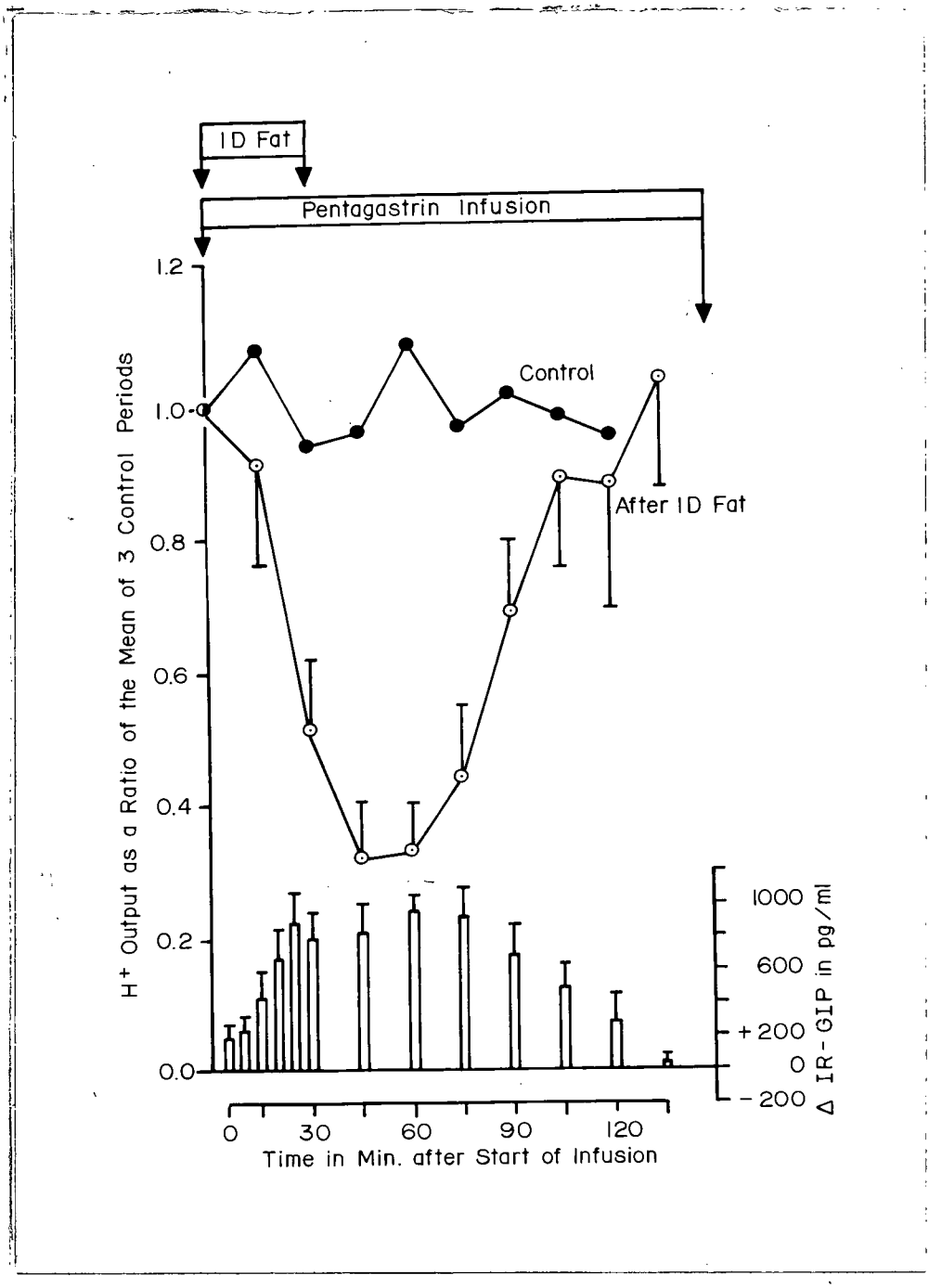


Fig. 66 The incremental IR-GIP response and inhibition of pentagastrin-induced gastric acid secretion caused by an intraduodenal infusion of 1.9 ml/min Lipomul over 30 min. The results represent the mean ± SE of 8 experiments in 3 dogs.

(II) After an intraduodenal infusion of glucose

A 30 min. duodenal infusion of 1.0g/kg 20% dextrose was performed when a plateau of gastric acid secretion had been achieved by pentagastrin administration. The H^+ output was reduced to 52% of the pre-infusion plateau levels with a concomitant increment in IR-GIP of 400 - 600 pg/ml. Each point represents the mean of 9 experiments in 3 dogs (Fig. 67, Table XXXV).

C. Effect of an intraduodenal infusion of acid on gastric acid secretion

A duodenal infusion of 1.91 ml/min 0.15M hydrochloric acid over 30 min. inhibited the H^+ output stimulated by pentagastrin to 48% of the pre-infusion levels. This reduction in the acid secretion was not accompanied by any significant change in IR-GIP, in 4 experiments in 2 dogs (Fig. 68, Table XXXVI).

D. Effect of an intraduodenal infusion of saline on gastric acid secretion

In 7 experiments in 3 dogs, a duodenal infusion of 0.9% saline at 1.91 ml/min. over 30 min. resulted in a small (27%) non-significant, inhibition of H^+ output, preceded by a slight, transient increase in IR-GIP (Fig. 69, Table XXXVII).

These results are summarized in Fig. 70, which compares the maximum inhibition of gastric acid achieved with the concomitant circulating level of IR-GIP,

TABLE XXXV

Effect of duodenal infusion of glucose[1] on pentagastrin-stimulated H^+ output[2] and IR-GIP release[3].

Experiment #		CONTROL			Duodenal Glucose Infusion						Post Infusion Period					
		0_1	0_2	0_3	5	10	15	20	25	30	45	60	75	90	105	120
Ma 1	H^+	203	202	182			82			42	33	55	197	107	163	
	IR-GIP	125	125	125	125	125	1020	460	500	300	680	235	145	150	430	
Ma 2	H^+	329	374	314			137			106	97	120	249	148	193	243
	IR-GIP	340	400	320	-	800	1475	1500	1650	1675	1750	1325	1225	1425	1020	920
Be 1	H^+	298	273	297			185			186	166	216	163	201	278	286
	IR-GIP	480	460	440	370	320	690	940	930	740	740	1150	1350	125	370	275
Be 2	H^+	145	156	124			119			167	90	130	217	172		
	IR-GIP	125	125	125	125	125	625	380	415	250	260	125	145	125		
Be 3	H^+	394	314	355			310			150	190	193	196	236	-	234
	IR-GIP	125	125	125	125	125	725	490	330	420	240	125	125	125	-	125
Di 1	H^+	277	270	260			161			199	243	208	186	235	229	287
	IR-GIP	350	340	220	140	340	480	480	650	1400	750	340	280	240	125	125
Di 2	H^+	326	272	298			232			232	202	112	103	206	143	135
	IR-GIP	125	125	125	125	170	200	680	820	600	540	190	125	125	160	125

1. 20% dextrose - 1 g/kg over 30 min.
2. μEq H^+/15 min.
3. IR-GIP in pg/ml.

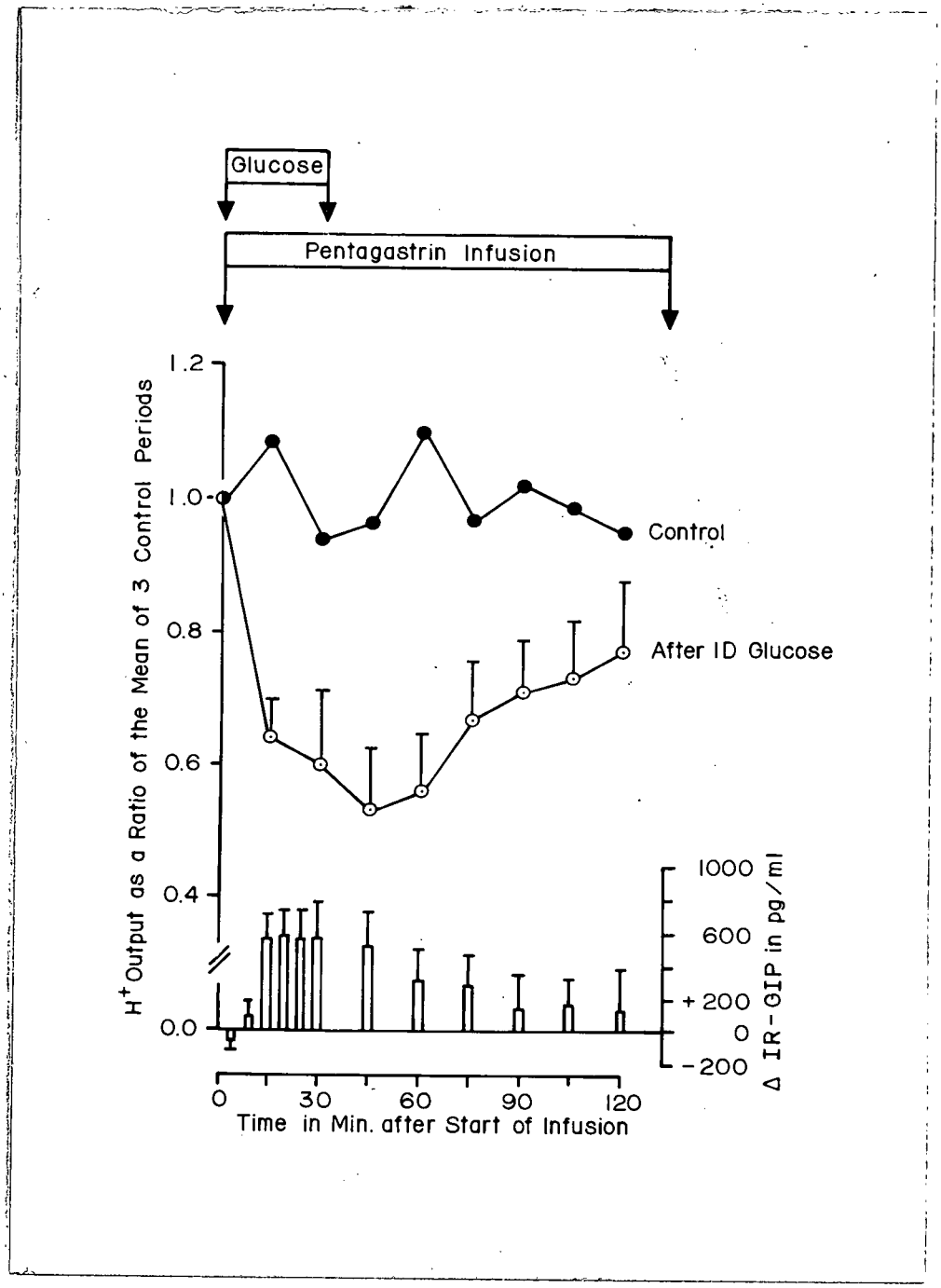


Fig. 67 The incremental IR-GIP response and inhibition of pentagastrin-induced gastric acid secretion caused by an intraduodenal infusion of 1 g/kg glucose over 30 min. The results represent the mean ± SE of 7 experiments in 3 dogs.

TABLE XXXVI

Effect of a duodenal infusion of acid[1] on pentagastrin-stimulated H^+ output[2] and IR-GIP release[3].

Experiment #		CONTROL			Duodenal Acid Infusion						Post Infusion Period					
		0_1	0_2	0_3	5	10	15	20	25	30	45	60	75	90	105	120
Be 1	H^+	2626	2608	2522			2060			1823	1944	1735	2013	2112	2257	2070
	IR-GIP	500	315	310			315			290	400	335	150	150	490	255
Be 2	H^+	2714	2831	2760			2173			1714	1802	1691	1990	2431	2461	2406
	IR-GIP	390	310	250			200			250	200	360	225	150	200	200
Ro 1	H^+	1534	1800	1746			1100			918	1120	1299	1415	1508	1368	1573
	IR-GIP	760	840	835			700			850	720	730	590	1100	1160	920
Ro 2	H^+	1780	1750	1830			1210			942	1100	1198	1460	1490	1600	1570
	IR-GIP	350	410	300			300			260	280	240	200	300	265	280

1. 0.15M HCl at 1.91 ml/min for 30 min.

2. In μEq H^+/15 min.

3. In pg/ml IR-GIP.

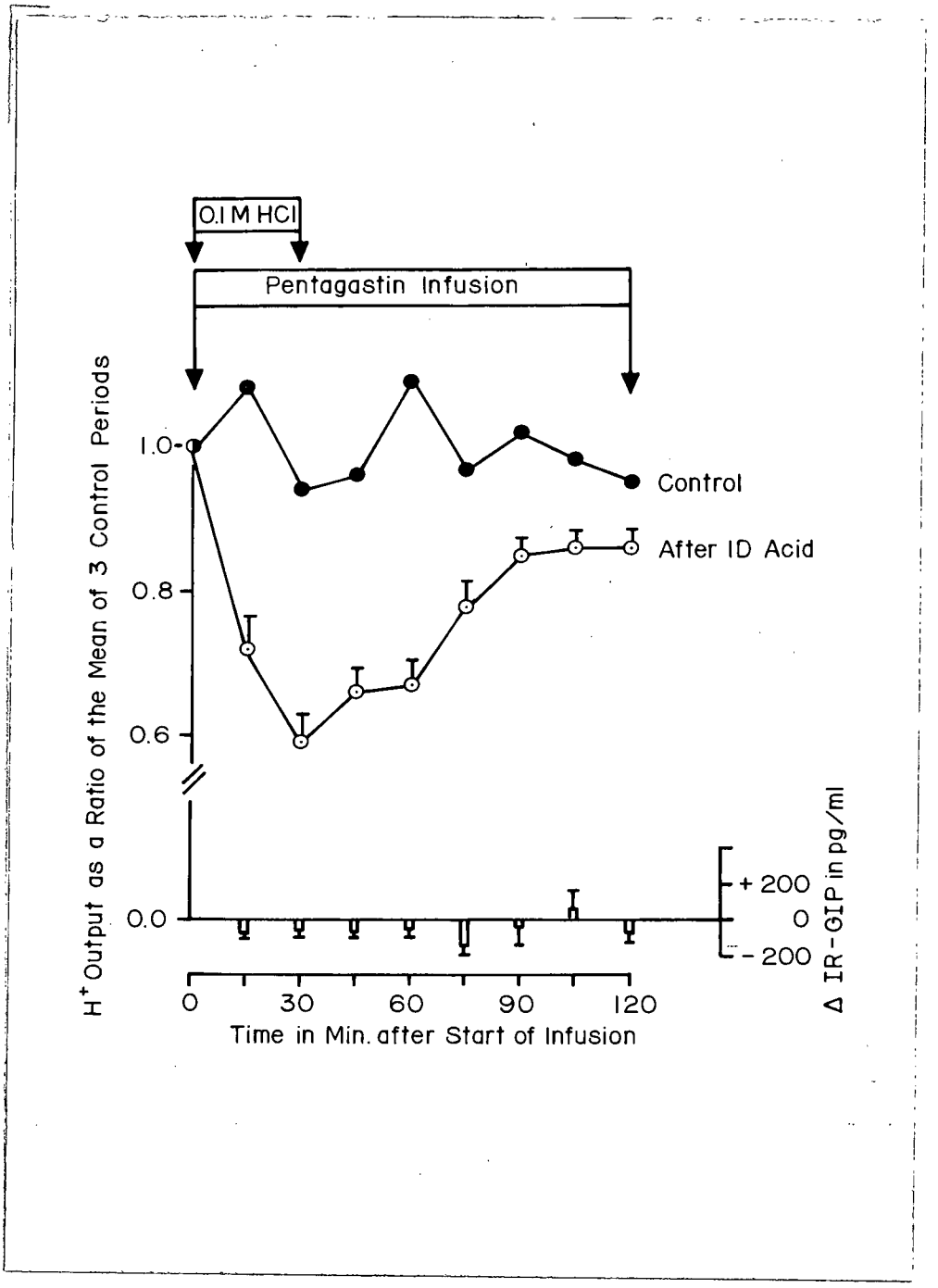


Fig. 68 The incremental IR-GIP response and inhibition of pentagastrin-induced gastric acid secretion caused by an intraduodenal infusion of 1.91 ml/min 0.1M Hcl. The results represent the mean $\pm$ SE of 4 experiments in 3 dogs.

TABLE XXXVII

Effect of a duodenal infusion of saline[1] on pentagastrin-stimulated H^+ output[2] and IR-GIP release[3].

Expt. #		CONTROL			Duodenal Infusion of Saline						Post Infusion Period					
		0_1	0_2	0_3	5	10	15	20	25	30	45	60	75	90	105	120
Ma 1	H^+	326	294	250			180			220	164	129	135	128	184	220
	IR-GIP	270	260	260			140			370	330	250	240	190	170	300
Ma 2	H^+	216	257	182			136			129	105	142	170	260	264	251
	IR-GIP	530	240	330			280			440	1150	670	630	360	340	410
Ma 3	H^+	275	256	249			195			134	159	232	216	280	168	141
	IR-GIP	350	330	370			380			300	340	320	320	-	-	-
Be 1	H^+	143	178	223			297			200	118	184	233	189	215	198
	IR-GIP	125	125	125			125			125	125	125	125	125	125	125
Be 2	H^+	256	242	264			222			192	198	133	169	121	159	168
	IR-GIP	125	125	125			125			125	125	125	125	125	125	125
Di 1	H^+	294	292	239			250			266	206	199	142	205	108	-
	IR-GIP	315	130	380			125			1100	125	125	125	125	125	125
Di 2	H^+	275	256	249			195			134	159	232	216	280	168	141
	IR-GIP	250	280	220			135			125	125	150	210	210	125	125

1. 0.15M saline, 100 mls over 30 min.
2. in μEq H^+/15 min.
3. IR-GIP in pg/ml.

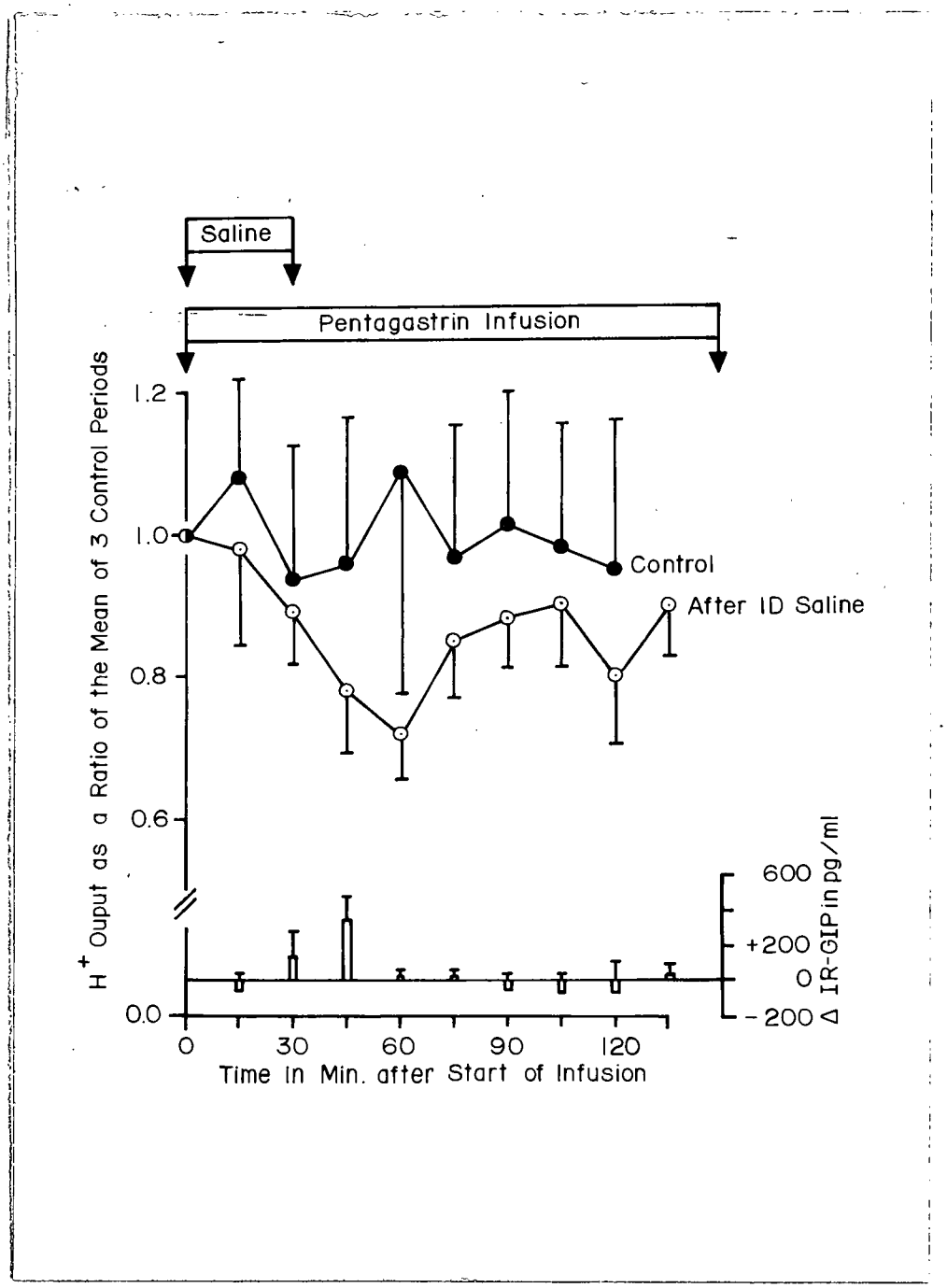


Fig. 69 The incremental IR-GIP response and inhibition of pentagastrin-induced gastric acid secretion caused by an intraduodenal infusion of 1.91 ml/min 0.15M saline. The results represent the mean $\pm$ SE of 7 experiments in 3 dogs.

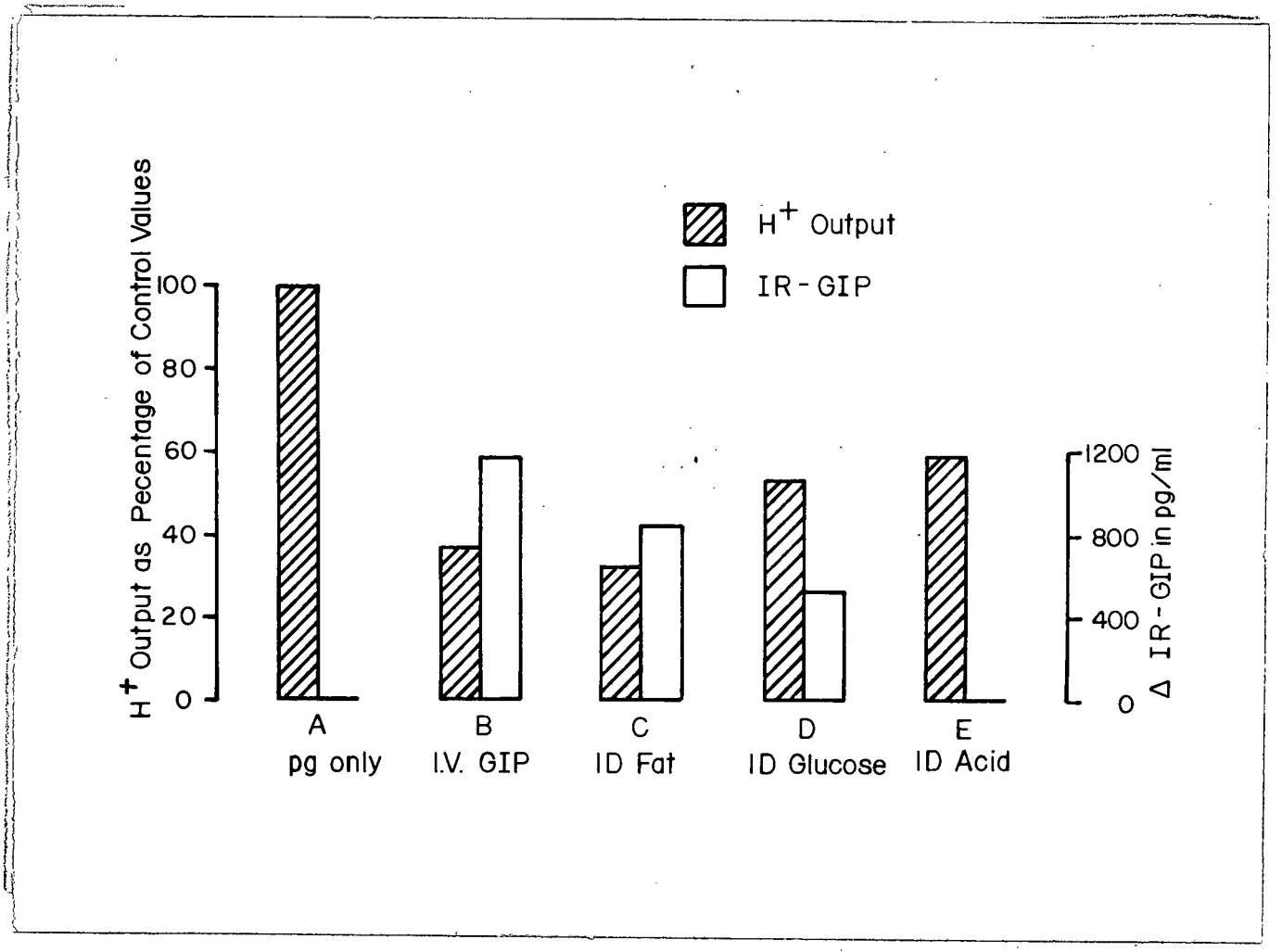


Fig. 70 The fundic pouch H^+ output and incremental serum IR-GIP response achieved after i.v. infusion of pentagastrin (pg) only - control - compared with these same parameters during a concomitant i.v. infusion of porcine GIP or intraduodenal infusions of fat, glucose or acid.

after intraduodenal infusion with fat, glucose and acid or intravenous infusion of porcine GIP.

STUDIES ON THE POSSIBLE HETEROGENEITY OF GIP

A. In serum

(I) Immunoreactivity of GIP released by glucose or fat

Serum samples were obtained from the same human subject (JRD) either 45 min. after ingestion of 100 ml 20% dextrose or 90 min. after ingestion of 100 ml of the triglyceride emulsion, Lipomul, representing the initial peak response of IR- GIP to either stimulus. They were serially diluted in diluent buffer and monitored by RIA.

Neither of the serum dilution curves showed any significant difference from the standard curve obtained by diluting pure porcine GIP. The antiserum used in the routine RIA did not, therefore, differentiate between the GIP released by glucose or fat given orally, as shown in Fig. 71.

(II) Immunoreactivity of GIP after column chromatography

The apparent immunoreactive homogeneity of the GIP released by glucose or fat was further examined by chromatography of 2 ml aliquots of these same serum samples on a 1x200 cm column of Sephadex G50 fine in 0.2M acetic acid. The column was calibrated prior to each run with dextran blue and ^{125}I- GIP (~ 60,000 cpm) in 2 mls charcoal-extracted plasma and the conductivity monitored to determine the position of the salt peak. One ml

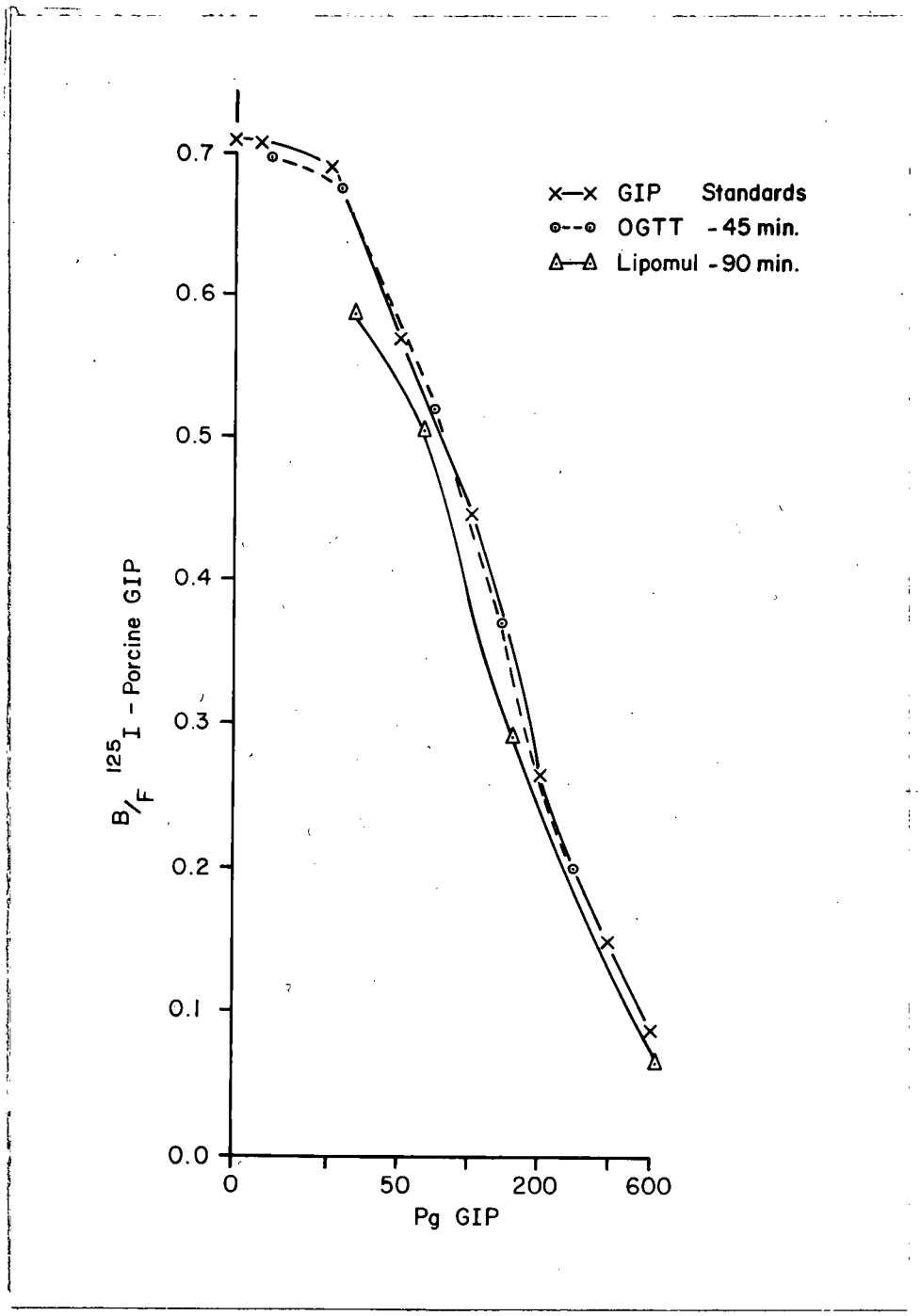


Fig. 71 A comparison of the immunoreactivities of porcine GIP and the IR-GIP in human sera after the ingestion of glucose or Lipomul. (Brown et al, Rec. Prog. Horm.Res. 31 : 487-532, 1975).

fractions were collected and the IR-GIP content of each fraction measured by RIA.

At least three immunoreactive regions were detectable after this treatment, one eluting in the void volume of the column GIP_{V^o} : and a significant immunoreactive component which eluted ahead of the GIP_{5000} arbitrarily named PROGIP. These three immunoreactive peaks were present in serum samples after either glucose or fat stimulation (as shown in Fig. 72 and Fig. 73).

The relative proportions of each IR-GIP component were given by expressing the area under each peak (approximately $^1/2$ height x width) as a percentage of the total (Fig. 74, Table XXXVIII).

TABLE XXXVIII

Proportions of IR-GIP components released by fat and glucose

	After glucose stimulation	After fat stimulation
Total IR-GIP content	1400 pg/ml	2700 pg/ml
% Total as GIP_{V^o}	14.4	31.4
% Total as ProGIP	35.3	40.8
% Total as GIP_{5000}	50.3	27.8

These results indicated that there might be a difference in the GIP response to glucose and fat, the major component of IR-GIP in the initial peak response to glucose being GIP_{5000} whilst that after fat was ProGIP.

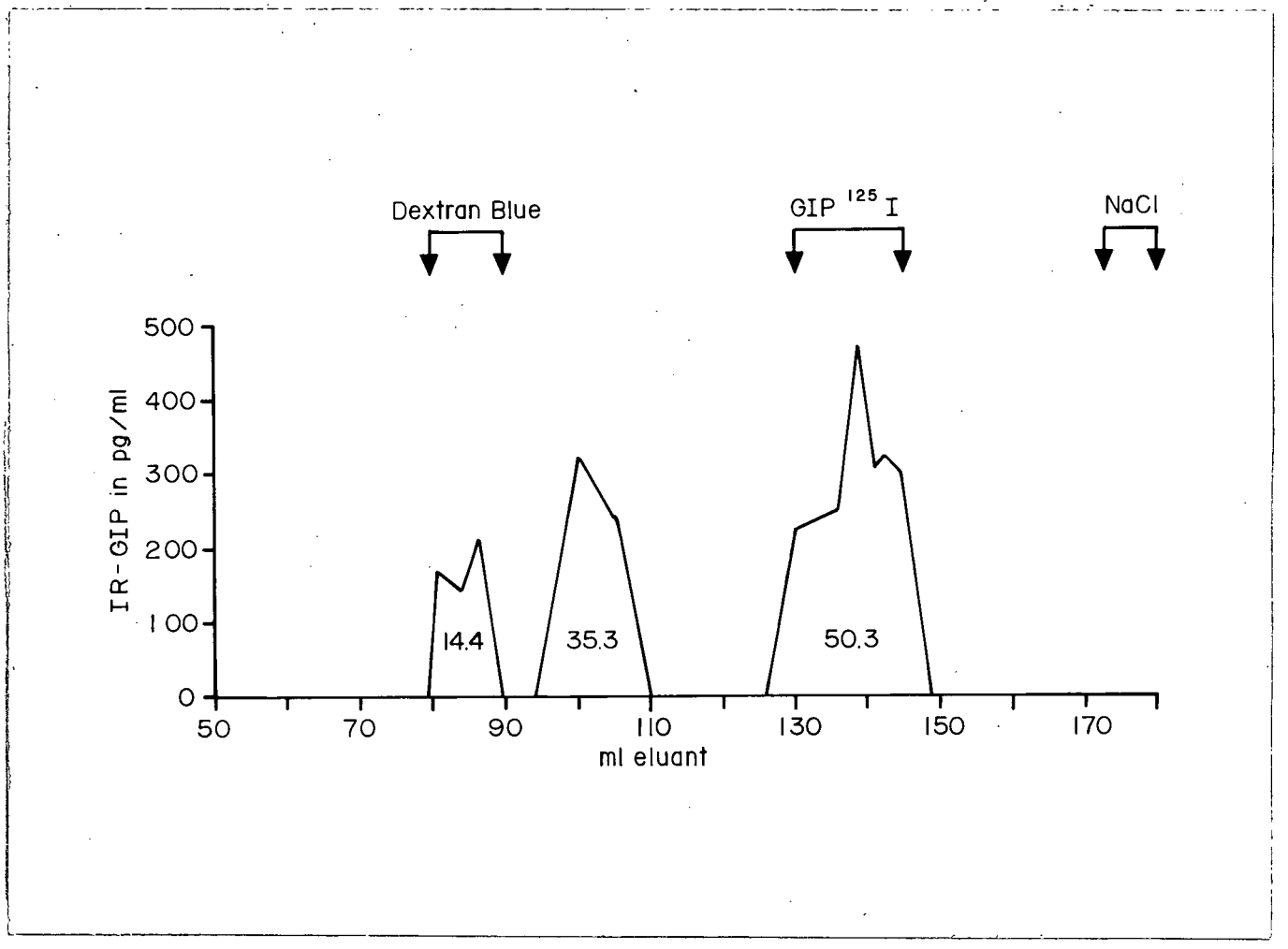


Fig. 72 Regions of IR-GIP observed after chromatography of 2 ml serum, obtained 45 min after ingestion of glucose, on Sephadex G50 (1 x 200 cm) in 0.2M acetic acid. The column was previously calibrated with dextran blue and ^{125}I-GIP. The enclosed numbers refer to the percentage of the total IR-GIP represented by that region.

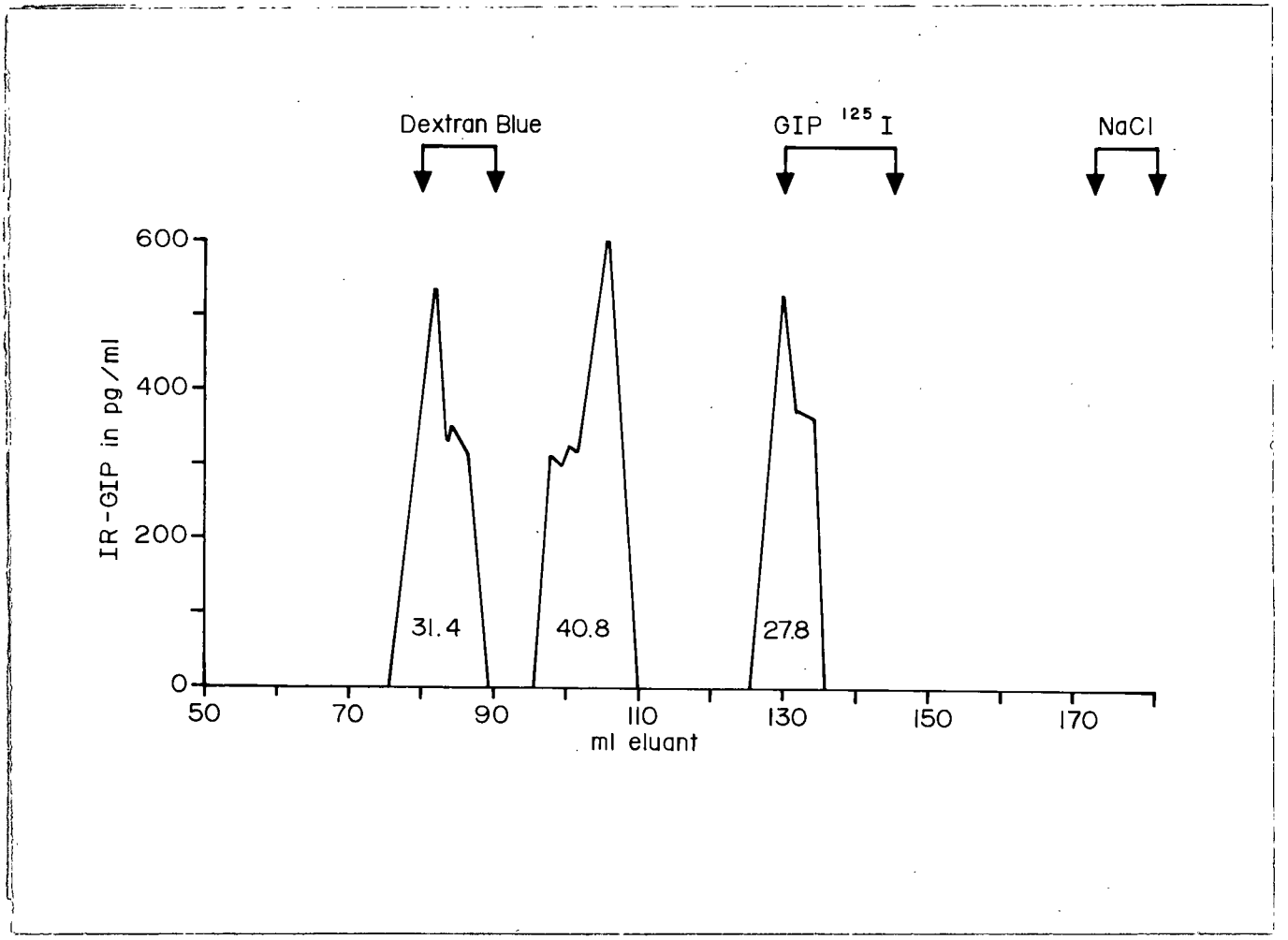


Fig. 73 Regions of IR-GIP observed after chromatography of 2 ml serum obtained 90 min after ingestion of Lipomul, on Sephadex G50 in 0.2M acetic acid. The column was previously calibrated with dextran blue and ^{125}I-GIP. The enclosed numbers refer to the percentage of total IR-GIP represented by that region.

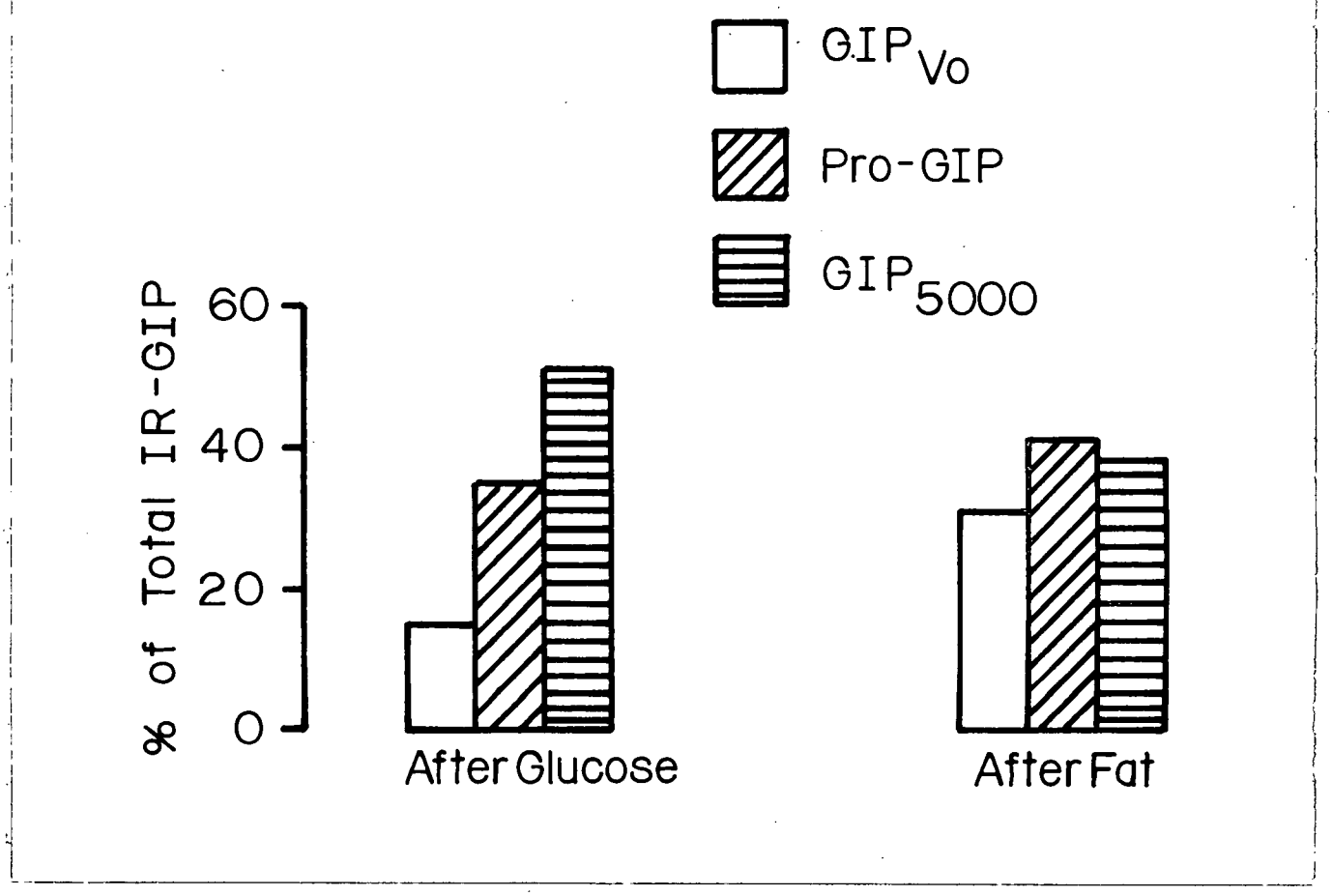


Fig. 74 The chromatograms from Fig. 71 and Fig. 72 expressed in histogram form, the heterologues of GIP being represented as percentages of the total IR-GIP response.

(III) Immunoreactive forms of GIP released by oral fat or glucose

(a) After oral fat

Serum samples were obtained from both normal human subjects and dogs at various time intervals after ingestion of fat in the form of 100 ml Lipomul. After routine RIA of these samples a 2 ml aliquot of each was chromatographed on Sephadex G50 fine in 0.2M acetic acid and 1 ml fractions collected. The column was routinely calibrated with ^{125}I- albumin, ^{125}I- GIP and ^{125}I in 2 ml extracted plasma.

The results obtained after RIA of the column fractions are typified in Fig. 75 and Table XXXIX. In both species the proportion of the total IR- GIP represented by the larger molecular form (ProGIP) increased with increase in time after the ingestion of fat.

(b) After oral glucose

Sera from human subjects 45 min and 100 min. after oral administration of 100 ml 20% dextrose were eluted from Sephadex G50 fine as previously described. The percentage of the total IR- GIP existing in the ProGIP form increased with increase in time after the ingestion of glucose (Fig. 75 Table XXXIX).

These results indicate that the important factor in determining the relative proportions of the different forms of IR- GIP is the time of sampling the serum after the stimulus and not the nature of the stimulus itself.

(IV) Immunoreactive forms of GIP after its exogenous administration

Normal, fasted, dogs were given intravenous infusions of 1.5 µg/kg/hour

TABLE XXXIX

Change in relative proportions of IR-GIP components with the time of serum sampling after oral fat and glucose.

Type of stimulus	Oral Fat					Oral Glucose	
Time (min) of sampling after stimulus	20	35	60	90	150	45	100
Oral fat in man							
*Total IR-GIP content	650	1680	1050				
% Total as GIP_{v^o}	100	28.0	47.3				
% Total as ProGIP	-	14.3	30.6				
% Total as GIP_{5000}	-	57.7	22.1				
Oral fat in dog							
*Total IR-GIP content				1250	1750		
% Total as GIP_{v^o}				41.6	40.1		
% Total as ProGIP				7.5	30.4		
% Total as GIP_{5000}				50.9	29.5		
Oral glucose in man							
*Total IR-GIP content						1400	680
% Total as GIP_{v^o}						14.4	16.1
% Total as ProGIP						35.3	42.5
% Total as GIP_{5000}						50.3	41.4

* Expressed in pg/ml IR-GIP.

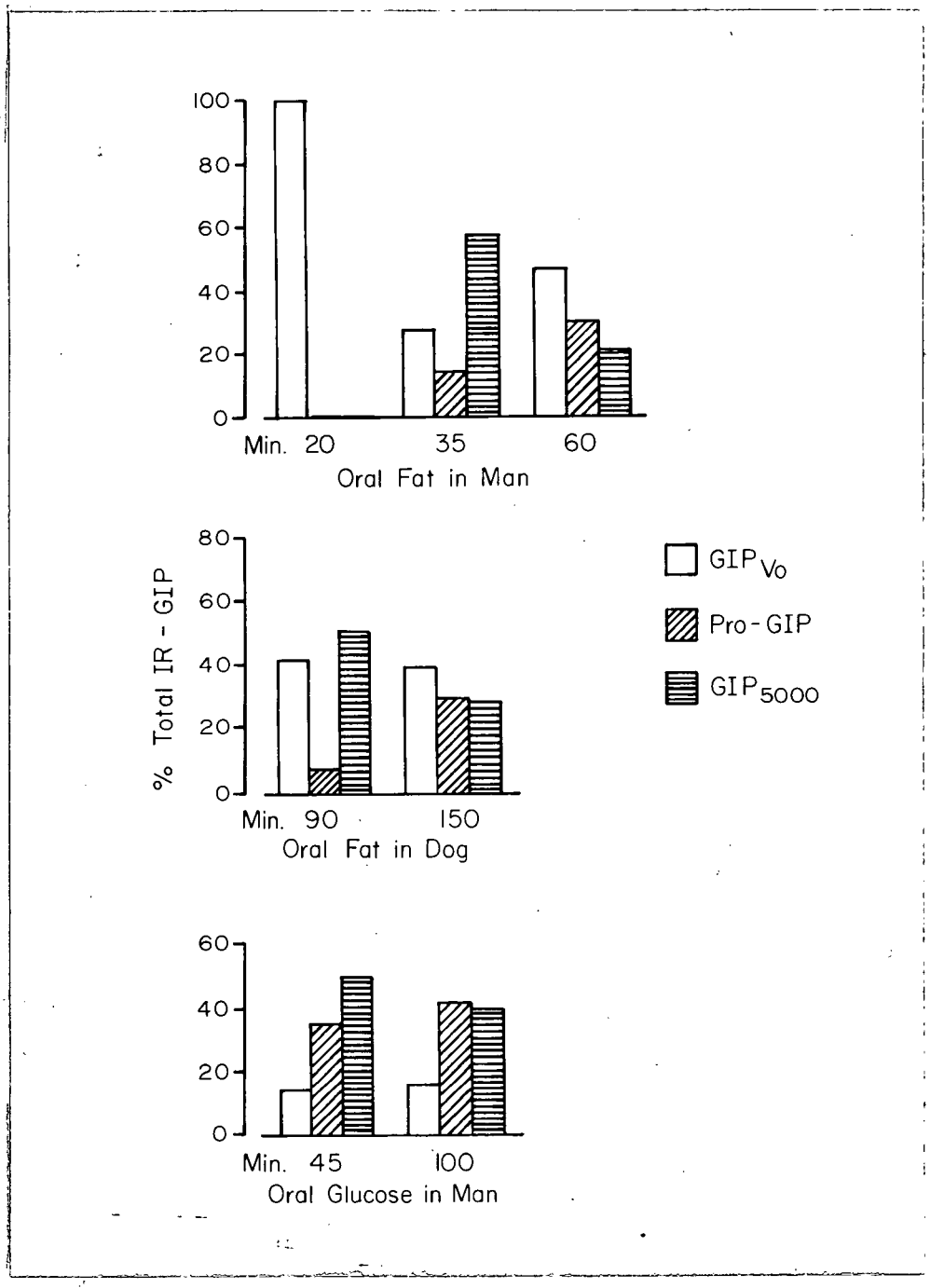


Fig. 75 The relative proportions of IR-GIP$_{Vo}$, IR-GIP$_{5000}$ and IR-ProGIP, expressed as percentages of the total IR-GIP response, observed (a) 20,35 and 60 min after fat ingestion in man, (b) 90 and 150 min after fat ingestion in dogs and (c) 45 and 100 min after glucose ingestion in man.

natural porcine GIP over one hour. Blood samples were taken after 15, 30, 50 min. and 15 min. after the infusion had ended. The total IR- GIP of each serum sample was determined by RIA and 2 ml aliquots were subjected to gel filtration on Sephadex G50 fine prior to further RIA.

Two major regions of immunoreactivity were observed, one corresponding to GIP_{5000}, as might be expected by reason of the procedures involve in the purification of GIP. The other immunoreactive peak eluted in the void volume of the column, i.e., GIP_{V^o}, representing a form or complex with a molecular weight >750,000, and was too large to be explained in terms of the circulating GIP present in the serum of the fasted dog, (Fig.76).

Pretreatment of serum samples from the series with 6.0M urea prior to chromatography resulted in the conversion of a significant proportion of the GIP_{V^o} to GIP_{5000} suggesting that the IR- GIP eluting in the void volume represents an immunoreactive complex formed by the binding of GIP_{5000} to a large molecular weight, serum protein, e.g., albumin or globulin (Fig.776).

B. In tissue extracts

(I) Initial tissue extraction

Extracts of the duodenal and jejunal mucosa of dogs were partially purified in the laboratory of Dr. V. Mutt (Karolinska Institutet, Stockholm, Sweden). The tissue was boiled briefly and extracted into acetic acid. The protein was adsorbed onto alginic acid, eluted with 0.2M hydrochloric acid and precipitated from solution with saturated sodium chloride. This precipitate contained secretin and cholecystokinin - pancreozymin, as well as GIP. After

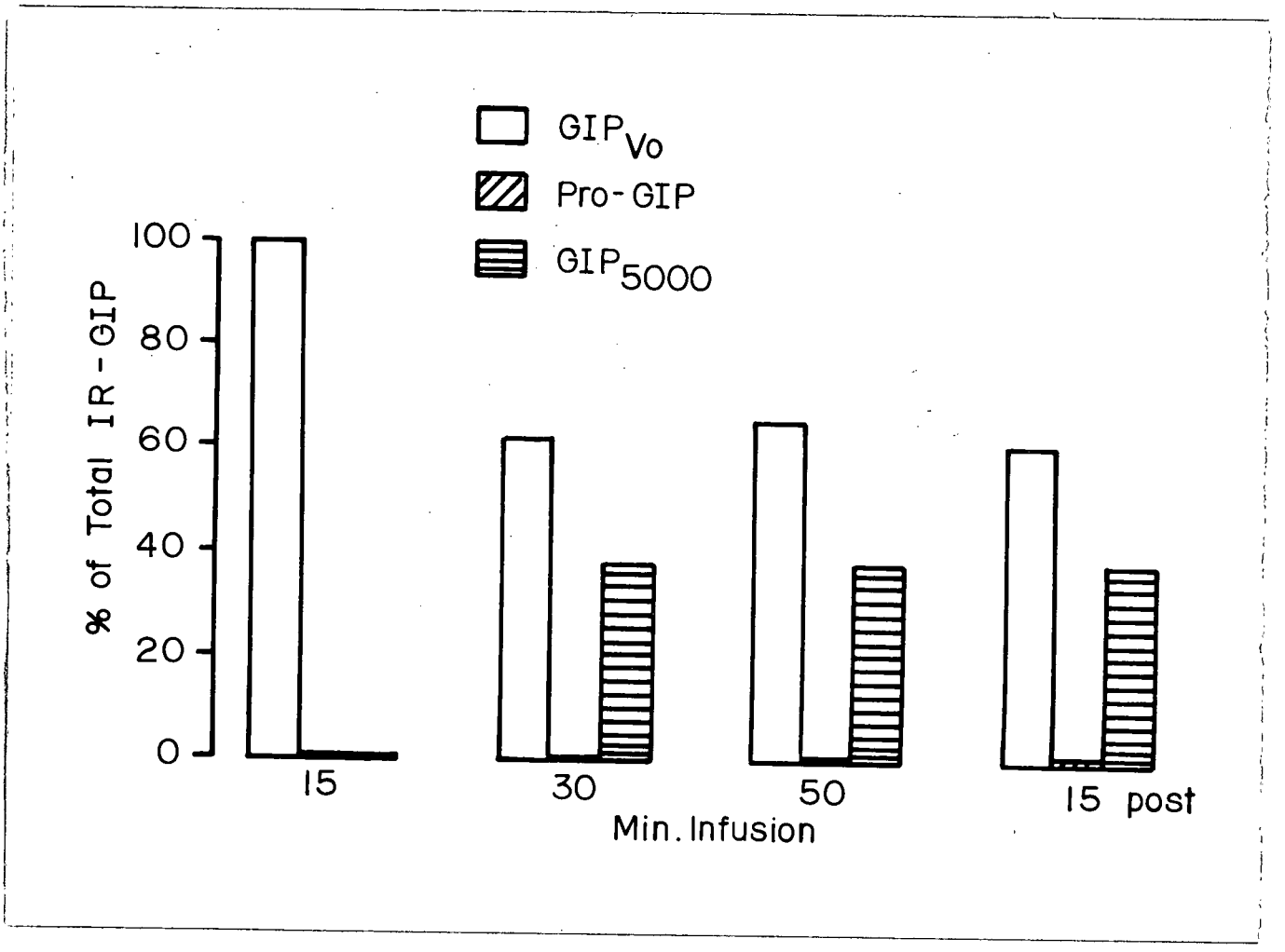


Fig. 76 The relative proportions of IR-GIP$_{V^o}$, IR-GIP$_{5000}$ and IR-ProGIP, expressed as percentages of the total IR-GIP response, in serum taken 15, 30 and 50 min after the start of an infusion of porcine GIP, 1 µg/kg/hour, and 15 min after the termination of the infusion.

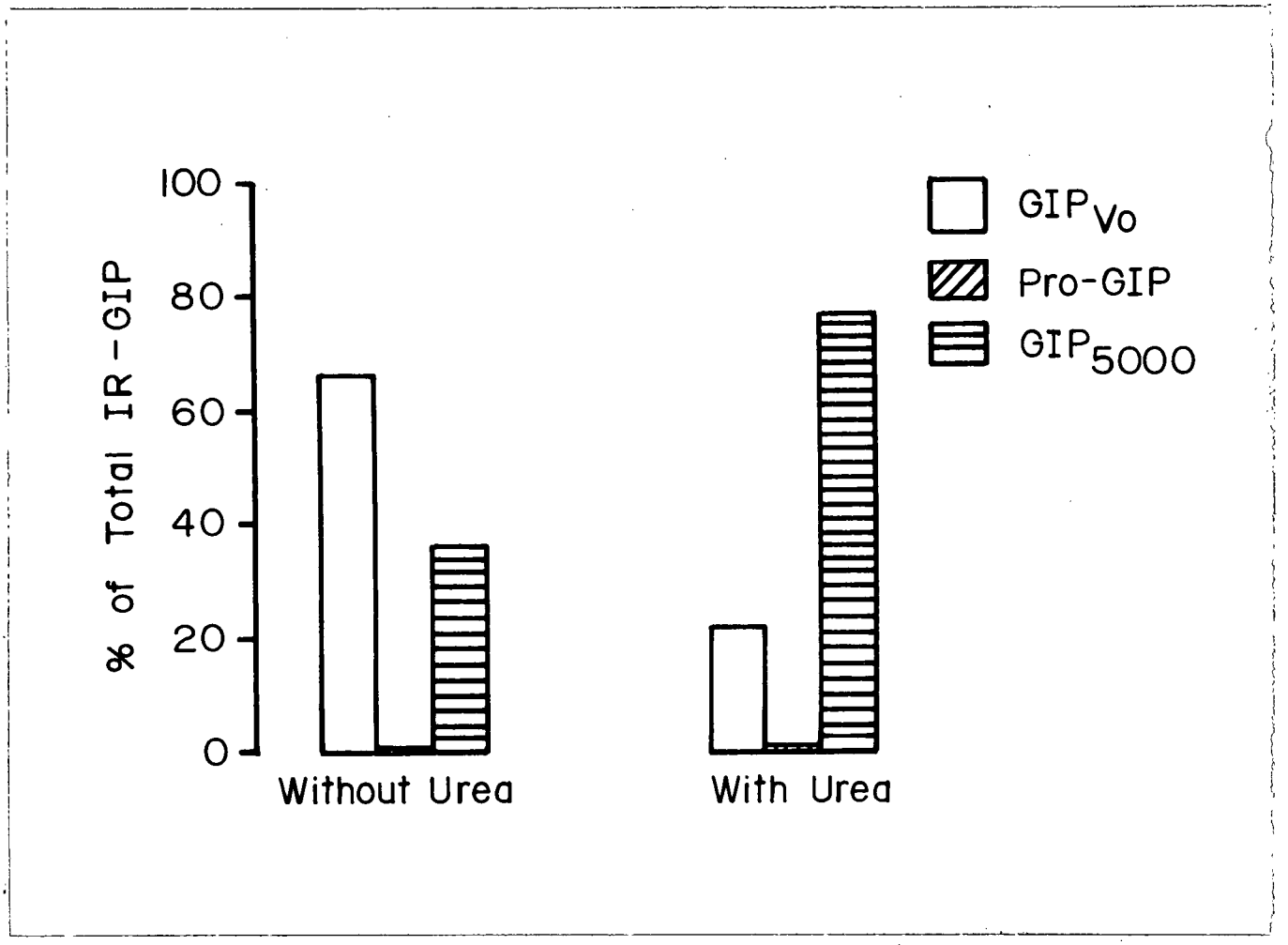


Fig. 77 The relative proportions of IR-GIP$_{V_o}$, IR-GIP$_{5000}$ and IR-ProGIP, expressed as percentages of the total IR-GIP response, in a serum sample containing exogenously administered GIP, with or without pretreatment with 6.0M urea.

desalting on Sephadex G25, a fraction preceding IR-GIP$_{5000}$ (Fr 1-8) was selected for further extraction. Ten g of this material (SPC I G25 Fr 1-8) was dissolved in 200 ml ammonium acetate, pH 6.5, and the pH corrected to 7.0 by addition of 2.0M ammonia. The mixture was centrifuged at 7000 rpm for 30 min at 4°C and the supernatant was decanted. The insoluble precipitate was redissolved im 0.1M acetic acid and lyophilized - neutral insoluble material. Methanol (9 volumes) was added to the supernatant and the insoluble precipitate removed by centrifugation, redissolved in 0.1M acetic acid and lyophilized - methanol insoluble material. The protein remaining in solution was precipitated by the addition of 4 volumes of acetone at 4°C, and the precipitate was filtered out on Whatman's 3MM paper, redissolved in 0.1M acetic acid and lyophilized - methanol soluble material. These procedures are summarized in Table XXXXI. The fractions designated neutral insoluble, methanol insoluble and methanol soluble were chromatographed on Sephadex G50 fine (1x100 cm) in 0.2M acetic acid and the IR-GIP content of each 1.0 ml fraction was measured by RIA. The relative proportions of each component as a percentage of the total IR-GIP content is shown in Table XXXX and Fig. 78.

Table XXXX

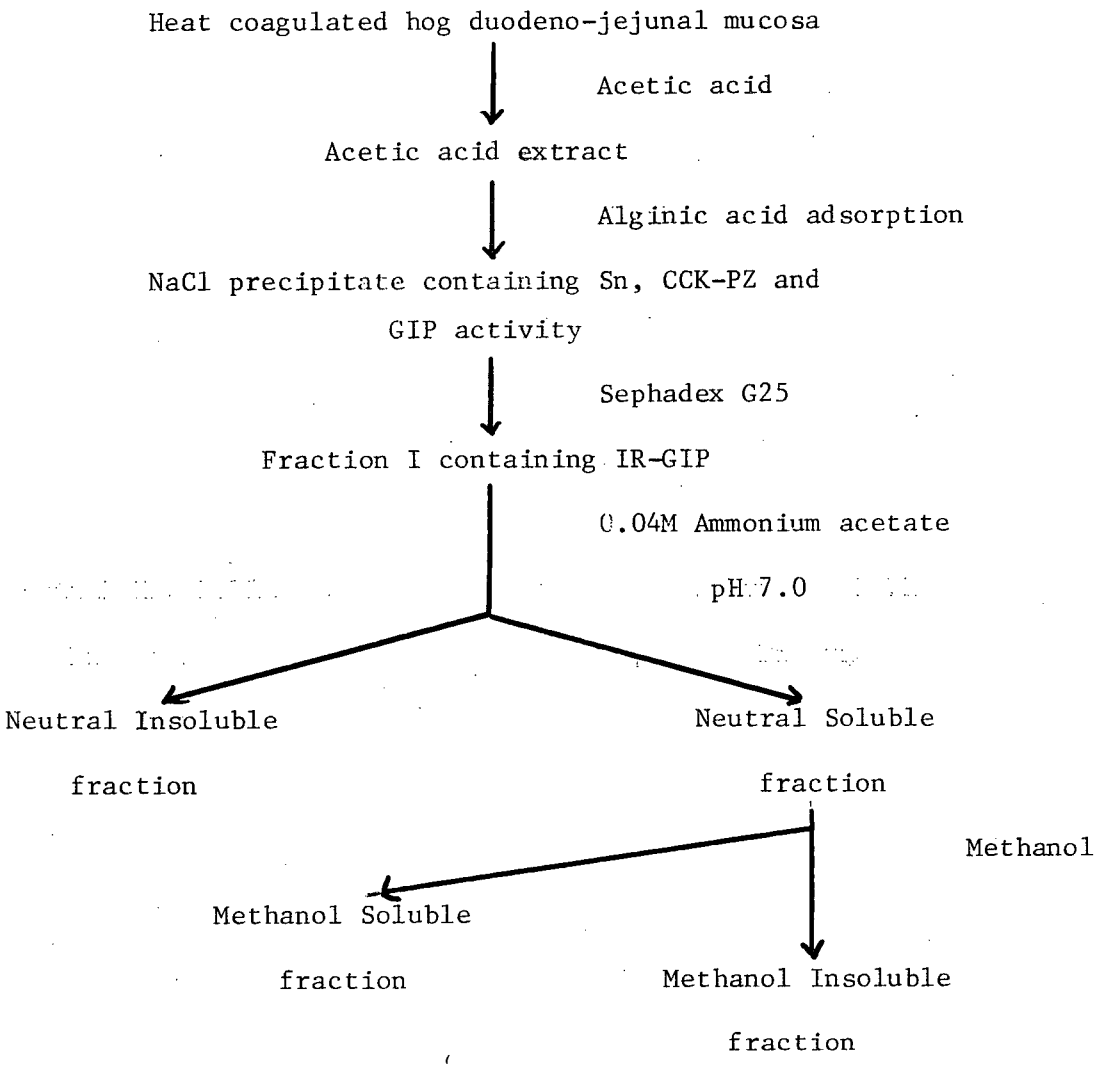
Relative proportions of the IR-GIP components in fractions obtained from an extract of hog intestinal mucosa

Fraction	% Total IR-GIP content		
	GIP$_{Vo}$	ProGIP	GIP$_{5000}$
Neutral insoluble	0	33.3	66.7
Methanol insoluble	0	72.2	27.8
Methanol soluble	0	0.0	100.0

Further purification was performed on the methanol insoluble fraction.

TABLE XXXXI

Summary of Tissue Extraction Procedure



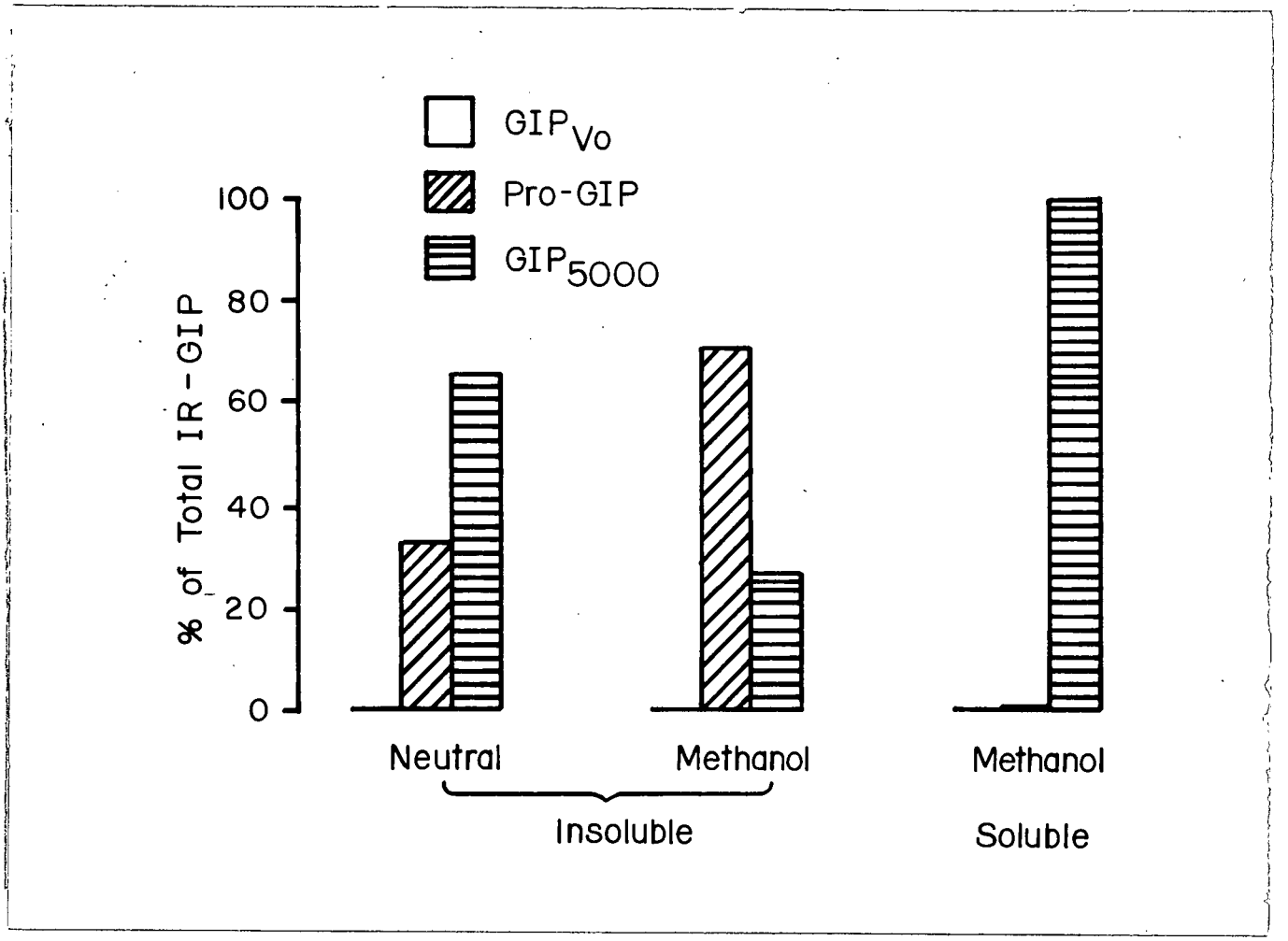


Fig. 78 The relative proportions of IR-GIP$_{Vo}$, IR-GIP$_{5000}$ and IR-ProGIP, expressed as percentages of the total IR-GIP, in partially purified extracts of the hog duodenal and jejunal mucosa.

(II) Purification

(a) Methanol insoluble on Sephadex G50

In a typical experiment 300 mg methanol insoluble was dissolved in 5 ml 0.2M acetic acid containing ^{125}I- albumin and ^{125}I- GIP. The column of Sephadex G50 fine (2.5x90 cm) was developed with 0.2M acetic acid and 5.0 ml fractions were collected at a flowrate of 80 ml/hour. The void volume of the column and the elution volume of GIP_{5000} were determined by counting 0.5 ml aliquots of each fraction for 1 min. in an automatic gamma counter. The region between these peaks was pooled, lyophilized and designated ProGIP I. The column profile of such a column, obtained by plotting absorbance at 280 nm in a 1 cm light path against ml eluant, is shown in Fig. 79, with the regions of GIP immunoreactivity determined by RIA, superimposed. This procedure yielded approximately 100 mg lyophilized material with an IR- GIP content of 30 ng/mg.

(Ib) ProGIP I on CM cellulose

Thirty mg ProGIP I was dissolved in 5 ml 0.01M ammonium bicarbonate and the pH adjusted to 7.05 with carbon dioxide. The solution was applied to a column of cellulose CM II (1.5x13 cm) which was developed with 0.01M ammonium bicarbonate, pH 7.8. The more strongly absorbed material was eluted with 0.2M ammonium bicarbonate. The eluate was collected in 5 ml fractions at a flow rate of 120 ml/hour. The column was calibrated by chromatographing porcine GIP under identical conditions.

The absorbance of each fraction was measured at 280 nm in a 1 cm light path and the IR- GIP content estimated by radioimmunoassay with two different antisera. There was no significant IR- GIP in this sample, the major immuno-

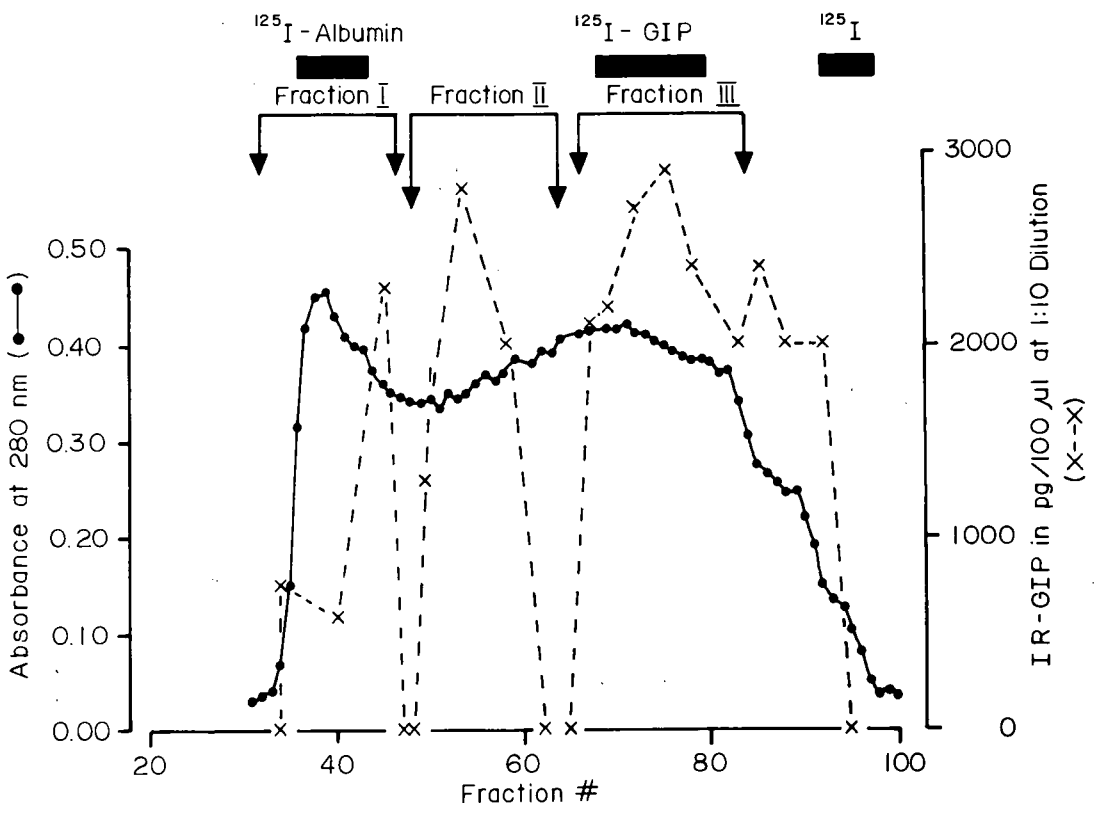


Fig. 79 Column profile obtained after elution of the methanol insoluble fraction from Sephadex G50 and measurement of absorbance at 280 nm. The regions of GIP immunoreactivity were determined by RIA. Fr II was designated ProGIP I. The column was calibrated with ^{125}I- albumin, ^{125}I-GIP and ^{125}Iodine.

reactive peak being less basic than GIP_{5000}. The alternative antiserum Ro 7, appeared to cross react to a different degree with this molecular form of IR- GIP, compared with the antiserum, Van 8, routinely used in the assay (Fig. 80). Both antisera seemed to cross-react with the standard GIP preparation to the same degree. The interassay control value was 254 ± 43 pg/ml IR- GIP (mean $\pm$ SE in 50 determinations) according to Van 8, and 242 ± 14 pg/ml IR- GIP (mean $\pm$ SE in 18 determinations) according to Ro7.

When Fraction III, from chromatography on Sephadex G50, i.e., that fraction corresponding to GIP_{5000}, was eluted from cellulose CM II under identical conditions, the major portion of the immunoreactivity eluted in the same position as natural porcine GIP_{5000} (Fig. 81).

(c) Stability of ProGIP

The material containing ProGIP, and selected to contain no GIP_{5000} was routinely lyophilized and stored at -20°C. After the yield from several columns had been pooled, 2 mg of the material was re-run on Sephadex G50 fine (1x100 cm) in 0.2M acetic acid as previously described. Radioimmunoassay on the fractions obtained revealed that a third of this material now existed in the GIP_{5000} form, as shown in Fig. 82.

(III) Molecular weight determination

A series of chromatograms were run on Sephadex G50 fine (1x100 cm) in 0.2M acetic acid. The samples were ~ 50,000 cpm ^{125}I- albumin and ~ 50,000 cpm of one of the following iodinated markers, ^{125}I- motilin, ^{125}I-GIP, ^{125}I-insulin or ^{125}I- parathyroid hormone, in 2 mls 0.2M acetic acid. The 1 ml

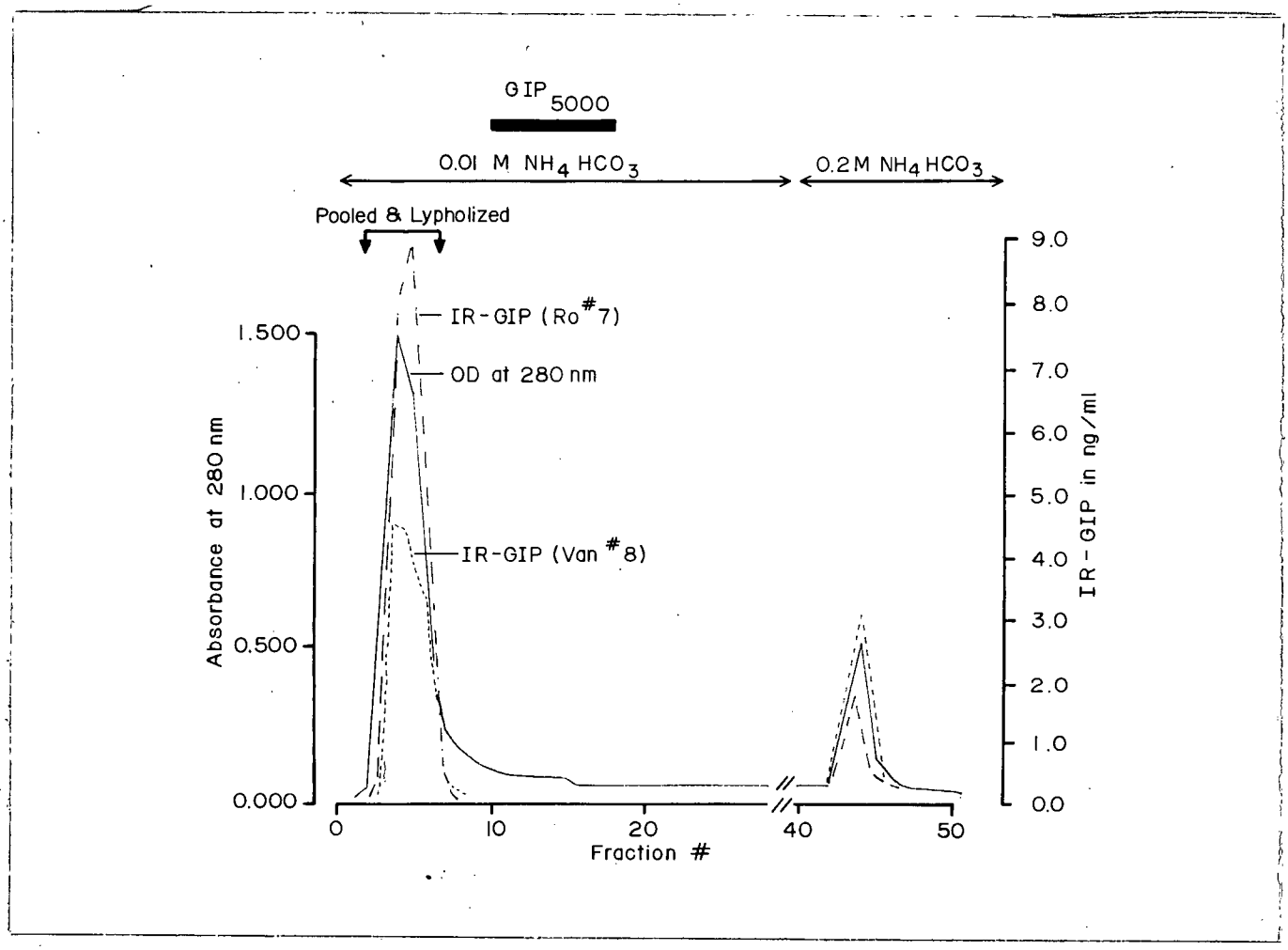


Fig. 80 Column profile obtained after elution of ProGIP I from CM cellulose. The IR-GIP content was determined by RIA with antisera Van 8 and Ro7. The column was calibrated with porcine GIP.

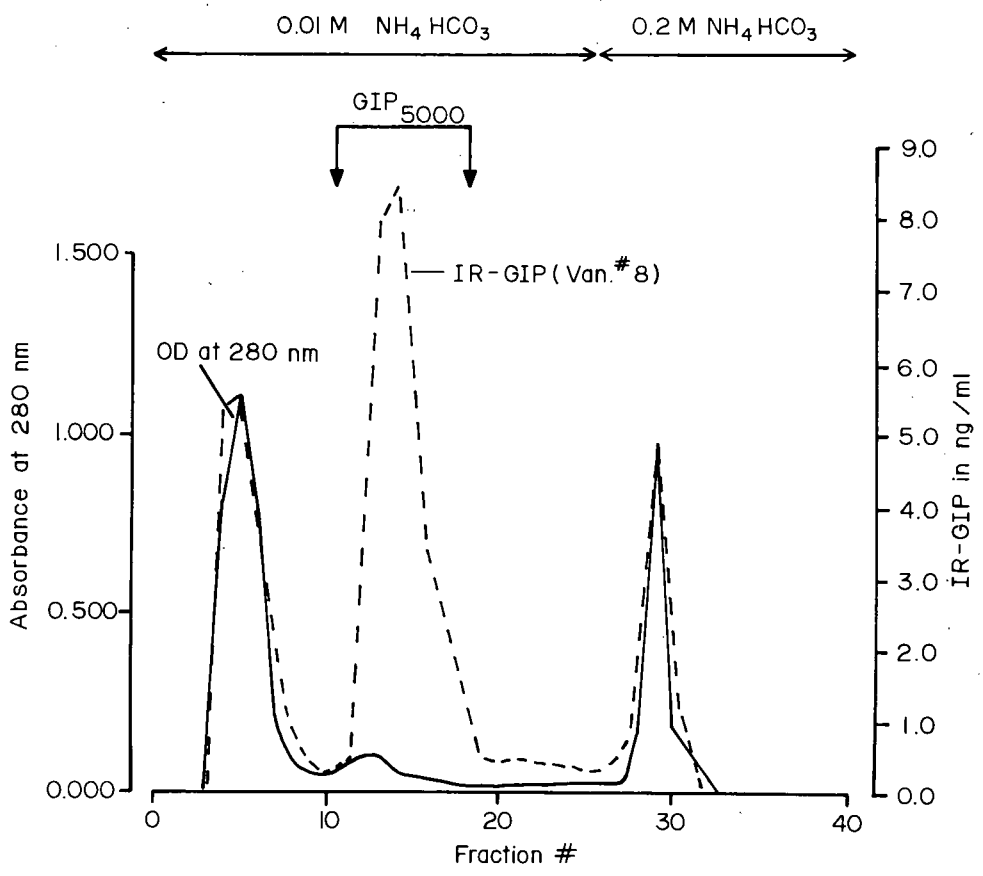


Fig. 81 Column profile obtained after elution of the GIP_{5000} - containing fraction from Sephadex G50, on CM cellulose. The column was calibrated with porcine GIP

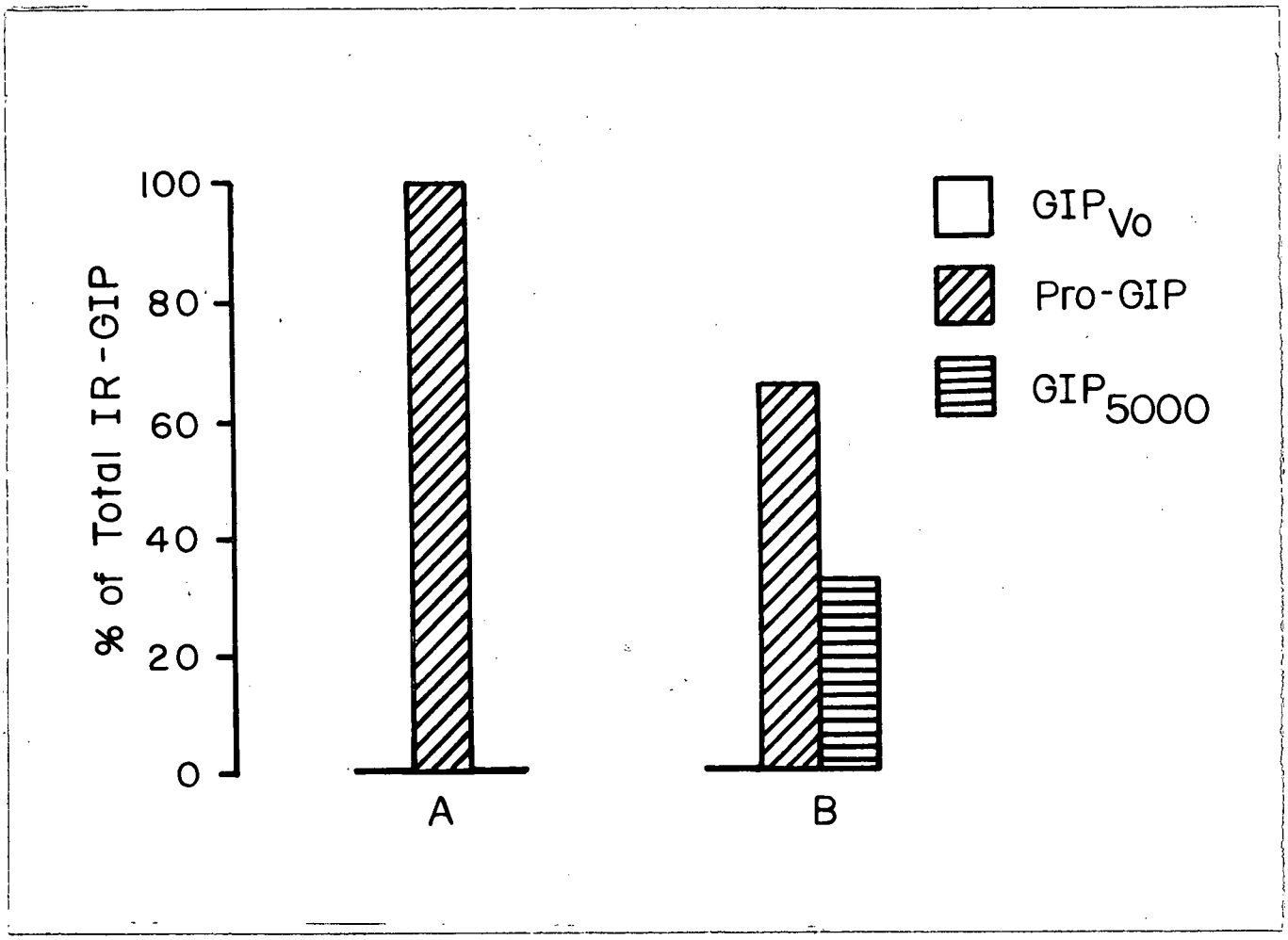


Fig. 82 Column profile obtained after rechromatography of ProGIP I on Sephadex G50 in 0.2M acetic acid, demonstrating the re-appearance of IR-GIP$_{5000}$.

fractions collected were counted for 1 min. in an automatic gamma counter and plotted against ml eluant. The void volume ($V°$) and elution volume (V_E) were taken as the volumes corresponding to the peak tube of the ^{125}I-albumin and ^{125}I marker respectively, and $V_E/V°$ versus molecular weight was plotted for each marker (Fig. 83). $V_E/V°$ was determined for the IR-GIP in ProGIP I and this was found to correspond to a molecular weight of 7500-8000 in four separate determinations.

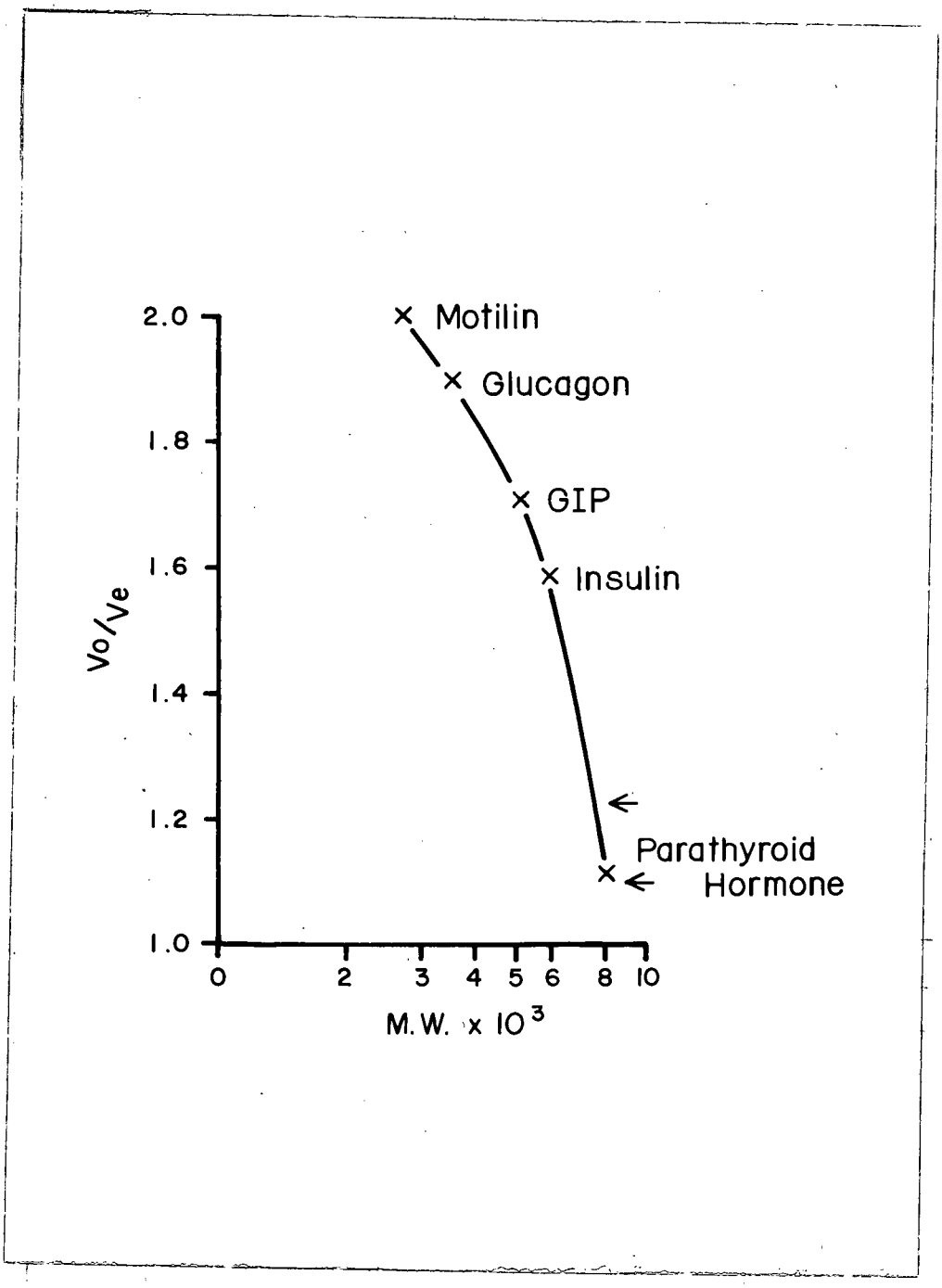


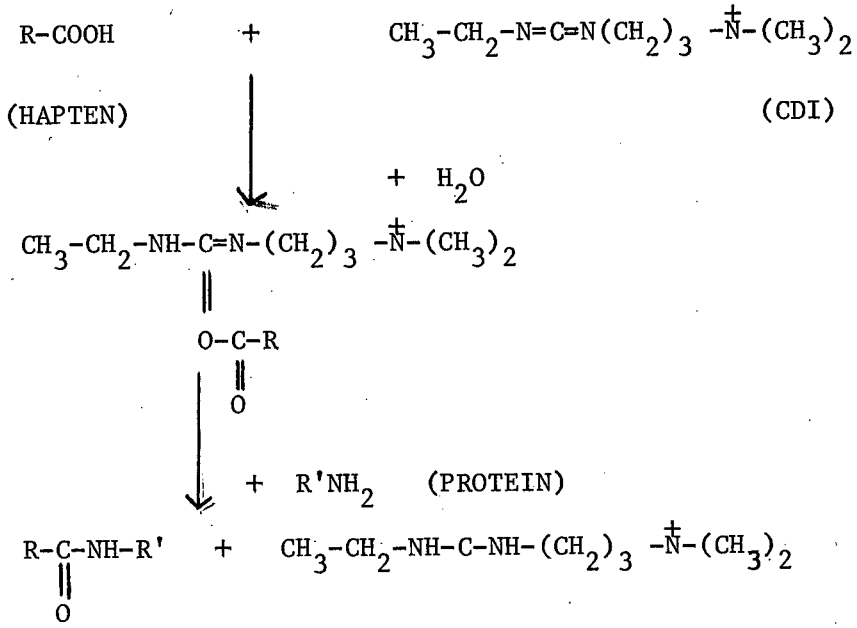
Fig. 83 Curve showing the relationship between V_o/V_e and molecular weight for various polypeptides, obtained by elution of the iodinated polypeptides from Sephadex G50 (1 x 100 cm) in 0.2M acetic acid and counting of these fractions for 1 min in an automatic γ counter.

DISCUSSION

Studies on the composition and structure of motilin revealed that it was quite distinct from the previously isolated and characterized gastrointestinal polypeptides of duodenal mucosal origin. The major property of motilin appeared to be its stimulatory effect on the motor activity of the stomach. Unlike gastrin and cholecystokinin-pancreozymin, motilin stimulated the motor activity of the fundus as well as that of the antrum. Another action of motilin, suggested by studies with exogenously administered polypeptide, was stimulation of pepsin secretion in dogs. It did not, however, appear to have any effect on the exocrine pancreas or on gastric acid secretion (Brown et al,1972). Recognition of the physiological role played by motilin in regulating gastric motor and secretory activities, and support for the hypothesis that motilin was the humoral agent released upon alkalinization of the duodenal mucosa both required the development of some method for the identification and measurement of changes in the levels of motilin in the systemic circulation.

A radioimmunoassay has been developed for the measurement of IR- motilin in sera and tissue extracts (Dryburgh and Brown, 1975). Antisera were raised in albino guinea pigs and New Zealand white rabbits. As a general rule, any substance with a molecular weight of < 1000 may be regarded as non-immunogenic, whilst those with molecular weights in the range 1000-6000 are poor immunogens. Motilin, with a molecular weight of 2700, falls into the latter group and no useful antisera to this polypeptide have been obtained by immunization with the polypeptide alone. This was overcome by covalently coupling the molecule (hapten) to a larger protein. The most commonly used method of achieving this is the carbodiimide condensation reaction. Carbodiimide will react with a

number of weak acids but the predominant reaction at room temperature is with carboxylic acid, usually provided by the hapten. The activated carboxyl group then reacts with the free amino groups on the protein to form a peptide bond as schematically shown by the formula:-



Some degree of condensation will occur between the protein molecules, via their activated carboxyl groups. This may be reduced somewhat by activating the haptenic carboxylic acids before the addition of the protein. Bovine and human serum albumin are the most commonly used proteins. Immunization of both rabbits and guinea pigs, the schedule of injections involving both motilin, conjugated to BSA, and non-conjugated motilin, resulted in the production of acceptable antisera in the majority of animals.

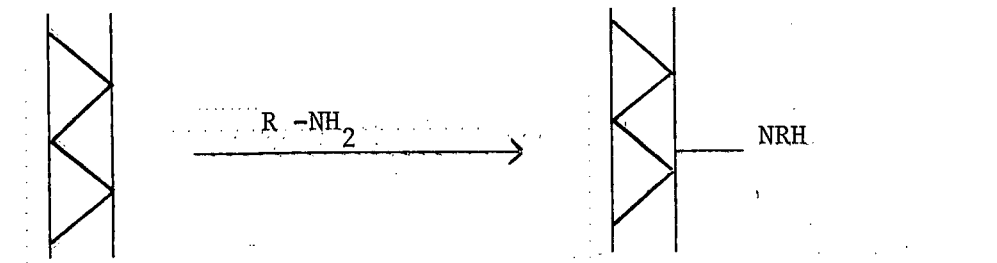
The range of affinity constants (K) of motilin antisera was 1×10^{12} to 1×10^{14} 1/mole, calculated as shown in Fig. 10. When a radioimmunoassay is being established it must be accepted that there is an inherent limit to the sensitivity

of the assay that is dependent on the affinity constant characterizing the predominant antibodies in the antiserum. The affinity of the antiserum has been determined to be a function of the dose of the immunogen employed (Parker et al, 1967), and the time interval since the immunization (Eisen and Siskind, 1964). It is assumed that the heterogeneity of antibodies present in an antiserum is the result of their production by a heterogeneous population of antigen-sensitive small lymphocytes. Low to moderate doses of immunogen will preferentially stimulate cells with high affinity receptors and their progeny will, in turn, produce high affinity antibodies. With the passage of time the antibodies produced will tend to neutralize some of the antigen and as the level falls, it will be the higher affinity cells which will continue to be stimulated. Antisera to motilin have been raised by immunization with 20-50 µg polypeptide, given at monthly intervals until a reasonable titre was achieved. Booster immunizations with low doses of immunogen, at 3 - 6 month intervals, maintained or increased the titre. Animals were bled 10 - 12 days after each immunization and at monthly intervals thereafter. Motilin antiserum Mo7 had a titre of 1: 10×10^4, 10 days after its fourth immunization, which had increased to 1: 20×10^4 two months later, with no intervening booster (Table II). The immunization schedules and results are summarized in Table I and Table II. No crossreactivity has been demonstrated between any motilin antiserum and gastrin, GIP, secretin, glucagon, cholecystokininpancreozymin, VIP or insulin, as shown in Figs. 8 and 9.

Theoretically the iodination of any polypeptide containing tyrosine requires only 3 basic ingredients:- the isotope, usually ^{125}I-Na, the pure polypeptide, and some method for oxidizing the iodide to iodine. Variables, such as the

relative ratios of the various reagents, the constituents of the diluent buffer, the final volume of the reaction mixture, and the method for separating the unincorporated isotope from the labelled polypeptide, have to be established for each individual polypeptide. The majority of iodination procedures, however, vary only slightly from the original method proposed by Hunter and Greenwood in 1963. Motilin was iodinated by a slight modification of their chloramine-T method and radioactive tracer with a specific activity of 400 mCi/mg was routinely produced. A theoretical iodination of this polypeptide, resulting in the incorporation of 1 atom of iodine into each mole of peptide, would produce ^{125}I- motilin with a specific activity of 626 mCi/mg, assuming an isotope abundance of 96%. The actual results obtained would suggest that the tyrosine in position 7 in motilin is relatively accessible to incorporation of iodine. Storage of ^{125}I- motilin, at a dilution of 1 x 10^6 cpm/ml, in 0.2M acetic acid, containing 0.5% B.S.A. at -20°C, resulted in a label, stable without repurification, for periods of up to 3 months. With the antiserum so far available, the most sensitive assay has resulted from an incubation which is allowed to reach equilibrium over 48-72 hours at 4°C. (Fig. 14).

The routine method of separating the free antigen from the antibody/antigen complex is adsorption of the antigen onto dextran-coated charcoal, 2.5 mg and 0.5 mg dextran being added per tube, as illustrated in Fig. 16. A solid phase antibody has been developed by coupling motilin antiserum to a beaded form of agarose, a cross-linked dextran with high porosity (Sepharose 4B). This matrix is activated by treatment with cyanogen bromide at high pH. It is then reacted with the unprotonated amino groups on the ligand, in this case the IgG in the antiserum, to form a stable complex by formation of hydrogen bonds.



No detectable antibody activity was measured in the washings of the solid after coupling to the antiserum was complete (Fig. 18). The coupled antibody could be used in the radioimmunoassay with no apparent loss in antibody activity or sensitivity potential. No significant difference was observed in the antiserum dilution curves or assay standard curves, obtained with coupled or uncoupled antiserum, all other assay conditions being the same (Fig. 42 and Fig. 43). Solid phase antibodies are a feasible prospect in the further development of the motilin radioimmunoassay. Pilot studies were performed on the extraction of motilin from sera and tissue extracts by chromatography of the motilin-containing material on columns of Sepharose 4B, coupled to motilin antiserum. The results were favourable for the development of this technique on a larger scale, both for the concentration of motilin from sera and the isolation of the peptide from its starting material, Presekretin, in a more economical fashion than can be achieved by the serial stages of chromatography, at present in use (Fig. 51 and Fig. 52). Motilin, adsorbed to this matrix can be eluted by a lowering of the pH with no apparent damage to the molecule, as illustrated by the similarity of the standard curves obtained with ^{125}I- motilin, before and after treatment by affinity chromatography (Fig. 49).

Antisera to porcine motilin appear to crossreact completely with porcine, canine and human motilin. The fasting serum motilin concentrations in man, measured in 45 normal subjects in the age range 20-35 years, was 190 $\pm$ 131 pg/ml IR motilin (mean $\pm$ S.D.). The mean $\pm$ S.D. fasting serum motilin concentration in 8 dogs was 294 $\pm$ 44 pg/ml IR- motilin. When serum samples, containing exogenous or endogenous motilin, were assayed at several dilutions, the results obtained could be fitted to the standard curve, as shown in Fig. 10. This antibody would not therefore appear to differentiate between unlabelled antigen in the form of the standard or antigen in the form of the endogenous polypeptide, satisfying one of the basic requirements for the development of a sensitive radiomunoassay. Addition of 5000 cpm per tube of a radioactive tracer with a specific activity of 400 mCi/mg entails the addition of only 7 pg motilin and still allows an efficient rate of counting. The final absolute essential, a high affinity antiserum, has also been approached, and with these conditions satisfied, as laid down in the rationale, the other variables have been established at their optimal values.

The apparent homogeneity of IR-motilin, suggested by the serum dilution curves, was further examined by chromatography of either alkali-stimulated IR- motilin in serum or an impure duodenal extract on Sephadex G-50. As illustrated in Fig. 31 and Fig. 32, only one region of IR- motilin was detectable, eluting in the same position as ^{125}I- motilin.

The motilin content of 2 different preparations, one the synthetic analogue, 13-norleucine-motilin, the other an impure duodenal extract, was measured by bioassay and immunoassay, in comparison with natural porcine motilin. The natural and synthetic motilins were found to be identical in both biological

and immunological activity (Fig. 39 and Fig. 41). The natural polypeptide represented a 10,000 fold purification of the crude extract, measured by either bioassay or immunoassay, as illustrated in Fig. 29 and Fig. 30. These results and the apparent homogeneity of IR- motilin, suggest that the biological activity of motilin may be fairly closely correlated with its immunological activity, as measured by radioimmunoassay.

The IR- motilin of various regions of the hog gastrointestinal tract was measured in partially purified acid-ethanol extracts of the gastrointestinal mucosa. The region with the highest motilin content (per g dry weight of the extract) was the jejunum, followed by the duodenum and upper ileum, as summarized in Table XIV. No detectable motilin was found in the oesophagus, stomach or lower ileum. These findings agree with the results of Pearse et al (1974). Using the indirect sandwich technique, with motilin antiserum as the first layer,and fluorescein-labelled goat antirabbit IgG as the second layer, they were able to detect fluorescent motilin-containing cells in the duodenum, jejunum and upper ileum of the dog, pig, baboon and man. The cell of origin was identified as the enterochromaffin (EC) cell, 85% of the motilin-containing cells showing positive argentaffinity. The remaining 15% of the cells were argyrophyl. No EC cell in the stomach or lower intestine could be demonstrated as containing motilin. The EC cells of the upper gastrointestinal tract have also been shown to contain serotonin, substance P and melatonin, all of which are also found in neural tissue. Although the EC cell is unlike the other cells classified as APUD in that it does not derive from the neural crest, it does appear to originate from neuroectodermally derived tissue. The possibility should be investigated that motilin has neural connections. The relationship, if any, between motilin and the other substances of EC cell origin is not clear.

Differential staining techniques, applied to mammalian duodenal tissue, have demonstrated that motilin and substance P are present in different EC cells (Polak et al, 1976). Forssman et al (1976) confirmed that not all serotonin-containing cells in this region contained motilin but were unable to rule out the possibility that motilin - containing cells do contain serotonin. One must postulate that EC cells fall into subpopulations, classified by the polypeptide they produce.

The production of a synthetic analogue (Wünsch et al, 1973) permitted some insight into the relationship betweent the structure of motilin and its biological potency. The methionine residue at position 13 was originally thought to be essential for the expression of biological activity. When the methionine was oxidized by treatment with hydrogen peroxide, 95% of the biological activity was lost. Full potency was restored, however, after reduction of the residue with cysteine (Cook, 1972). During the synthesis leucine or norleucine were substituted for the methionine, because of the difficulties involved in a synthesis containing a central arginyl-methionyl bond. No loss in either biological or immunological activity was observed (Fig. 39 and Fig. 41) suggesting that it was not the methionine residue per se which was important in conferring full biological potency. It was more likely that some conformational change, which resulted from the oxidation of the sulphur-containing residue, was responsible for the loss of activity. In the course of the synthesis and purification of 13-norleucine-motilin, the intermediate compounds MoB_2 and MoC_2 were found to be inactive. MoB_2 resulted from a failed synthesis, in which the amino acids, threonine and tyrosine, at positions 6 and 7, were not incorporated. MoC_2 was determined to be a diastereomeric form of the active polypeptide, the

phenylalanine at position 5 being in the D-configuration, rather than the L-configuration found in the active molecule.

Differences were observed in the electrophoretic mobilities of the tryptic digestion products of the natural and synthetic motilins. The acidic tryptic peptide 3 (Tr 3), reported by Brown et al (1973) was absent in the synthetic material. When the tryptic digestion was repeated on freshly prepared porcine motilin, Tr 3 was isolated and subjected to electrophoresis at pH 6.5. It now ran as a neutral peptide. Kinetic studies with leucine aminopeptidase and a 3-cycle Edman degradation, indicated that the peptide contained glutamine at position 14 and not glutamic acid as originally stated. It must be supposed that deamidation of the residue had occurred during the early preparation of motilin (Schubert and Brown, 1974). This deamidation had no effect on biological activity.

Studies on the synthetic fragments 9-22 and 13-22 or on fragments of natural motilin produced by either chemical or enzymatic digestion have not resulted in the isolation of a fragment containing any significant biological or immunological activity. The immunological activity observed after cyanogen bromide cleavage of motilin, seen in Fig. 40, can be accounted for by the presence of uncleaved material remaining in the reaction mixture.

Modification of the naturally-occurring molecule by acylation drastically reduced the potency of motilin. Acetylation neutralized the positive charges of the ε -amino groups and the N-terminal phenylalanine, whilst succinylation produced a net negative charge on the molecule. One cycle of the Edman's degradation procedure also resulted in a loss of biological potency. It is

unclear whether this was due to the loss of the N-terminal aromatic residue, phenylalanine, or to the acylation of the Σ-amino groups on the lysine residues, caused by exposure of the molecule to phenylisothiocyanate. The loss of activity associated with the change in the net charge on the molecule would suggest that motilin binds to its receptor site by formation of ion pair bonds. The biological activity of motilin was seemingly unimpaired by the removal of the C-terminal and penultimate amino acids, after treatment with carboxypeptidase A. It should be remembered, however, that this is not a complete degradation procedure and until synthetic peptides 1-20 and 1-21 can be prepared, no firm conclusions may be drawn, regarding the importance of these amino acids in the biological activity of motilin. From these results, summarized in Table XV, it must be concluded that virtually the intact molecule is required for the expression of full biological potency. The individual amino acids are important inasmuch as they contribute to the charge distribution and probable confirmation of the molecule.

Brown et al (1966) reported an increase in the motor activity of an extrinsically denervated or transplanted pouch of the fundus of the stomach after duodenal infusion of isotonic alkali or fresh pig pancreatic juice, sufficient to raise the pH from the basal level of 7.5 to 8.2. Intravenous infusion of a pure polypeptide, isolated from hog duodenal mucosa, mimicked this response. No other gastrointestinal polypeptide has been discovered which will produce the reported increase in fundic motor activity, gastrin and cholecystokinin-pancreozymin having their motor effect only on the antrum of the stomach. The development of a radioimmunoassy, specific for motilin, confirmed the supposition that the increased fundic motor activity observed after duodenal alkalinization was accompanied by a concomitant increase in the circulating levels of IR- motilin,

comparable to those achieved during exogenous administration of the polypeptide (Fig. 34). An increase in serum IR- motilin levels was also reported by Hellemans et al (1976) after instillation of bicarbonate into the antrum of the stomach in man, but no such response was detected if the perfusate was sodium hydroxide. Mitznegg et al (1976) were unable to detect any increase in circulating IR- motilin levels after intraduodenal Tris buffer, pH 10.2, in human volunteers. They claimed a fall in plasma motilin levels occurred after duodenal alkalinization but examination of their results failed to reveal that the depression of IR- motilin levels was significant in view of the variation in IR- motilin levels measured during the pre-infusion control period. Control studies in dogs, with an intraduodenal infusion of 0.15M saline showed no increase in fundic pouch motor activity and no change in IR- motilin from the basal levels, throughout the duration of the experiment, as shown in Fig. 35. Similar findings in man have been reported by Mitznegg et al (1976).

In two experiments, the dogs exhibited spontaneous increases in fundic pouch motor activity, very similar to those obtained after alkali. An increase in IR- motilin accompanied this increased motor activity. The cause of this motilin release is unclear. The activity occurred after the dogs had exhibited a period of normal basal motor activity for at least 20 mins. and was therefore unlikely to be caused by either distension of the fundic pouch whilst it was being filled with water or by insertion of the Foley catheter into the Mann-Bollman fistula.

A somewhat anomalous observation was that duodenal acidification appeared to produce an increase in serum IR- motilin in dogs. This elevation was less than that seen after duodenal alkalinization, but was more prolonged, as illustrated in Fig. 37. No increase in fundic pouch activity was observed in these animals. This result was unexpected in view of the finding by Brown et al (1967) that intraduodenal acid suppressed alkali-induced fundic pouch motor activity. Recent studies by Itoh et al (1976) supported this observation. They demonstrated that the inter-digestive pattern of gastric motor activity in the fundus, antrum and lower oesophageal sphincter in dogs was exactly mimicked by infusion of 0.1 - 2.7 μg/kg/hour synthetic motilin (13-methionine-motilin) when compared with respect to duration, amplitude and frequency of the contractions, and their velocity at different distances along the gastrointestinal tract. This interdigestive pattern, whether natural or motilin-induced, was interrupted by feeding, duodenal acidification or an infusion of pentagastrin. One possible explanation of these contradictory findings is that the substance released by duodenal acidification is not motilin, but some other substance which shares immunological but not biological identity with motilin. It has already been noted that although acylation almost completely abolished the biological activity of the molecule, approximately 50% of the immunological activity still remained (Table XXV). Investigation of this acid-released motilin-like immunoreactivity by electrophoresis or column chromatography would provide more information relative to any size or charge difference between it and the alkali-induced, motor stimulatory motilin. It is interesting to speculate whether the anti-motilin effect observed after duodenal acidification is due, in part, to competitive antagonism by this motilin-like material.

The physiological role(s) of motilin will remain a subject of controversy until a physiological secretagogue or other mechanism for its release can be demonstrated. The lack of any significant increase in the systemic levels of IR- motilin after ingestion of either glucose or a mixed meal (see Fig. 38) would suggest that motilin played little role in the normal digestive processes. The apparent increase in IR- motilin reported by Mitznegg et al (1976) after ingestion of fat, with the accompanying inhibition of fundic motor activity, may be due to the same motilin-like material released by duodenal acidification.

In 1966, when Brown et al first described the alkali-induced increase in fundic pouch motor activity, they postulated that, under these conditions, some humoral agent was being released which would counteract the inhibitory effect of the other gastrointestinal polypeptides, gastrin, cholecystokinin-pancreozymin (and, of course, GIP) which were released by the ingestion of various nutrients. Studies by Hoelzel (1925) and Reinke et al (1969) in dogs, indicated that the duodenal contents tended to an alkaline pH during the fasting periods. As previously described by Itoh et al (1976) exogenous motilin exactly reproduced the normal pattern of mechanical interdigestive activity. This consisted of bands of contractions, arising simultaneously in the fundus and duodenum and travelling aborally the length of the ileum. Each cycle took approximately 20 mins. to pass one point and consecutive cycles were about 90 mins apart. This pattern was interrupted by feeding. Preliminary studies have suggested that motilin levels were depressed initially after feeding, as shown in Fig. 38. The purpose of this interdigestive cyclic activity is postulated to be the cleaning from the digestive and absorbent, e regions of the upper gastrointestinal tract of the extra mucus and desquamated epithelial cells resulting from the processing of the previous meal. This "cleaning up" operation is

probably mediated in part by humoral means, in part by neural mechanisms. The appearance of a single cycle of activity at a time in the upper 70% of the gastrointestinal tract may be due to some inhibitory reflex between the upper and lower regions of the tract. The tachyphylaxis described by Cook (1972) as occurring when a second injection of motilin was given less than 40 mins after the first, might be explained by an inhibitory reflex of this type.

The fact that gastric alkalinization, with or without prior gastric acidification, has been reported as causing an increase in the pressure of the lower oesophageal sphincter (LESP) (Castell and Levine, 1971) raised the possibility that motilin was involved in the control of this region. The increased pressure was initially postulated to be the result of an increased gastrin output from the antrum. This was not however supported by the work of Debas et al (1974) or Kline et al (1975) who could show no increase in serum IR-gastrin after antral alkalinization. Jennewein et al (1975) measured changes in LESP in dogs after bolus injections or intravenous infusions of motilin over 30 mins. The most effective doses were 30 ng/kg or 100 ng/kg/hour respectively. The motilin resulted in increased activity in both the antrum and the fundus, and low frequency phasic activity in the LES, related to the activity in the fundus. When motilin was administered as an infusion, the phasic activity in the LES ceased within 10 mins of the end of the infusion. Duodenal alkalinization resulted in an increase (non-significant) in the LESP in 4 dogs. Similar results were obtained in studies in man, the LES responding to graded doses of the synthetic nor-leucine analogue in a dose-related manner. This response was depressed by infusion of atropine sulphate, suggesting a cholinergic involvement. (Rösch et al, 1976).

An attempt to correlate increased LESP with endogenous release of motilin was performed in normal subjects and patients who had undergone truncal vagotomy with Billroth I or Billroth II antrectomies (McCallum et al, 1977). The subjects ingested either 0.4M sodium bicarbonate or a commercial antacid preparation. Increased LESP was observed in the normal subjects and those with Billroth I anastomoses. No increase in either serum IR- motilin or serum IR- gastrin was detected in association with this pressure increase. The lack of any response in the Billroth II patients indicated the importance of the duodenum in eliciting this response. Hellemans et al (1976) were able to demonstrate a slight increase in IR- motilin after either gastric alkalinization or acidification. The motilin response to alkali was rapid in onset and slightly preceded the peak response in the LES. This finding was in agreement with the postulate of Jennewein that motilin exerted its effect on the LES indirectly, via its effect on fundic motor activity and gastric intraluminal pressure. The increase in intraluminal pressure in the fundus was then the direct cause of the increased LESP. In contrast, the motilin response to antral acidification was much slower in reaching its peak (approximately 45 mins.) and correlated directly with the peak LESP. These differences were not remarked upon by the authors. If the different rates of motilin release were due to different rates of passage of the acid or alkali into the duodenum, the authors' postulate that motilin, like acid, has a role in the inhibition of gastric emptying loses support.

Increased LESP after exogenous administration of low doses of natural or synthetic motilin has been conclusively demonstrated in both dogs and man. The results obtained after endogenous released motilin are far less conclusive. Direct instillation of alkali into the antrum or duodenum has been shown to

result in an increased LESP, which correlated with the release of IR-motilin. However, the increase in LESP associated with oral ingestion of alkali could not be related to any detectable increase in IR- motilin. This finding does not necessarily preclude motilin from playing a role in regulating LESP. It may exert its effect at levels not detectable by radioimmunoassay.

In 1967 Brown and Parkes postulated the existence of a duodenal, pH dependent, dual hormonal mechanism for the control of gastric motor activity. In such a mechanism, low duodenal pH would inhibit gastric motor activity and delay gastric emptying, by the release of some humoral agent, whilst a high duodenal pH would increase gastric motor activity and possibly increase the rate of gastric emptying. The effect of motilin on the rate of gastric emptying was investigated in dogs equipped with gastric fistulae. Intravenous infusion of porcine motilin was found to accelerate the emptying of a neutral test meal (Fig. 53). It had no effect on the rate of emptying of solids (Fig. 54). The site of action of the motilin during the emptying of the liquid was determined to be the fundus, antrectomy having little effect on the response, as shown in Fig. 56. The decrease in sensitivity of this response to motilin after truncal vagotomy (shown in Fig. 55) suggested that the response depended on some interaction between motilin and a cholinergically-mediated neural reflex, at the level of the fundic musculature (Debas et al, 1977). These results were in direct contradiciton to those obtained by Ruppin et al (1975) in man. They described a decrease in the rate of gastric emptying of an acid test liquid, after an infusion of 13-norleu-motilin. The lack of any effect on the rate of emptying of the solid meal may be due to an over-riding or an inhibition of the motilin-induced response by the humoral factors released from the duodenum by the constituents of that meal. As yet, an increased

release of endogenous motilin has not been demonstrated to be part of the pattern associated with increased rates of gastric emptying, and the physiological importance of this action is uncertain.

Investigation of the mode of action at the cellular level has involved the development of an in vitro preparation for the assay of motilin activity. Domschke et al (1976) have reported the development of such a preparation utilizing either rabbit duodenal muscle or strips of human fundus. They were able to demonstrate a dose-related contractile response to natural or synthetic motilin. This response was unaffected by ganglionic blockade, axonal blockade, atropine or antihistamine, suggesting that motilin acted at a receptor on or in the muscle cell. Motilin may exert its effect on muscle by influencing the transport of calcium (Ca^{++}) within the cytosol. The action of motilin was blocked by the Ca^{++} antagonist, verapamil. Increased cyclic guanosine 3'5' monophosphate (cGMP) levels, associated with the rapid release of Ca^{++} from microsomal fractions, were observed during the response to motilin.

Strunz et al (1976) found that the response of isolated rabbit pyloric muscle to acetylcholine was enhanced by pretreatment with subthreshold levels of motilin. This augmentation was not associated with either increased acetylcholine synthesis or decreased acetylcholine degradation. The doses of motilin required to stimulate the in vitro preparations were relatively larger than those which promoted gastrointestinal motility in the intact animal. It is possible that circulating, subthreshold levels of motilin in the intact animal may be contributing to the cholinergically-maintained tone in the gastrointestinal musculature and that functionally significant changes in serum IR- motilin levels

are not detectable in the radioimmunoassay system. A second possibility is that motilin is not transported to its target cell via the circulation but is merely passed via the extracellular fluid to the adjacent cell where it acts in a paracrine fashion. The inability of other groups to establish an in vitro assay using tissue from rats, guinea pigs and dogs can only be explained at this time by species differences.

It is now suggested that there is no evidence that motilin normally plays any physiological role at the levels of IR- motilin achieved by infusion of 1 µg/kg/hour of polypeptide or after duodenal infusion with alkali at pH 10.2. The hypothesis is that the role of motilin is to maintain the interdigestive pattern of gastric motor activity, the effective levels being much lower than those achieved during these experiments. A study of IR- motilin levels during a continuous recording of the interdigestive and digestive patterns of motor activity would help confirm or deny this supposition. A second question which should be answered is whether motilin is acting independently in controlling contractile activity or whether it is acting in concert with some other humoral or neural reflex, probably cholinergically-mediated. If this is the case, the effective increases in motilin levels may only occur at the cellular level and may never be reflected by changes in the systemic levels of the polypeptide. The physiological stimulant for motilin release would be the rise in duodenal and jejunal pH occurring in the fasted state. The absence of increased systemic levels of IR- motilin associated with any specific part of the digestive cycle and the lack of evidence regarding the existence of any hypersecretory syndrome set motilin apart from the other known gastrointestinal hormones and would suggest that motilin may be acting as a local hormone or in a paracrine manner, rather than as a classical endocrine hormone.

The polypeptide isolated from the intestinal mucosa of hogs by Brown et al (1969) was named gastric inhibitory polypeptide (GIP) because of the early observation that the pure porcine material, infused intravenously in dogs, inhibited stimulated gastric acid secretion, in a dose related manner (Pederson and Brown, 1972). A second, at least equally important, biological activity of this polypeptide was demonstrated by Dupre et al (1973). They infused porcine GIP in men and were able to measure an enhanced insulin response to an intravenous glucose infusion. Pederson et al (1975A, 1975B) confirmed this finding in dogs. Their results would suggest that GIP might also be interpreted as glucose-dependent insulinotropic polypeptide (Brown and Dryburgh, 1977). A radioimmunoassay, specific for GIP, was developed by Kuzio et al (1974) to determine the physiological secretagogues for GIP and the relative importance of the endogenously-released polypeptide in gastric acid secretion or insulin release under normal or pathological conditions.

Antisera to GIP have been obtained in guinea pigs and rabbits after immunization with GIP conjugated to BSA. GIP would not appear to be a good immunogen, no satisfactory antisera having been produced in animals immunized with non-conjugated GIP, and even the conjugate has produced good antisera in only a small percentage of the animals injected. All the studies reported from this laboratory, up to the present time, have been based on a radioimmunoassay utilizing one antiserum, Van 8, and the assay conditions have been established to produce the most sensitive assay for this particular antiserum. When an alternative antiserum, Ro7, was introduced, the assay system had to be changed to the disequilibrium type to achieve a satisfactory degree of label displacement, as shown in Fig. 59.

When it was revealed that IR-GIP did not exist in a homogeneous form, the crossreactivity of the available antisera was examined with the various heterologues of IR-GIP. The original antiserum, Van 8, crossreacted with 3 different immunoreactive forms of GIP: GIP_{Vo}, GIP_{5000} and ProGIP (Fig. 72 and Fig. 73) and antiserum GB 5 behaved in a similar fashion. Antiserum Ro7 and antiserum Van 8 crossreacted with GIP_{5000} in the interassay control preparations to the same degree, but did not agree in the measurement of ProGIP, as illustrated in Fig. 80. Antiserum Ro7 appeared to have a greater affinity for this form of IR-GIP than did antiserum Van 8. The total IR-GIP response to any stimulus consists of varying proportions of at least 3 components, as determined by these antisera. It is obvious that comparisons between IR-GIP responses to stimuli may only be made if the studies are performed with antisera whose behaviour with the various IR-GIP heterologues is also comparable, or better still, with the same antiserum throughout.

The usual technique used in the iodination of GIP has been a modified version of the chloramine-T method. In an attempt to reduce the damage done to the GIP molecule by the oxidising agent, pilot studies were performed, varying the concentration of chloramine-T and the length of the exposure time. The specific activity of the resulting label, calculated from the percentage incorporation of ^{125}I into the molecule was 350 mCi/mg when GIP was exposed to a chloramine-T concentration of 4 µg for 120 sec. This compares well with the routine label, whose specific activity, calculated in the same way, was 120 mCi/mg, (from the radiochromatogram in Fig. 21). An improved label is likely to result from further studies with reduced concentrations of chloramine-T and varied periods of exposure. It should be noted that this method of calculating the specific activity is much less reliable for GIP than it was for motilin. With motilin there was little difference in the values obtained, whether they were calculated

from the radiochromatogram or from a separate assay, requiring the addition of label at several dilutions. This is not the case with GIP. The label obtained in the procedure illustrated in Fig. 21 had an apparent specific activity of 120 mCi/mg. The same label, after a label dilution assay, had an estimated specific activity of 68 mCi/mg. This indicates that a smaller proportion of the labelled polypeptide eluting in the first peak represented mono-iodinated ^{125}I-GIP. The molecule has two tyrosyl residues, one N-terminal, the other at position 10. At present, there is no way of knowing which tyrosine is the more available to the incorporation of iodine, or whether the tertiary structure of the molecule is such that the N-terminal portion of the peptide is indeed readily accessible to the incorporation of iodine at all. The lack of improvement in the specific activity of the label after the gentler lactoperoxidase method of iodination would suggest that neither tyrosine is readily accessible to iodine incorporation.

Purification of the label by gel filtration on Sephadex G-25 will separate the iodinated polypeptide from the unincorporated isotope but fails to separate the labelled and unlabelled peptides from each other, and is barely adequate for separation of mono-iodinated GIP from the di- and tri-iodinated forms. The presence of uniodinated GIP in the labelled preparation is a contributing factor to the low specific activity, blunting the sensitivity of the upper end of the standard curve and limiting the concentration of labelled antigen which may be added to the assay. Subsequent ion exchange chromatography on QAE Sephadex A-25 of ^{125}I-GIP, initially isolated by gel filtration, has produced an iodinated GIP with a specific activity in the range 200-250 mCi/mg (estimated by the label dilution method). This improvement is probably due to the removal of the unlabelled polypeptide, which elutes

ahead of the labelled material in the system employed, (Fig. 22). The routine inclusion of interassay controls in the assay have made it easier to monitor the performance of the assay system and acts as a reliable index for the estimation of the effect of variations in procedure on the sensitivity of the assay.

Antisera to porcine GIP, raised in rabbits, were covalently coupled to Sepharose 4B and tested as an alternative to dextran-coated charcoal in the separation of bound antigen from free antigen in the incubation mixture. The binding of the antibodies to the agarose was complete, as indicated by the lack of antibody activity in the wash, (Fig. 19). The coupled antiserum, tested in the radioimmunoassay, appeared to possess only a small percentage of the original antibody activity. This was indicated by the reduction in the titre of the coupled antiserum, required to produce a maximum binding of 30% of the labelled tracer, shown in Fig. 46. The coupled antiserum at this lower titre also showed a diminished sensitivity to the addition of unlabelled antigen. (Fig. 47)

The results obtained with Sepharose-coupled antisera to the steroid hormones and the low molecular weight polypeptide hormones, e.g., gastrin, would suggest that the antibody activity, in the radioimmunoassay system, was unaltered by the presence of the solid matrix or the coupling process (Bolton and Hunter, 1973). The Sepharose-coupled antiserum in the radioimmunoassay for motilin, another small polypeptide, also demonstrated a high recovery of antibody activity and no loss in sensitivity potential. The same was not true for insulin, human growth hormone, human thyroid stimulating hormone or GIP. All these radioimmunoassay systems showed a loss in antibody activity and sensitivity when the antibody was coupled directly to the Sepharose matrix. Bolton and Hunter suggested that there was a critical size of antigen, above which steric hindrance prevented the molecule

from having complete access to the binding sites on the antibody. This critical size must lie between 2700 (motilin) and 5105 (GIP). Direct coupling of GIP antisera to Sepharose was an uneconomical way of utilising the antisera, and this technique has not been used in studies on the extraction and purification of GIP.

This problem may well be overcome by interposing a hydrocarbon chain between the ligand and the solid matrix. The use of such a hydrocarbon spacer has dramatically improved the effectiveness of several Sepharose systems in the purification of enzymes (Cuatrecasas, 1970), especially in low affinity systems. The reaction involves the coupling of the spacer, such as ethylene diamine or the tripeptide GLY-GLY-TYR to the activated Sepharose, followed by the coupling of the ligand to the spacer by the carbodiimide reaction. The increase in distance between the solid and the antibody reduces the steric hindrance imposed by the presence of the matrix and increases the flexibility and mobility of the ligand.

It would be advantageous to pursue the possibility of a coupled ligand for GIP for the following reasons: The separation procedure in the routine assay becomes more rapid, entailing no extra addition step, as in the dextran-charcoal separation, and no further incubation, as in the double antibody system. Centrifugation for 5 min. at 2000 rpm is adequate for packing the solid, allowing the supernatant liquor to be decanted. The system is unaffected by the plasma concentration in the normal radioimmunoassay and is less disruptive to the primary antigen/antibody reaction than charcoal addition. Reduction of the incubation volume to a minimum obviated the need for rotation of the incubation tubes and therefore they do not require stoppering.

With the advent of a radioimmunoassay it became possible to investigate the physiological function of GIP, firstly as an inhibitor of gastric acid secretion and strong candidate for the role of enterogastrone. The term "enterogastrone" was defined by Kosaka and Lim to describe the humoral agent postulated to be released from the duodenal mucosa by fat or fat digestion products and responsible for the inhibition of gastric acid secretion and the delay in gastric emptying. Pederson and Brown (1972) demonstrated that porcine GIP was effective in dogs in inhibiting gastric acid secretion, whether that secretion was stimulated by infusion of pentagastrin or histamine, or by vagal stimulation (induced by insulin-mediated hypoglycaemia). In studies where the extrinsically denervated fundic pouch was stimulated to produce ~75% of its maximum secretory capacity, a significant degree of inhibition was observed at doses of 1 µg/kg/hour.

When serum samples, obtained from human volunteers after ingestion of a normal breakfast, were subjected to radioimmunoassay, they were found to rise from a mean fasting level of 237 $\pm$ 14 pg/ml IR-GIP (mean $\pm$ SE) to a mean level of 1200 pg/ml IR-GIP, and they remained elevated above basal levels for periods in excess of 3 hours, (Kuzio et al, 1974). When the various components of the meal were tested individually, oral ingestion of both glucose (Cataland et al, 1974) and fat, in the form of a triglyceride emulsion (Brown et al, 1974) were found to produce a significant elevation in the circulating levels of serum IR-GIP. Ingestion of protein, in the form of either a meat extract or a fat-trimmed filet steak, produced no such increase in the circulating levels of IR-GIP (Brown et al, 1975). When the original studies were duplicated in dogs, the circulating levels of IR-GIP, achieved during the exogenous infusion of GIP, sufficient to produce a significant inhibition of the gastric acid output, were determined to lie within the range of serum IR-GIP levels released by ingestion of fat. Similar IR-GIP responses were obtained in dogs when the stimulus, either fat or glucose, was

administered as an intraduodenal infusion.

Confirmation of the inhibitory action of endogenous GIP was obtained in studies in dogs. Acid, stimulated by a continuous infusion of pentagastrin, was inhibited by intraduodenal infusion of fat (Fig. 66), glucose (Fig. 67) or hydrochloric acid (Fig. 68) but not saline (Fig. 69). The inhibition of fundic pouch acid secretion by fat or glucose was accompanied by a concomitant elevation of serum IR-GIP. There was no significant change in IR-GIP levels related to the acid-induced inhibition and intraduodenal perfusion with saline produced neither inhibition of gastric acid secretion nor change in the serum IR-GIP levels. It would seem that GIP is, in strong likelihood, the enterogastrone postulated by Kosaka and Lim, according to the evidence obtained in dogs. The evidence in man, regarding the inhibitory role played by GIP released by fat digestion, is less strong. Cleator and Gourlay(1975) found that exogenous GIP would inhibit gastric acid secretion in man at a dose of 2 µg/kg/ 30 min. which resulted in circulating IR-GIP levels well above those achieved by ingestion of fat in the same subjects.

GIP does not appear to play any part in the autoregulation of gastric acid secretion by duodenal acid. This finding was supported by the lack of any IR-GIP response to ingestion of protein or alcohol, or the passage of pentagastrin-stimulated acid into the duodenum (Cleator and Gourlay, 1975).

The evidence supporting the claims of the other gastrointestinal polypeptides to be enterogastrone has gradually been diminished. Secretin is not released in any significant amounts by ingestion of fat, and has been shown to have little inhibitory effect on the secretory or motor activity of the stomach when infused in

doses which mimicked the circulating levels of IR- secretin achieved by duodenal acidification. Cholecystokinin-pancreozymin is released by fat, but much of the inhibitory activity reported as occurring after infusion of the polypeptide can be accounted for by the GIP contamination in the impure preparation of cholecystokinin-pancreozymin used in these studies. A third possible enterogastrone, VIP, is a potent inhibitor of gastrin- or histamine-stimulated acid secretion, when it is administered exogenously (Barbezat and Grossman, 1971) but no mechanism for the physiological release of VIP has yet been described. GIP may not be the only enterogastrone but it would appear to be a major factor in the humoral reflex so designated. The actual mechanism of GIP release after ingestion of fat remains to be elucidated. The reduced IR-GIP response to a test meal in patients with coeliac disease would suggest that the rate of absorption of the nutrients is an important factor. As yet, no significant reduction in the absolute number of GIP-producing cells has been detected in biopsy samples from the jejunal mucosa of these subjects (Creutzfeldt et al, 1976).

The search for the duodenal factor involved in a second humoral reflex dates back to the work of Moore et al (1906). They were able to relieve the glycosuria of patients with diabetes mellitus by orally administering an extract of the duodenal mucosa. A hypoglycaemic fraction was separated from a crude secretin preparation. It had no secretin-like effect on the exocrine pancreas and was not insulin. This fraction was named "incretin" by La Barre (1932) when he postulated a possible role for this duodenal factor in the treatment of diabetes mellitus.

With the advent of the radioimmunoassay for plasma insulin (Yalow and Berson, 1958) it became possible to compare the IR- insulin response to glucose adminis-

tered orally or intravenously (Elrick et al, 1964). The greater insulin response and improved glucose tolerance which accompanied glucose administration by the oral route was postulated to be due to the release of some insulotropic factor from the duodenal-jejunal mucosa (McIntyre et al, 1965). This postulated intestinally-mediated regulation of endocrine pancreatic function has been termed "the enteroinsular axis".

If a gastrointestinal polypeptide is to be seriously considered as a candidate for the role of incretin in this enteroinsular reflex it must satisfy the following criteria. It must be demonstrated that glucose is a stimulant for its release. It should be shown that exogenous administration of this polypeptide, in doses achieving circulating immunoreactive levels within the physiological range, administered in parallel with an intravenous glucose load, will mimic the pattern of serum IR- insulin release and glucose tolerance observed after oral or duodenal administration of glucose alone. In light of these requirements most of the established gastrointestinal peptides have been ruled out as possible candidates. Secretin and cholecystokinin-pancreozymin are not released by ingestion of glucose in physiologically effective levels, as demonstrated by the lack of effect on the exocrine pancreas (Mahler and Weisberg, 1968) whilst only a slight elevation in serum IR- gastrin levels were observed (Rehfeld and Stadil, 1973). When gastrin, secretin (Lerner and Porte, 1972) or the synthetic octapeptide of cholecystokinin-pancreozymin (Frame et al, 1975) were infused intravenously, in conjunction with intravenous glucose, all three peptides produced a transitory, enhanced insulin response, characteristic of the initial phase of insulin release. The response was over within 10-15 min. even when the polypeptide infusion was continued.

In 1973, Dupre et al measured the IR- insulin response in normal volunteers to intravenous infusion of 0.5 g/min glucose alone, glucose infusion with the addition of 1 μg/min pure porcine GIP and GIP infusion alone. The addition of GIP resulted in an enhanced IR- insulin response to the glucose infusion, during both the initial phase of insulin release and the later sustained phase. This same dose of GIP, without the glucose, had no insulinotropic action. The levels of circulating IR-GIP reached during this infusion were comparable to those achieved in the same subjects after the ingestion of 50 g glucose. The effect of endogenous GIP on insulin release in man was reported by Brown et al, (1975). The IR- insulin response to intravenous glucose was potentiated by GIP released after the ingestion of fat, in the form of a triglyceride emulsion.

Although GIP- mediated insulin release has been demonstrated in the fasted dog (Pederson et al, 1975b) it is probable that the effective levels of IR-GIP achieved were pharmacological rather than physiological. In man, some degree of hyperglycaemia was essential if physiological levels of GIP were to be insulinotropic. Studies in the isolated rat pancreas preparation have confirmed that GIP is capable of augmenting the sustained insulin response to glucose, in a dose-related manner, (Pederson and Brown, 1976). Their findings suggest that GIP is only effective as an insulinotropic agent in the presence of a glucose concentration which is itself capable of stimulating insulin release from the pancreas. In the presence of 8.9 mM glucose, GIP was effective in doses as low as 1 ng/ml perfusate and in the presence of a fixed GIP concentration, increasing glucose concentrations stimulated insulin release in an exponential manner.

GIP has also been shown to potentiate the insulin response to glucose in the isolated rat pancreatic islet preparation (Schauder et al, 1975) at glucose concentrations above a threshold level, which lay between 6-8 mM. The effective dose of GIP, however, was 10 µg/ml incubate at the lower glucose concentration, and 1 µg/ml at the higher concentrations. The reason for this much greater GIP requirement in this preparation, compared to that of the isolated pancreas or the intact animal, is not degradation of GIP during the incubation period. It is possible that exposure of the islets to collagenase and pancreatic proteolytic enzymes, during their isolation, causes some alteration to their membrane structure and reduces their sensitivity to the action of GIP. An alternative explanation is that GIP requires the presence of some intermediate for full expression of its biological potency and this substance has been destroyed or lost in the isolation procedure. In both in vivo and in vitro systems GIP has satisfied the criteria for its establishment as a major factor in the entero-insular axis. Teleologically, it is desirable that GIP should have no insulinotropic action in the fasted animal. It would be inappropriate for insulin to be released when serum glucose levels were not elevated.

In 1973, Raptis et al reported that an intraduodenal infusion of a mixture of amino acids was a stimulant for insulin release, whilst intravenous infusion of these same acids was far less effective. This same intraduodenal amino acid perfusate was found to be a stimulant for GIP release, whereas an intravenous infusion resulted in no detectable IR-GIP production, although the serum α-amino nitrogen levels achieved were much higher, (Thomas et al, 1976). In this situation GIP was shown to be insulinotropic in the absence of measurable hyperglycaemia and the authors suggested that GIP will also act to enhance the

pancreatic insulin response in the presence of hyperaminoacidaemia. However, they did not measure serum IR- gastrin levels in this study and it is possible that the gastrin response to an amino acid infusion would contribute to the insulinotropic response observed after protein ingestion.

Evidence supporting the claims of GIP to be the major humoral factor in both the enterogastrone and entero-insular reflexes is gradually accumulating. IR-GIP released after ingestion of a mixed meal demonstrated a biphasic pattern, with the early peak occurring approximately 45 min. after ingestion of the meal, and a second, more prolonged response being seen between 120-180 min. (Brown et al, 1975). Ample evidence exists relating the initial response to the glucose content of the meal, correlating it with the increase in serum glucose and the period just prior to the peak response of IR- insulin. The second peak compares well with the IR-GIP response to orally administered fat. The GIP released by either fat or glucose appeared to be effective as an enterogastrone or an insulinotropic agent. However, if the serum glucose and IR-GIP levels achieved after oral glucose were duplicated by an intravenous glucose infusion with either intraduodenal fat infusion or intravenous porcine GIP infusion, the IR- insulin response after fat was significantly lower than that produced by either oral glucose or exogenous GIP, (Pederson et al, 1975B). From the teleological angle it is desirable to have an immediate insulin response to a carbohydrate-containing meal but it would be most inappropriate for the gastric acid secretion to be inhibited this early in the digestion of the meal. In light of these findings it seemed advisable to investigate the nature of the IR-GIP released after these different stimuli.

The antiserum routinely used in the GIP assay, Van 8,was unable to differentiate

between the serum IR-GIP released by fat or glucose (Fig. 71) but if these same serum samples were subjected to chromatography on Sephadex G-50, three regions of immunoreactivity were detected in the fractions obtained, (Fig. 72 and Fig. 73)(Dryburgh and Brown, 1976). The first region ($IR\text{-}GIP_{vo}$) eluted in the void volume of the column and was significantly diminished if the serum was pretreated by boiling or with 6.0M urea. (Fig. 77) This would suggest that GIP_{vo} represented a complex formed by the non-specific binding of GIP to a serum protein. A second region corresponded to the elution pattern of natural porcine GIP (GIP_{5000}) and a third immunoreactive region eluted ahead of the normal GIP and was determined to have a molecular weight of 7500-8000, as illustrated in Fig. 83. This form of IR-GIP was designated ProGIP. No attempt has yet been made to examine the relative ratios of GIP_{vo}, ProGIP and GIP_{5000} in fasting serum. The early GIP response to either glucose or fat was characterized by the proportions of the IR-GIP components bearing the relationship $GIP_{vo} > GIP_{5000} >$ ProGIP. As the stimulation was continued, the percentage of GIP_{vo} remained relatively constant, whilst that of GIP_{5000} increased. Still later, the relative proportions of GIP_{5000} and ProGIP were reversed. An all studies, in man or dog, after glucose or fat, the percentage of the total IR-GIP represented by ProGIP increased with increase in time after the stimulus, as typified in Fig. 75.

Chromatography of partially purified extracts from the duodenal mucosa of hogs demonstrated that they also contained IR-GIP in the ProGIP and GIP_{5000} forms. The highest ratio of ProGIP : GIP_{5000} was found in the neutral soluble, methanol insoluble extract (Fig. 78). Attempts which have been made to purify ProGIP from this extract have not been successful. Rechromatography of material, supposedly containing no GIP_{5000}, resulted in approximately 30% of the total IR-GIP recovered being in the GIP_{5000} form, as shown in Fig.82,

indicating that ProGIP had yet to be isolated in a stable form. From its behaviour on CM-cellulose, ProGIP was determined to be less basic than GIP_{5000}, (Fig. 80). One might expect a functionally different molecule to be more stable than ProGIP has so far proved to be. It is possible that this molecule represents a precursor form of GIP_{5000}. In this case, the initial IR-GIP response to any stimulus might then consist, predominantly, of already pre-formed GIP_{5000}. As the stimulus persisted the IR-GIP response would gradually change to contain increasing amounts of the precursor form, as the preformed pool of GIP_{5000} diminished and increased precursor was released as the synthesis of GIP accelerated. Biosynthesis studies, with tritiated amino acids, would help to answer some of these questions about the actual rate of GIP synthesis, under various conditions, and might also elucidate the relationship between GIP_{5000} and ProGIP. An alternative explanation for this phenomenon might be that the different forms of IR-GIP are being produced by different populations of APUD cells, spatially separated. This hypothesis would require that GIP_{5000} be synthesized and secreted by cells predominantly located in the upper region of the duodenum, whilst the ProGIP cells would be situated more distally. As the stomach contents pass into the upper intestine they will initially stimulate primarily GIP_{5000}- containing cells. Later this mixture of nutrients would come into contact with the lower, ProGIP-producing cells. The biological potency of ProGIP, relative to that of GIP_{5000} is difficult to estimate in view of the instability of the material.

A second unknown factor is the biological potency of that proportion of the total IR-GIP complexed to serum protein. This phenomenon has been demonstrated with other polypeptide hormones, e.g., gastrin (Yalow and Berson, 1972) and insulin (Sramakova et al, 1975). The function of this type of complex was

studied by Simon and Antoniades (1975). They measured the transport of insulin across the isolated rat mesentery, in the presence of human serum-bound insulin. They found that the insulin transport was inhibited in a specific manner. One might extend this finding to postulate that naturally-occurring serum protein/polypeptide complexes would limit the transport of the polypeptide across the membrane of the target cell or reduce its activity at that membrane by competitively binding with the receptor sites thereon. The complex might also act by sequestering, temporarily, some of the polypeptide in the serum. "Big, big" gastrin was found to be a major component of the total IR- gastrin in the fasting serum of men, dogs and pigs. Its release was not stimulated by feeding (Yalow, 1974), leading one to suspect that it did not play an active role in the gastrin-mediated acid response to feeding. The existence of a similar "big, big" insulin in normal subjects is less well documented. It has, however, been demonstrated to comprise a large percentage of the total IR- insulin in the fasting and stimulated serum in certain pathological conditions, (not insulinomas). These patients have extremely high basal and stimulated IR- insulin levels but rarely experience hypoglycaemic attacks after prolonged fasting or limited food intake. The most common time for hypoglycaemia to occur in these subjects was a few hours after a substantial meal. Sramkova et al (1975) postulated that these findings could be accounted for if the IR- insulin was predominantly in an inactive form, the hypoglycaemic attacks being due to disruption of this complex and the liberation of the biologically active insulin after the stress of the large meal. In the light of these observations, it was postulated that GIP_{vo} is either biologically inactive or has reduced potency. The biological activity of GIP would not then correlate directly to the total IR-GIP response measured. Bearing this in mind, the experiments performed by Pederson et al, comparing

the IR- insulin response to matched serum glucose and IR-GIP levels, obtained by various means, were reconsidered. The peak levels of IR-GIP and the integrated insulin response achieved after intravenous glucose and porcine GIP were arbitrarily considered to be 100% of the possible response. The various IR-GIP components of the total IR-GIP response to oral fat were determined by column chromatography and were expressed as percentages of the total IR-GIP response. The integrated IR- insulin response achieved after intravenous glucose and oral fat was expressed as a percentage of the integrated IR- insulin response obtained with intravenous glucose and GIP. When these values were plotted in histogram form (Fig. 84), the closest correlation to the biological activity (i.e., the insulin response) was obtained by combining the IR-GIP_{5000} and IR-ProGIP, and ignoring the IR-GIP_{vo} component. (Fig. 84)

The existence of several molecular forms of IR-GIP cannot yet account for the apparently different functions of GIP, i.e., its initial incretin-like effect and the later enterogastrone effect. Another, as yet unexplained, phenomenon is the reduced IR-GIP response stimulated by oral fat in the presence of an intravenous glucose infusion, compared to that produced by oral fat alone, (Crockett et al, 1976). The answer to these problems may lie in a study of the other humoral mechanisms acting at the same time, or in the identification of some factor which inhibits the action of GIP at the level of either the parietal or the pancreatic β cells. The well documented effect of intravenously administered somatostatin on the pancreatic secretion of both insulin and glucagon *in vivo*, (Alberti et al, 1973 : Mortimer et al, 1974 : Koerker et al, 1974) and *in vitro*, (Gerich et al, 1975) stimulated interest in the possible effects of somatostatin on the insulin response to GIP and on GIP release after physiolo-

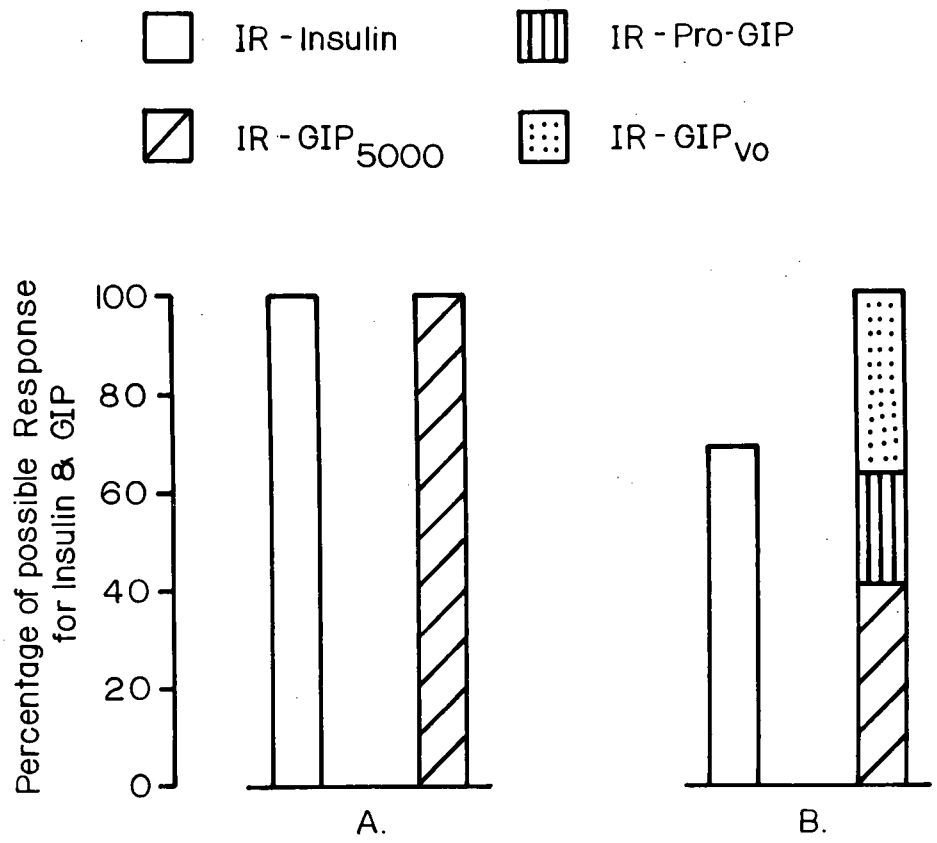


Fig. 84 The insulin response and serum IR-GIP levels associated with that response, showing the relative proportions of the different heterologues of IR-GIP. The serum glucose and IR-GIP levels were comparable after (A) i.v. glucose and i.v. GIP infusions (considered as producing 100% of the possible response) and (B) i.v. glucose and oral fat administration.

gical stimulation. Somatostatin was administered as a bolus injection, immediately prior to the exogenous administration of an intravenous GIP infusion, normally insulinotropic in the fasted dog (Pederson et al, 1975). The insulin response was delayed and the serum glucose values measured reflected this insulin inhibition, as shown in Fig. 63. The effect of a bolus injection of the synthetic somatostatin was to delay the release of IR-GIP, stimulated by either oral glucose or fat. In the case of the studies with oral glucose the insulin response was also delayed (Fig. 60 and Fig. 62). If the somatostatin was administered as an infusion, the IR-GIP and IR-insulin responses were delayed until the end of that infusion.

The IR-GIP response was also diminished, when compared to the control values achieved after oral glucose alone. The insulin response, however, rebounded to values significantly above the control values, a phenomenon not explicable in terms of the prevailing serum glucose levels (Fig. 61). A similar rebound response of insulin was observed by Mortimer et al, (1974) and the same phenomenon was observed with gastrin (Bloom et al, 1974), when an infusion of somatostatin, administered during the ingestion of a provacative meal, was terminated. Somatostatin, therefore, appears to block the endogenous release of GIP and also to inhibit the action of circulating GIP at the level of the cell.

The effect of somatostatin on GIP- mediated gastric acid inhibition has not been examined but the presence of somatostatin-containing cells in the gastric mucosa has been demonstrated by Dubois (1975). It would not be unexpected if intravenous infusion of somatostatin was also found to have a modulating influence on the response of the parietal cell to endogenous GIP. The possibility should be considered that the disparate actions of GIP on the gastric parietal

and pancreatic β cell are being influenced separately by somatostatin of gastric and pancreatic origin respectively. The stimulus for, and the time of release of the peptide from these different regions need not be identical. No information is available about the endogenous release of somatostatin at the present time.

The possibility that some inhibitory feedback mechanism existed between the endocrine pancreas and GIP release was suggested by the observation that subjects with maturity onset diabetes exhibited an abnormally elevated IR-GIP response to oral glucose or fat. The inhibitory factors implicated were insulin, glucagon or the degree of hyperglycaemia achieved. When an insulin injection was administered to normal human volunteers, the serum glucose levels being clamped in the fasting range, the IR-GIP response to fat ingestion was significantly less than that observed in the control situation. The time course of the peak serum IR- insulin response did not, however, correlate well with the IR-GIP depression (Brown et al, 1975). It would have been preferable if the insulin had been given as an infusion rather than a bolus injection.

Ebert et al (1976) infused glucagon intravenously for 2 hours during the ingestion of a provocative test meal and recorded a significant depression of the IR-GIP response. This effect could not have been due to the insulin released, there being no significant difference in the circulating IR- insulin response to the test meal, whether the glucagon was being infused or not. The hyperglycaemia stimulated by the glucagon was also unlikely to be the modulating influence. Diabetics with significantly higher levels of serum

glucose demonstrate an exaggerated GIP response to the same challenge. In normal subjects, the evidence so far accumulated, regarding endocrine pancreatic control of GIP release is most persuasive with respect to glucagon. Creutzfeldt and Ebert (1976) have also confirmed the inhibitory effect of somatostatin on GIP release and GIP-mediated response in man.

Further information about the mechanism of the action of GIP and its control was obtained from studies performed in subjects with abnormal digestive metabolism, or who had undergone gastrointestinal surgery. The stimulated IR-GIP response was significantly reduced in patients with coeliac disease, with a concomitant reduction in the IR- insulin response (Creutzfeldt et al, 1976). The most likely cause of the diminished GIP output is the defective absorption of nutrients, symptomatic of this disorder. Creutzfeldt and Ebert (1976) studied the importance of adequate nutrient absorption in rats, by comparing their IR-GIP response to glucose, administered with or without phlorizin. The addition of the phlorizin virtually abolished the release of IR-GIP. An alternative explanation for the low GIP levels measured is the possibility that the absolute number of GIP-producing cells has been reduced because of the villous atrophy, characteristic of coeliac disease. The majority of the GIP cells are found, however, in the crypts of the intestinal mucosa and villous atrophy would probably result in only an insignificant reduction in the GIP cell population..

Exaggerated GIP responses to stimulation by a mixed meal were observed after any surgical procedure which resulted in accelerated gastric emptying (dumping) e.g., gastrojejunostomy or vagotomy and pyloroplasty. This mechanism has also been put forward to explain the elevated IR-GIP levels measured in duodenal

ulcer patients (Creutzfeldt and Ebert, 1976). A similar elevation in stimulated IR-GIP levels was the general rule in chronic pancreatitics, possibly due to the loss of some feedback control by glucagon or insulin (Botha et al, 1976 : Ebert et al, 1976). The most marked IR-GIP response, however, was observed in patients with moderate hypoinsulinaemia, whereas those with severe insulin depression had an IR-GIP response which approached the normal values. This apparent anomaly is probably due to a combination of factors, affecting the release of GIP in different ways. The lack of insulin would lead to an overproduction of GIP but the associated gross exocrine pancreatic deficiencey would result in an abnormal fat metabolism, leading to malabsorption of fat (with associated steatorrhea) and a reduction in the release of IR-GIP. The relative hyperglucagonaemia, reportedly occurring in severe cases of chronic pancreatitis (Kalk et al, 1974) could also contribute to the reduction in the GIP response. In the final outcome these factors would balance each other, and the IR-GIP response would appear to be fairly normal. The further exaggeration in the levels of IR-GIP released after a partial duodenopancreatectomy would result from the accelerated rate of gastric emptying, following surgical interference.

The situation, in the cases of maturity onset diabetes (Brown et al, 1975) and obesity (Ebert et al, 1977) is slightly different. In these situations the GIP response to a test meal is abnormally high, in the presence of relatively high insulin levels. The insulin deficiency may be regarded as functional rather than absolute, the GIP-producing cell being unresponsive to the inhibitory action of that insulin. The possibility exists, and should be investigated, that this lack of sensitivity to the insulin may be due to high percentage of the total IR-insulin measured being in the form of the relatively

inactive proinsulin. This insensitivity would appear to be reversible, by sulphonylureas in the diabetics or by diet-mediated weight reduction in the obese subjects.

In all the previously mentioned abnormal conditions, only the stimulated IR-GIP levels were abnormal. In juvenile onset diabetes the fasting IR-GIP levels were in excess of 1 ng/ml. The fasting IR-GIP levels in the obese subjects, after prolonged starvation, approach this value. Both these situations are characterized by insulin deficiency and a high level of circulating ketone bodies. Treatment with insulin or food, respectively, reverses both these symptoms and reduces the IR-GIP output. The role of hyperketonaemia in the control of GIP release requires further investigation.

In summary, several factors have been implicated in the regulation of GIP production. The response of the GIP cell may be depressed by glucagon, insulin, somatostatin or a reduction in the absorption of nutrients. The IR-GIP output would appear to be increased by surgical disruption of gastrointestinal continuity, absolute or functional deficiency in insulin or glucagon and possibly by the presence of elevated levels of ketone bodies in the circulation.

If the GIP release is being inhibited by some endocrine pancreatic factor, either insulin or glucagon, this could explain the reduced IR-GIP response to oral fat in the presence of an intravenous glucose infusion, relative to that response observed when fat was administered alone and there was no pancreatic stimulation. It could also account for the fact that the IR-GIP response to an intraduodenal infusion of glucose is not only delayed but is also diminished when it is preceded by a somatostatin infusion. As shown in Fig. 62, the IR-insulin response rebounded to levels significantly above the control

values at the termination of the somatostatin infusion and this could be responsible for the reduction in the GIP response. It is interesting to speculate whether hyperinsulinaemia or hyperglucagonaemia might not only effect an inhibition of IR-GIP release but also inhibit the activity of circulating GIP at the level of the β cell (cf somatostatin).

Another facet of the role played by GIP in carbohydrate metabolism is illustrated in obesity. The observation that complete starvation or a low caloric diet resulted in an eventual decrease in both the IR-GIP and IR- insulin responses in obese individuals suggested that carbohydrate intake played a role in regulation of the sensitivity of the GIP- producing cell. It is also possible that the number of GIP cells is controlled by the nature of the diet. If this is so, one might question if any pathological condition, characterized by malabsorption of nutrients (e.g., coeliac disease) might not also result in a reduction of the absolute number of GIP- producing cells, which would contribute to the reduction in IR-GIP output observed in this circumstance. (see page 243).

GIP alone was found to have a weak lipolytic effect on isolated rat adipocytes, but GIP, administered in conjunction with glucagon, was found to be strongly antilipolytic. It was possible to block the lipolytic action of glucagon and to displace glucagon from its binding sites on the adipocyte plasma membrane by the addition of GIP to the incubate (Dupre et al, 1976 : Ebert and Brown, 1977). GIP was, however, much less effective in antagonizing the lipolytic action of secretin, and was ineffective against adrenocorticotrophic hormone (ACTH), adrenaline, noradrenaline and theophylline. Obesity is a pathological condition of many aetiologies, but an impaired carbohydrate metabolism,

resulting in or from an increased carbohydrate intake, is likely to produce obesity directly via the elevated GIP response and indirectly via the elevated insulin response induced by GIP.

The status of GIP as a hormone is established. Its predominant role would appear to be that of the major gastrointestinal regulator of carbohydrate metabolism. This is supported by the altered IR-GIP response measured in clinical conditions related to impaired carbohydrate metabolism. GIP also plays a part in controlling the secretory and motor activity of the stomach in dogs and probably in man, but the clinical evidence for GIP involvement in hypersecretory conditions, e.g. duodenal ulcer and Zollinger-Ellison syndrome, or the hyposecretory states, e.g., Werner-Morrison syndrome and achlorhydria, is virtually non-existent. In all the pathological conditions so far investigated, abnormal GIP responses are symptomatic, rather than causative, of the disorder.

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