ASPECTS OF

STRUCTURE-ACTIVITY RELATIONSHIPS

AND PHYSIOLOGY

OF GASTRIN AND RELATED PEPTIDES

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by

BEVERLEY JEAN NAPIER (née GRANT)

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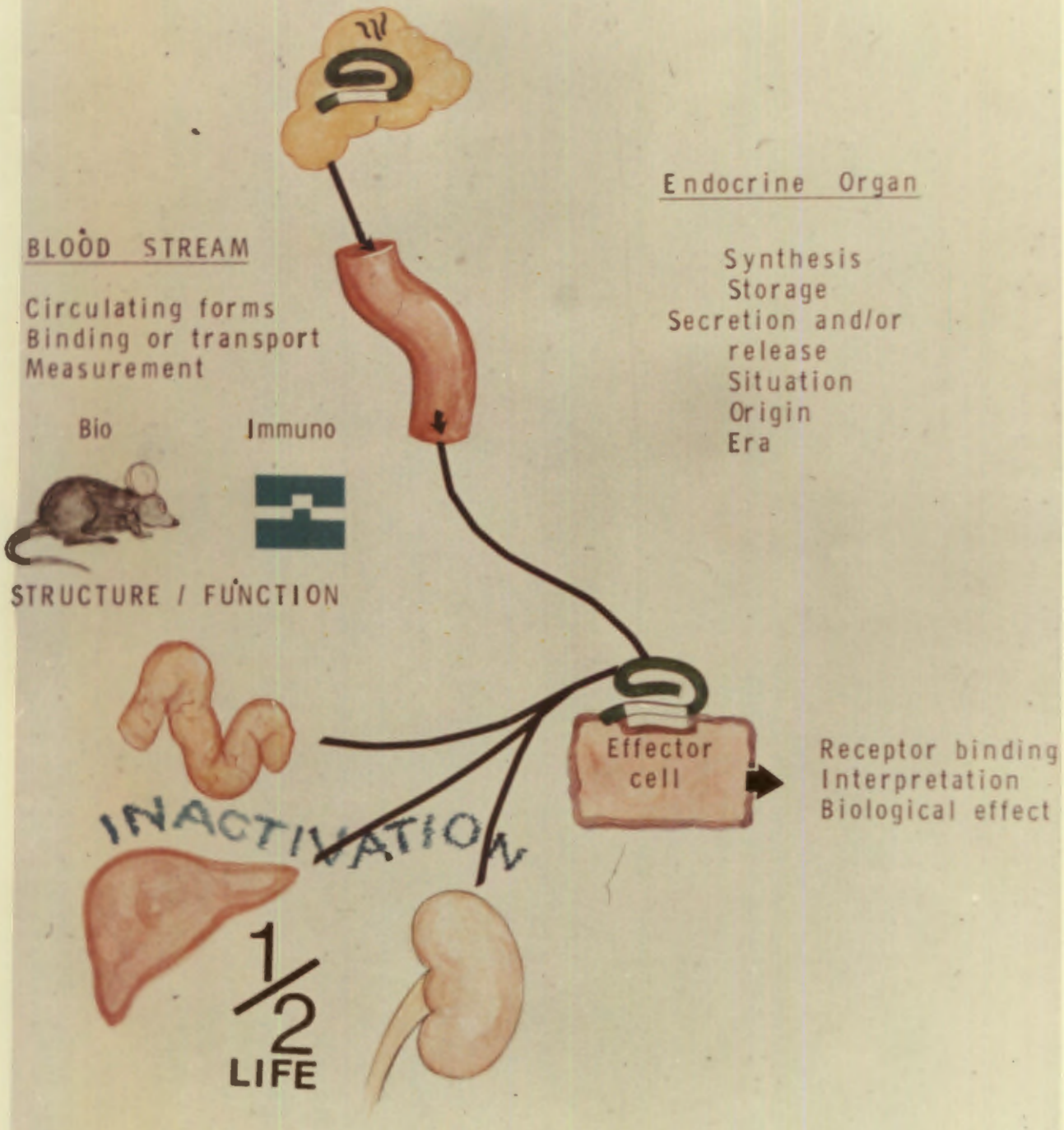
To CRAIG

KNOW YOUR HORMONE WELL



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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ADH	Antidiuretic hormone
APUD-FIF	Amine precursor uptake and decarboxylation-formaldehyde-induced fluorescence
BAC	Bromoacetylcellulose
BG	Big gastrin
BBG	Big big gastrin
BGP	Brain gastrin peptide
BOC	Butyloxycarbonyl
BSA	Bovine serum albumen
BSP	Bromsulphthalein
B. cincta	Burnupena cincta
^{14}C	^{14}Carbon
CCK-PZ	Cholecystokinin-pancreozymin
cm	centimetre
c.p.m.	counts per minute
c.p.s.	counts per second
C.U.R.E.	Centre for Ulcer Research and Education
DEAE	Diethyl-aminoethyl
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
D.U.	Duodenal ulcer
EDAP-CDI	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ep-HGH	extracted pituitary human growth hormone
fg/ml	femtograms per millilitre

FSH	Follicle stimulating hormone
g	gram
G.U.	Gastric ulcer
G-17 I	Heptadecapeptide gastrin type I
G-34 I	Big gastrin type I
HCG	Human chorionic gonadotrophin
HGH	Human growth hormone
HSA	Human serum albumen
^{125}I	^{125}Iodine
^{131}I	^{131}Iodine
ICG	Indocyanine green
I.C.I.	Imperial Chemical Industries
ID_{50}	Inhibition $Dose_{50}$
kg	kilogram
KIU	Kallikrein Inactivator Units
KRB	Krebs-Ringer Bicarbonate
l/kg	litres per kilogram
l/mol	litres per mole
LGI	Large glucagon immunoreactivity
LH	Luteinizing hormone
LRF	Luteinizing hormone releasing factor
m	metre
ml	millilitre
mm	millimetre
mCi	millicurie
mEq/l	milliequivalents per litre

mg	milligram
mg/kg	milligrams per kilogram
mg/min	milligrams per minute
min	minute
mM	millimolar
mCi/μg	millicuries per microgram
mCi/μmol	millicuries per micromole
ml/min	millilitres per minute
ml/min/g	millitres per minute per gram
ml/min/kg	millilitres per minute per kilogram
mol/l	moles per litre
M.C.R.	Metabolic clearance rate
ng	nanogram
ng/g	nanograms per gram
ng/ml	nanograms per millilitre
nmol/g	nanomoles per gram
nm	nanometre
nM	nanomolar
NHG	natural human gastrin
O.D.	Optical density
P.A.	Pernicious anaemia
PAH	Para-amino hippuric acid
pg	picogram
pg/ml	picograms per millilitre
pg/g	picograms per gram
pg/mg	picograms per milligram
pmol/l	picomoles per litre

pmol/kg/hr	picomoles per kilogram per hour
PG	Porcine gastrin
PTH	Parathyroid hormone
rev./min	revolutions per minute
RNA	Ribonucleic acid
S.A.I.M.R.	South African Institute of Medical Research
S.D.	Standard deviation
S.E.M.	Standard error of the mean
SHG I	Synthetic human heptadecapeptide gastrin type I
SHG (1-17)	Synthetic human heptadecapeptide gastrin
SHG (2-17)	Synthetic human hexadecapeptide gastrin
$T\frac{1}{2}$	Half-life time
TSH	Thyroid stimulating hormone
U.C.L.A.	University of California Los Angeles
µEq	microequivalent
µg	microgram
µl	microlitre
µg/g	micrograms per gram
µl/g	microlitres per gram
µCi/µg	microcuries per microgram
µl/hr	microlitres per hour
µg/kg/hr	micrograms per kilogram per hour
V	Volume of distribution
VIP	Vasoactive intestinal polypeptide
w/v	weight per unit volume
$\bar{X}$	mean
Z.E.S.	Zollinger-Ellison syndrome

CHAPTER I

INTRODUCTION

When, in 1973, I embarked upon this study, the hormone gastrin seemed a suitable hormone to study with regard to investigation of structure-activity relationships and physiology of a peptide hormone. Gastrin was best known at that stage as a linear peptide comprising 17 amino acids, which was stable at room temperature and was resistant to degradation by vigorous treatments such as boiling (Gregory and Tracy, 1964; Berson and Yalow, 1971; Yalow and Berson, 1972) and pH extremes (Piszkiewicz, 1974). Such a molecule could thus be studied at room temperature, using conventional biochemical techniques, without the need for any special precautions to preserve its integrity.

Heptadecapeptide gastrin had been isolated and purified from porcine (Gregory and Tracy, 1964) and human antral mucosa (Gregory, Tracy and Grossman, 1966) twelve to fourteen years previously, and its primary structure in each case had been determined (Gregory, Hardy, Jones, Kenner and Sheppard, 1964; Bentley, Kenner and Sheppard, 1966), so that at the time of commencement of this study pure preparations of synthetic human heptadecapeptide gastrin were freely available commercially. This meant that a radioimmunoassay for the measurement of gastrin levels in the circulation could be developed, which entails:

(i) immunizing rabbits with purified gastrin preparations to raise antisera,

(ii) labelling the peptide with a radioactive tracer such as ^{125}Iodine, and

(iii) preparing a series of standard concentrations of the hormone with which samples containing unknown levels of gastrin could be compared.

In addition, the availability of pure preparations of gastrin allowed estimations of gastrin activity by the technique of bioassay, studies of the effect of administration of this peptide hormone to human subjects and experimental animals, and investigation of its behaviour in vitro under various conditions.

Studies aimed at elucidating the physiological role of a hormone are designed to answer such questions as:

(i) where in the body is the hormone synthesised and secreted, or more specifically, in which cell of which organ, and which sub-cellular organelle is responsible for the manufacture of the hormone?

(ii) what is the stimulus for secretion of the hormone, how is its effect on the hormone-secreting cell mediated, and how is the secretion of the hormone regulated?

(iii) in what molecular form is the hormone secreted; is it pre-formed and stored in the cell or is it synthesised in the cell on demand?

(iv) does the hormone exist in the circulation in an inactive precursor form which is cleaved to a smaller biologically active form, and if so, where is the site of this conversion?

(v) what is the target organ of the hormone and how does the hormone produce its effect on the target cell?

(vi) in the case of the gastrointestinal hormones, one must ask whether they are secreted into the lumen of the gut or into the bloodstream and by which route they reach the target cell, whether it be via the lumen of the gut or via the bloodstream;

(vii) after the hormone has produced its effect it is degraded and removed from the circulation; the organ or organs responsible for metabolism

of the hormone, and the half-time of disappearance of the hormone from the circulation must be defined.

Some of the answers to these questions were known in the case of gastrin in 1973. It was known that gastrin is secreted by the G-cell in the antral mucosa, and to a lesser extent by the duodenal mucosa, in the human. Physiological stimuli known to cause release of gastrin in the stomach included gastric distension (shown in the dog), ingestion of food, especially protein, the presence of certain amino acids in the lumen of the stomach, blood-borne calcium and catecholamines, and vagal cholinergic stimuli. The primary action of gastrin is stimulation of the parietal cell in the gastric fundus to secrete hydrochloric acid for digestion and a negative feedback system appeared to regulate the secretion of gastrin, in that the presence of acid in the stomach inhibited further release of gastrin. It was not known how gastrin elicited this response in the parietal cell, but on the basis of findings relating to other peptide hormones, it seemed likely that the first stage involved binding of gastrin to a receptor in or on the target cell.

Preliminary studies had revealed that serum gastrin was heterogeneous, in that several molecular forms of immunoreactive gastrin of different molecular sizes had been observed following Sephadex gel filtration of hypergastrinaemic serum (Yalow and Berson, 1970b; 1971a; 1972; Rehfeld, 1972). The precise number of serum gastrin components, their interrelationships and whether or not they all possessed biological activity had not been conclusively established. Which form of gastrin was secreted by the antral G-cell, whether the same form was secreted by the duodenal mucosa, and whether the secreted form was an inactive large precursor form, were all unanswered questions. Although the antral G-cell had been shown to

communicate both with the lumen of the stomach and with the capillaries bathing the basement membrane of the mucosa (Creutzfeldt, Arnold, Creutzfeldt, Feurle and Ketterer, 1971; Greider, Steinberg and McGuigan, 1972), the proportions of lumenal and humoral secretion of gastrin were not known, nor the route to the fundic parietal cell.

Regarding the removal of gastrin from the circulation, reports had suggested the importance of the kidneys in this connection (Jaffe and Newton, 1969; Dent, Hirsch, James and Fischer, 1972; Korman, Laver and Hansky, 1972; Hjelmquist, Reeder, Brandt and Thompson, 1972) and one study had implicated the liver in this role (Thompson, Reeder, Davidson, Charters, Brückner, Lemmi and Miller, 1969). Early studies of the disappearance of heptadecapeptide gastrin from the circulation had shown a half-life of between 2 and 10 minutes, depending on the design of the experiment (Blair, Harper and Reed, 1962; Jaffe and Newton, 1969; Yalow and Berson, 1970a; Ganguli, Elder, Smith and Hunter, 1970; McGuigan, Isaza and Landor, 1971; Reeder, Jackson, Brandt and Thompson, 1972).

Turning to the structure-function relationships of gastrin and the related peptides, cholecystokinin-pancreozymin (CCK-PZ), caerulein and the C-terminal pentapeptide of gastrin, one is struck by the close similarities in the amino acid sequences displayed by these peptides. All four peptides contain within their primary structure the C-terminal pentapeptide amide sequence: Gly-Trp-Met-Asp-Phe-NH_2, which includes the C-terminal tetrapeptide amide sequence shown to be responsible for the biological action of stimulation of gastric acid secretion by Tracy and Gregory in 1964. The structural similarities between the peptides CCK-PZ and caerulein

extend as far as eight residues from the C-terminus, with identity in all positions except one. Gastrin, CCK-PZ and caerulein all bear a tyrosine residue, which is situated six positions from the C-terminus in the case of gastrin and seven positions from the C-terminus in the latter two cases. Both caerulein and CCK-PZ require the presence of a sulphate group attached to the tyrosine ring for biological activity, whereas gastrin occurs naturally in both the sulphated and non-sulphated forms and both are biologically active.

The structural similarities in these peptides are accompanied by overlap in the pharmacological effects produced by their administration in man and experimental animals. All four peptides can elicit responses such as increased output of gastric juice, hydrochloric acid and pepsin secretion, contraction of the gall bladder and other smooth intestinal muscle, alterations in blood pressure by their action on the vessel musculature, increased production of pancreatic juice, pancreatic enzymes and bicarbonate secretion, as well as affecting the endocrine pancreas and causing release of insulin and glucagon. The differences between the actions of gastrin and these related peptides lie in the dose required to elicit a particular response; thus at physiological levels each peptide is more potent in producing its individual response, although at pharmacological levels the overlap in actions is observed.

Similar overlaps in primary structure and pharmacologic effects are found in the case of the gut peptides, secretin and glucagon; it is becoming increasingly apparent that structural resemblances occur among many other gut peptide-hormones as well.

This overlap in primary structure of gastrin and the related peptides strongly suggests a common molecular ancestry. It was considered of interest to trace the origin of the gastrin molecule down the evolutionary tree by investigating the occurrence of this peptide in various mammalian, vertebrate and invertebrate species, in the hope of providing answers to such questions as:

(i) is the tissue distribution of this peptide in mammals the same as in the human, and

(ii) how far back in evolutionary development did the gastrin molecule arise?

Although a sprinkling of reports on the occurrence of the gut peptide hormones insulin, glucagon, CCK-PZ and secretin in lower species had appeared, there was a dearth of information regarding this aspect of gastrin in the literature in 1973. Lai (1964b) had reported finding a gradient of gastrin activity on descending the human gut, as determined by bioassay of tissue extracts. Elwin and Uvnäs (1966) and Emås, Borg and Fyrö (1971) had studied the relative gastrin bioactivity found in the antrum and duodenum of the cat and the human. In 1973 two reports of measurements of immunoreactive gastrin in mammals appeared (Nilsson, Yalow and Berson, 1973; Thompson, Reeder, Davidson, Jackson and Clendinnen, 1973), and Kenner and Sheppard (1973) determined the primary structures of heptadecapeptide gastrin isolated from pig, cow, sheep, dog and cat. No information pertaining to investigation of gastrin distribution in lower species was found.

The aims of this study, accompanied by an outline of the way in which the investigations were undertaken, will now be described.

(i) A sensitive radioimmunoassay, capable of measuring basal circulating levels of gastrin, was established in our laboratory. The reliability

and validity criteria of the assay, and the specificity of three antisera used in the assay were determined;

(ii) The assay was used to establish a normal range of serum gastrin levels and a survey of serum gastrin concentrations in patients belonging to different categories was carried out;

(iii) The molecular heterogeneity of gastrin in human serum was investigated using Sephadex column chromatography;

(iv) The distribution of tissue gastrin in the gastrointestinal tract of the human and five mammalian species was studied, and characterisation of human antral and duodenal gastrins was attempted by gel filtration and investigation of their immunochemical behaviour;

(v) The occurrence of immunoreactive gastrin among nineteen invertebrate species was investigated by radioimmunoassay measurements of serum, and, in some instances, tissue gastrin content;

(vi) More detailed studies aimed at characterisation of a gastrin-like material found in the serum and skin extracts of the frog, Xenopus laevis, were performed;

(vii) Tissue extracts prepared from nine different invertebrate species were tested for the presence of immunoreactive gastrin. It was hoped that these investigations would throw some light on the evolutionary origin of the gastrin molecule;

(viii) Transhepatic measurements of basal endogenous gastrin levels in the pig were carried out to assess the importance of the liver in relation to possible removal of gastrin from the circulation;

(ix) The kinetic parameters of half-life, metabolic clearance rate, and space of distribution of infused exogenous heptadecapeptide gastrin in the pig were determined, since no information of this kind

regarding the pig was found in the literature;

(x) Serum samples following infusion of synthetic human heptadecapeptide gastrin into the pig were chromatographed to assess whether the molecular species of gastrin was altered by prolonged circulation *in vivo*;

(xi) The effect of the isolated rat liver on physiological and supraphysiological levels of gastrin perfused cyclically through this organ in a plasma-free medium was studied. Column chromatography of perfusate samples was performed to assess whether the rat liver had any effect on circulating gastrin;

(xii) Finally, the *in vitro* binding of heptadecapeptide gastrin to the basic liver cytosol protein, ligandin, was studied to assess the importance of this protein in relation to gastrin transport and/or inactivation.

In setting out this thesis I have reviewed the relevant literature (including that which has postdated many of my studies) pertaining to the aspects of gastrin which I investigated, and grouped this into ten different sections in Chapter 2. Chapter 3 contains an account of all the methods and materials used in these studies, and is divided into five sections, which in turn comprise several sub-sections. The results of these studies are contained in Chapter 4, in which case the same sequence of the various sub-sections is maintained. The findings in this study are discussed in the light of the findings of other groups working in the same field in Chapter 5, and as far as possible the sequence set out in the two preceding chapters is adhered to. Chapter 6 contains a summary of the results of this investigation and suggestions for future research arising from the findings of this study. In Chapter 7, the appendix, several methods relevant to the thesis, although not carried out by myself, are described.

CHAPTER 2

LITERATURE REVIEW

Section I Historical Aspects

The birth of endocrinology occurred with the discovery by Bayliss and Starling in 1902 of an active principle in the upper intestinal mucosa which on extraction and intravenous injection into anaesthetised dogs, caused the flow of pancreatic juice. They called this active principle "secretin" and on the basis of their experiments they concluded that contact of the acid chyme with the epithelial cells of the duodenum causes the production of a body (secretin) which is "absorbed from the cells by the blood current and is carried to the pancreas, where it acts as a specific stimulus to the pancreatic cells, exciting a secretion of pancreatic juice proportional to the amount of secretin present " (Bayliss and Starling, 1902). This was the first demonstration of the existence of a humoral mechanism in the body, by which is meant a process demonstrated to be independent of nervous connections between the site of stimulation and the effector site (Grossman, 1950). Three years later, in 1905, Starling introduced the word "hormone" to designate this class of chemical coordinators (Walsh and Grossman, 1975a).

In the same year Edkins suggested that a "gastric secretin" mechanism operated by means of a substance produced by the presence of partially digested food in the stomach, which passes into the blood or lymph and later stimulates the secretory cells of the stomach to functional activity. He showed that extracts of pyloric mucous membrane made in boiling water or 0,4% HCl, prepared from the cat or the pig, contained an active substance which on intravenous injection into anaesthetised cats led to the

secretion of acidic gastric juice. This effect was not observed with an extract prepared from the mucous membrane of the fundus (Edkins, 1905a). He suggested that this active principle, which was not destroyed by boiling, be named "gastrin" (Edkins, 1905b).

Edkins' findings regarding pyloric extracts and their effect in causing gastric secretion were confirmed by Lim (1923), who reproduced his experiments essentially as Edkins had done them. Sacks, Ivy, Burgess and Vandolah (1932) isolated histamine from hog pyloric mucosa and showed that this gastric secretory excitant was active when injected subcutaneously, leading them to conclude that "histamine is the gastric hormone, or if not, there is no gastric hormone, or the gastric hormone has never been extracted from pyloric mucosa". Boller and Pilgerstorfer (1937, cited by Grossman, 1950, p. 42) modified Edkins' procedure either by using different methods of extraction, or by injecting their extracts subcutaneously as opposed to intravenously, and found that subcutaneously effective extracts could be prepared from both pyloric and fundic mucosa as well as from many other tissues. We now know that these observations were due to the presence of that ubiquitous substance, histamine, in these extracts.

This controversy as to whether the extracts contained a stimulant other than histamine raged for many years, until the work of Komarov in 1938, which radically changed the general belief that the activity of Edkins' extracts was probably attributable to the presence of histamine. Komarov, recognising that the antral hormone might be of a protein nature, succeeded in precipitating a protein fraction from an acid aqueous extract of canine pyloric mucosa with 10% trichloroacetic acid. On injection into anaesthetised cats this protein fraction stimulated the copious secretion

of gastric juice with high acidity and low peptic power (Komarov, 1938). He found this active principle only in the pyloric region of the stomach, and he also detected a small amount in duodenal extracts prepared in the same manner. Komarov's preparations were of low potency and non-toxic when injected intravenously. Subcutaneous injection of his extracts did not stimulate secretion, providing further evidence that the active principle was of protein nature and thus not readily absorbed when administered subcutaneously (Komarov, 1942 (a) and (b), cited by Gregory and Tracy, 1961, p. 523).

Uvnäs was able to confirm Komarov's results using the same method of preparing gastrin with some modifications (Uvnäs, 1942). In the same year Uvnäs demonstrated that vagal impulses caused the release of gastrin (Uvnäs, 1942). Further studies by Uvnäs (1945), Harper (1946) and Jorpes, Jalling and Mutt (1952) established that a potent gastric acid secretory stimulant could be extracted from the pyloric mucosa of dogs, cats, pigs and humans. The histamine content of these active extracts was usually too small to be detected (Uvnäs, 1943) and incubation with histaminase failed to reduce the effectiveness of potent extracts (Bauer and Uvnäs, 1944).

Although these investigations established beyond doubt that these gastrin extracts stimulated gastric secretion in anaesthetised cats, there was little evidence that such preparations were active when injected into conscious animals (Gregory and Tracy, 1961). Prompted by the need to find a suitable gastrin preparation for such studies, Gregory and Tracy (1961) developed a method of extraction of gastrin from hog antral mucosa which strongly stimulated gastric acid secretion when injected subcutaneously as well as intravenously or intramuscularly into conscious dogs with gastric

fistulae or gastric pouches. The preparation was pure enough to be suitable for experiments in humans (Gregory and Tracy, 1961). However, the extraction procedure was tedious, expensive and inefficient, making it unsuitable for the isolation of the hormone in amounts large enough for structural studies (Gregory and Tracy, 1964).

In 1964 Gregory and Tracy evolved a new method of preparation of gastrin from hog antral mucosa which could be worked easily and cheaply on a very large scale (Gregory and Tracy, 1964). They identified the final product as consisting of two almost identical peptides which they called gastrin I and gastrin II. The extraction procedure was commenced by boiling batches of 300 hog antra in tap water for 30 minutes. The final yield from 600 antra after the extraction procedure, which culminated in fractionation on a Sephadex G-50 column and two fractionations on aminoethylcellulose columns, produced two well defined peaks containing gastrin activity, comprising 17 mg gastrin I and 22 mg gastrin II (Gregory and Tracy, 1964). High voltage electrophoresis did not alter either the amino acid constitution or the physiological activities of either gastrin, and both gastrins ran as single compact spots on the paper (Gregory and Tracy, 1964), indicating biochemical purity. The absence of a terminal amino group in the molecule was confirmed by negative fluorodinitrobenzene and ninhydrin tests (Gregory and Tracy, 1964). Amino acid analyses of gastrins I and II after acid hydrolysis showed that both molecules had the same amino acid constitution, and the minimal molecular weight for each gastrin, calculated on amino acid composition, was found to be 2114 (Gregory and Tracy, 1964). Both gastrins were shown to be many times more potent than histamine when injected as single subcutaneous doses in

conscious dogs with denervated fundic pouches (Gregory and Tracy, 1964). On a molar basis gastrin II was shown to be 500 times more effective than histamine in stimulating gastric secretion in human subjects (Makhlouf, McManus and Card, 1964).

Working in collaboration with Gregory and Tracy on the hormones they had isolated, Gregory, Hardy, Jones, Kenner and Sheppard (1964) determined that porcine gastrin II was a heptadecapeptide amide with the structure:

$$\square\text{Glu - Gly - Pro - Try - Met - Glu - Glu - Glu - Glu - Glu - Ala -}$$
$$\text{Tyr - Gly - Try - Met - Asp - Phe - } NH_2$$
$$|$$
$$SO_3H$$

and they showed that gastrin I differs from gastrin II only in that it lacks the sulphate group esterified to the tyrosine in position 12. This group of workers succeeded in synthesising gastrin in the same year, thus confirming the sequence deduced from the degradative investigations (Anderson, Barton, Gregory, Hardy, Kenner, MacLeod, Preston, Sheppard and Morley, 1964), and they were able to show that their purified product of synthetic gastrin I was indistinguishable from natural gastrin I on column chromatography, high-voltage electrophoresis and thin-layer chromatography. The same was true of their preparation of gastrin II. In addition their synthetic gastrin I preparation gave full biological responses when injected subcutaneously into a conscious dog (Anderson, Barton et al., 1964).

The actions of the pure natural gastrins were summarised by Tracy and Gregory in 1964. By using the peptides produced during the programme of synthesis of gastrin and testing their activity, Tracy and Gregory (1964) were able to show that all the physiological actions displayed by the gastrins were possessed by the C-terminal tetrapeptide amide, and that

these activities were largely abolished by removal from this structure of the amide group masking the C-terminal phenylalanine residue.

Extending this work, Gregory, Tracy and Grossman (1966) applied the same extraction procedure as used for hog antra to isolate gastrin from human antral mucosa. Antral mucosa was collected from human subjects undergoing partial gastrectomy, boiled in tap water for 5 minutes, and gastrin isolated in the same manner as for porcine gastrin (Gregory and Tracy, 1964). Gradient elution of the final product on an aminoethyl-cellulose column produced separate peaks of human gastrin I and II, but in this case the yield from 23 antra gave 1,35 mg gastrin I and 0,79 mg gastrin II (Gregory, Tracy and Grossman, 1966), in contrast with the ratio of approximately 3:4 found for porcine gastrins I and II (Gregory and Tracy, 1964).

Bentley, Kenner and Sheppard (1966) found the amino acid sequence of human gastrin to differ from that of porcine gastrin only in the substitution of methionine in position 5 by leucine, while the presence or absence of a sulphate group esterified to the tyrosine residue in position 12 was the same as for porcine gastrins II and I respectively. Human gastrins I and II displayed the same physiological activity as did porcine gastrins (Gregory and Tracy, 1964; Gregory, Tracy and Grossman, 1966), which was not surprising since the differences in primary structure between the two molecules occurred in the region of the molecule which is not essential for biological activity (Tracy and Gregory, 1964). Synthetic human gastrin prepared according to the structure determined by sequential analysis was shown to be biologically active and its identity with the natural human gastrin I was confirmed by degrading both peptides in parallel with papain and comparing the peptides thus obtained using thin-layer chromatography (Beacham, Bentley, Gregory,

Kenner, MacLeod and Sheppard, 1966).

With the advent of purification of gastrin from hog and human antral mucosae and the demonstration of their biological activity by injection into test animals came the need to quantitate the biological activity of these preparations. Early measurements of gastrin activity were performed using bioassay techniques, as described in the next section.

Section II Measurement of Gastrin Activity by Bioassay

The technique of bioassay of gastrin involves comparing the response in gastric acid secretion evoked by administration of a test substance to an experimental animal with that produced by a standard reference preparation of known biological activity. Animals used in bioassay techniques which have been described include the cat, rat and dog, either in the anaesthetised or conscious state, although dogs have the disadvantage that a larger total dose of the test material is required to stimulate acid secretion (Emås and Uvnäs, 1973). Histamine was used as the reference standard before preparations of gastrin were available, but the disadvantage of using a reference standard which differs from gastrin is obvious. A crude gastrin extract prepared by the method of Blair, Harper, Lake, Reed and Scratcherd (1961) was used as the standard reference preparation in some earlier bioassays, whereas pure gastrin or synthetic human gastrin were used in more recent methods.

For a reliable bioassay certain basic requirements must be met: the animal used must be sensitive to gastrin, it must respond predictably to different doses of the test material, the reference standard must be of a constant potency, and it must behave as gastrin (Emås and Uvnäs, 1973). The first method of gastrin bioassay that fulfilled these requirements was described by Uvnäs and Emås (1961). They used conscious cats with gastric fistulae, since it had been shown by Emås (1960) that smaller doses of stimulant were required to stimulate acid secretion than in anaesthetised cats, and conscious cats responded more uniformly to stimulation than did anaesthetised cats. Histamine was used as the reference standard; it was shown that the slopes of the dose-response curves for gastrin and histamine did not differ (Uvnäs and Emås, 1961). Two identical doses of a gastrin

extract were infused for 15 minutes per hour, interposed between a small and a large dose of histamine, so that as long as the gastrin doses fell within the range of the two histamine doses, the mean secretory activity of a gastrin extract could be expressed in histamine units per milligram preparation. Only one gastrin extract could be assayed in each experiment, the duration of which was 4, 5 hours, but the cats could be used repeatedly. Once pure synthetic human gastrin was available this was used instead of histamine as reference standard, and the smallest effective dose, or sensitivity of the assay, was found to be 0,08 - 0,2 micrograms (µg) synthetic human gastrin infused for 15 minutes (Emås and Uvnäs, 1973).

Blair, Harper and Reed (1962) described a gastrin bioassay method using anaesthetised cats in which they overcame the great variation in acid response to 15-minute infusions of gastrin extracts or histamine, described by Emås (1960), by injecting a standard gastrin extract every 15 minutes, to maintain a continuous secretion of acid. The activity of a test extract was measured by replacing one injection of the gastrin standard with the test extract and comparing the response with that expected to the standard extract. A reliable assay required that the response fell within $\pm$ 25% of the anticipated response to the standard. This technique was further refined by Blair, Keenlyside, Newell, Reed and Richardson (1968). As many as ten extracts could be assayed with good precision in a single experiment. Using this technique, Blair and Wood (1968) estimated the half-life of gastrin activity in plasma to be less than 4 minutes between the second and tenth minutes after an intravenous injection of a gastrin extract. This value is remarkably close to the half-life value for gastrin in the circulation found by later investigators, as discussed in section IX(iii) of this chapter.

Ghosh and Schild (1958) developed a method for the continuous recording of gastric acid secretion in the anaesthetised rat by perfusing the stomach with a dilute sodium hydroxide solution (1/4000 N) via the oesophagus, and monitoring the pH of the fluid emerging from a cannula in the pylorus which passed over a glass electrode connected to a recording pH meter. Test substances were given as a rapid intravenous injection, and the response was measured as a pH change in the perfusate passing through the stomach. This system was suitable for the bioassay of secretory stimulants, and 10 or more drug doses could be administered in succession in the same preparation, provided that successive doses of drugs were only administered once the effect of a previous dose had subsided (Ghosh and Schild, 1958). The advantage of this method was that each animal served as a self-contained assay unit. Although this method measured total acid secretion it did not allow quantitation of the volume or acidity of the gastric juice secreted in response to various stimulants. In addition, the gastric secretion may have been affected by the artificial conditions prevailing during the study, such as the body temperature which was kept at 30°C, the alkaline perfusate in the stomach, and the effect of anaesthesia on acid and mucous secretion (Ghosh and Schild, 1958).

Two modifications of the method of Ghosh and Schild (1958) have since been used for the bioassay of gastrin in rats. Amure and Ginsburg (1964) described an assay based on the observation that a linear relationship existed between the maximal pH change of the gastric effluent and the dose of gastrin extract given in the anaesthetised rat, prepared according to the method of Ghosh and Schild (1958). They used a crude gastrin extract as standard, and the assay involved injection of a high and a low dose

of both standard and test extract. However, the method only applied to a narrow range of doses where this relationship held true, which meant that preliminary tests were necessary before the final assay. Only one test extract could be assayed per experiment.

Lai (1964a) modified the method of Ghosh and Schild (1958) by washing the stomach of an anaesthetised rat clean in a slow stream of tap water and then perfusing it in situ with 0,9% saline, warmed to 37°C, and collecting 10-minute samples of gastric effluent. These samples were titrated against 1/100 N-NaOH so that the acid secretion could be expressed as μ Eq/10 minutes. Test substances were injected intravenously over a period of 15 minutes and the mean rate of acid secretion, corrected for the continuous basal acid secretion, was determined as the mean acid output per 10 minutes, calculated over several 10-minute periods. The response to the first dose was excluded as it was unreliable. The effects of test preparations were determined by comparison of the mean rates of acid secretion which they elicited with the response to graded intravenous doses of gastrin extracts (Lai, 1964a). However, the assay of one extract required 6 infusions of both test and control extract and 4 rats had to be used (Emås and Uvnäs, 1973). According to Emås and Uvnäs (1973), the sensitivity of the assay methods of Amure and Ginsburg (1964) and Lai (1964a) cannot be evaluated from available data.

An improved bioassay for gastrin, using the perfused rat stomach, was described by Smith, Lawrence, Colin-Jones and Schild (1970), who developed a method based on reperfusion of the rat stomach, in which the acid output could be accurately quantitated. The anaesthetised rat stomach was reperfused continuously, stimulants were injected intravenously, and the accumulation of hydrogen ions in the recirculating buffer was integrated as it occurred, so

that the amount of acid secreted in a given time, and the time course of secretion could be continuously followed. Correction for baseline acid secretion was made, and the results of the first test dose were disregarded as they were inconsistent. The reperfusion solution was changed before each dose of secretagogue was administered. The assay had a sensitivity of 10-20 nanograms (ng) of synthetic human gastrin, and the steepest part of the dose-response curve lay between 40 and 200 ng synthetic human gastrin. This assay was used to measure gastrin activity in the plasma and tumour extract of a patient with Zollinger-Ellison syndrome (Z.E.S.)

From this discussion it is apparent that the technique of bioassay poses certain limitations. Only a very small number of unknown or test specimens, and in some cases only one, could be assayed at a time, and most of the methods were tedious and time consuming to execute. Variations in the methods and the animals used, which included rats, dogs and cats, made comparison between different assays difficult. This problem was compounded by the fact that pure gastrin preparations were not available in the early days of bioassay, and in some cases histamine was substituted for gastrin as the standard reference preparation. In addition, these methods were very insensitive, with the lowest detectable level in the range of 1 ng (Walsh and Grossman, 1975a); this is not sensitive enough to measure normal circulating gastrin levels, which are usually less than 100 picograms per millilitre (pg/ml) serum. Bioassay techniques are only useful in measurement of serum gastrin levels in hypergastrinaemic patients with gastrinoma (Walsh and Grossman, 1975a). However, bioassays can be performed with good precision, so that repeated measurements of the same sample agree to within 10% of each other (Walsh and Grossman, 1975a; Loraine and Bell, 1971).

Once pure gastrin preparations became available, the technique of radioimmunoassay of gastrin could be developed. This technique is about a thousand times more sensitive than bioassay, allowing detection of gastrin levels as low as 1 picogram (Walsh and Grossman, 1975a), and large numbers of serum gastrin estimations can be performed simultaneously, with high sensitivity and specificity (Loraine and Bell, 1971; Walsh and Grossman, 1975a).

Bioassay is still very useful currently for the measurement of the physiological activity of secretory stimulants, compared to their immunological reactivity, which is detected by radioimmunoassay, and for comparison of the biological and immunochemical activity of natural and synthetic gastrin preparations. Ideally the two techniques should be used together, although this is impractical in most instances.

Since the development of radioimmunoassay a further technique for the bioassay of gastrin has been described, which allows exquisitely sensitive measurements of gastrin activity as low as 5 femtograms per millilitre (fg/ml). Loveridge, Bloom, Welbourn and Chayen (1974) described the cytochemical bioassay of gastrin, which entails measuring the hydrochloric acid secretion by the parietal cells of the gastric fundus of the guinea pig in response to stimulation by graded concentrations of gastrin in the range 5 pg/ml to 5 fg/ml. The response is quantitated by recording the intensity of staining in the parietal cells, since the optical density is directly proportional to the gastrin activity. This technique has not yet been widely used, and is rather time consuming and laborious to execute, so at present radioimmunoassay remains the most practical method for gastrin determinations on large numbers of samples.

Section III Radioimmunoassay of Gastrin

The observation that serum of human diabetic subjects who had been treated with mixtures of beef and pork insulin contained insulin-binding antibodies, and that increasing concentrations of unlabelled beef insulin produced a progressive decrease in the binding of ^{131}Iodine-labelled beef insulin by these antibodies (Berson, Yalow, Bauman, Rothschild and Newerly, 1956) was fundamental in the development of the technique of radioimmunoassay. Radioimmunoassay was first developed for the assay of insulin in plasma by Yalow and Berson in 1960, using antisera raised in guinea pigs immunised with beef insulin, and ^{131}I-beef insulin as tracer (Yalow and Berson, 1960; Berson and Yalow, 1972).

The first radioimmunoassay for gastrin was described by McGuigan in 1968 (McGuigan, 1968a). He produced antisera by immunizing rabbits via foot pad injections with synthetic human gastrin (SHG) (2-17) coupled to bovine serum albumen (BSA) with carbodiimide (1-Ethyl-3-(3-dimethylamino-propylcarbodiimide)) (CDI) according to the method of Goodfriend, Levine and Fasman (1964), and purified the gamma globulin fraction by ammonium sulphate precipitation before use in the assay. He used SHG (2-17) as standard, and his tracer was prepared by iodinating SHG (2-17) with ^{131}Iodine to a specific activity of 32-136 microcuries per microgram (μCi/μg), according to a modification of the method described by Hunter and Greenwood for the iodination of human growth hormone (Hunter and Greenwood, 1962). The assay diluent was 0,15M-NaCl-0,01M-potassium phosphate pH 7,4 containing 2,5 mg/ml ovalbumen. The total incubation volume was 0,5 ml and the assay was incubated for 24 hours at 4^{o}C. Separation of antibody-bound and free labelled hormone was effected using anti-rabbit gamma globulin

raised in goat. McGuigan (1968a) reported that it was possible to detect as little as 5 picograms (pg) gastrin I with this assay system.

In the years that followed a flurry of techniques for the radioimmunoassay of gastrin were published. Rather than attempt to describe these different assays in detail they are presented in table (2.III.1) with respect to the standard, tracer, antiserum, assay diluent and separation methods employed, as well as the sensitivity of the assay and normal fasting serum gastrin levels where available. Where details such as the molarity of the buffer used and whether the gastrin standard used was the (1-17) or the (2-17) sequence are missing in the table, the relevant information was not given in the references quoted.

Antisera

Antisera were raised chiefly by immunising rabbits (McGuigan, 1968a; Hansky and Cain, 1969; Stadil and Rehfeld, 1971; Ganguli and Hunter, 1972) and guinea pigs (Yalow and Berson, 1970a; Stadil and Rehfeld, 1971; Ganguli and Hunter, 1972), although in one case chickens were used for immunisation (Young, Byrnes, et al., 1969). Due to its small molecular size gastrin is not a good antigen (Walsh, 1974) so generally it is conjugated to a large carrier protein such as BSA by a coupling reagent such as carbodiimide before injection. The injection may be administered via the foot pad, subcutaneously, intraperitoneally, or intramuscularly. Synthetic human gastrin I (1-17) and (2-17) have been used for conjugation and immunisation; the rationale behind using SHG (2-17) being that removal of the N-terminal pyroglutamyl group reveals a free amino group to react with the carbodiimide. However, since CDI cross-links equally well through acidic side chains, of which gastrin has six, it is unlikely that a high proportion of conjugation

TABLE (2.III.1)

Details of Radioimmunoassays for Gastrin Previously Published

Abbreviations

SHG I : synthetic human heptadecapeptide gastrin I

gastrin I : non-sulphated gastrin

gastrin II : sulphated gastrin

numbers following a peptide in brackets indicate the amino acid sequence employed

^{131}I : ^{131}Iodine

^{125}I : ^{125}Iodine

$\mu Ci/\mu g$: microcuries per microgram

BSA : bovine serum albumen

HSA : human serum albumen

CDI : 1-Ethyl-3(3-Dimethyl-amino-propyl)-carbodiimide

pg/ml : picograms per millilitre

TABLE (2.III.1)

Authors	Date published and Reference	Standard	Tracer and Specific Activity	Animals Immunised	Antigens used for Immunisation	Assay Details: assay diluent, incubation volume incubation time	Separation of Bound and Free	Assay Sensitivity	Fasting Serum Gastrin in Control Subjects
McGuigan	1968 Gastroenterology 54 : 1005	SHG I (2-17)	^{131}I-SHG I (2-17) 32-136 μCi/μg	Rabbit. Foot pad injections	SHG I (2-17) coupled to BSA with CDI	0,15M-NaCl-0,01M-Potassium phosphate pH 7,4 containing 2,5 mg/ml ovalbumen. 0,5 ml. 24 hr x 4°C	Goat anti-rabbit gamma globulin	5 pg	
McGuigan & Trudeau	1968 New Engl.J.Med. 278 : 1308								Mean 425 pg/ml (n=24) Range 245-668 pg/ml
McGuigan & Trudeau	1970 (a) New Engl.J.Med. 282 : 353								Mean 165 pg/ml (n=102)
Trudeau & McGuigan	1971 New Engl.J.Med. 284 : 408								Mean 85 pg/ml (n=35)
Young, Byrnes et al.	1969 J.Nucl.Med. 10 : 746	SHG I	^{131}I-SHG I 200-500 μCi/μg	Chicken	Pentagastrin conjugated to rabbit serum albumen with CDI	0,15M-Phosphate pH 7,5 containing 0,5% BSA and 0,005% mercuric chloride	Amberlite anion exchange resin	50 pg/ml	Mean 400 pg/ml (n=41)
Hansky & Cain	1969 Lancet II : 1388	SHG I	^{125}I-SHG I 100-150 μCi/μg	Rabbit Intraperitoneal, intramuscular, subcutaneous	SHG I conjugated to BSA with CDI	Charcoal-adsorbed serum. 1 ml. 24 hrs, 72 hrs.	Dextran-coated charcoal	5 pg/ml	Mean 113 pg/ml Range 5-290 pg/ml
Yalow & Berson	1970 Gastroenterology 58 : 1	Porcine gastrin I	Highly purified ^{125}I-porcine gastrin I	Guinea Pig Subcutaneous	Crude porcine gastrin, unconjugated	0,02M-Veronal pH 8,4, containing 2,5 mg/ml HSA + 1% control guinea pig plasma. 2,5 ml. 4-5 days x 4°C	Amberlite resin CG-4B 200-400 mesh	Better than 5 pg/ml	<75 pg/ml (n=30)
Chan Yip & Jordan	1970 Proc.Soc.Exp. Biol.Med. (N.Y.) 134 : 380	SHG I	^{131}I- SHGI	Rabbit	Mixture partially purified porcine gastrins I and II conjugated to BSA with CDI	0,15M-NaCl-0,01M-potassium phosphate pH 7,4 containing 0,25% ovalbumen 24 hrs x 4°C	Goat anti-rabbit globulin	Less than 10 pg/ml	391 pg/ml Range 200-630 pg/ml

Continued overleaf

TABLE (2.III.1) (continued)

Authors	Date published and Reference	Standard	Tracer and Specific Activity	Animals Immunised	Antigens used for Immunisation	Assay Details : assay diluent, incubation volume incubation time	Separation of Bound and Free	Assay Sensitivity	Fasting Serum Gastrin in Control Subjects
Stadil & Rehfeld	1971 Scand.J.Gastro-enterol. Suppl. 9 : 61	SHG (1-17)	^{125}I-SHG (1-17) 300-850 μCi/μg	Rabbit Subcutaneous Guinea Pig	SHG (1-17) or (2-17) coupled to albumen with CDI	Veronal pH 8,4 containing 2,5 mg albumen/ml. 2,5 ml. 4-6 days x 4°C	Amberlite resin CG-4B 200-400 mesh	Better than 5 pg/2,5 ml	93 pg/ml Range 0-240 pg/ml
Schrumpf & Sand	1972 Scand.J.Gastro-enterol. 7 : 683	SHG I	^{125}I-SHG I 300-700 μCi/μg	Used Rehfeld's antiserum		Sodium phosphate 0,1 mol/l pH 7,5 0,02% sodium azide in 0,2% BSA. 1 ml. 48 hrs x 4°C	1,5% Norit A charcoal, 0,75% Dextran 70 in buffer without BSA	1,5 pg/tube i.e. 6 pg/ml	62 pg/ml
Ganguli & Hunter	1972 J. Physiol. 220 : 499	Highly purified natural porcine gastrin I, SHG I	^{125}I-porcine gastrin I 350-800 μCi/μg	Rabbit Guinea Pig	Partially purified porcine gastrin I, unconjugated	0,05M-Phosphate pH 7,5 16-24 hrs x 4°C	Anti-rabbit gamma globulin	25 pg/ml plasma	Mean 105 pg/ml (n=113) Range 31-270 pg/ml
Hayes, Ardill et al.	1972 Lancet I: 819		^{125}I-SHG I	Rabbit	SHG I		Dextran-coated charcoal	50 pg/ml	96 ± 57 pg/ml (mean ± 2 S.D.)
Feurle, Ketterer et al.	1972 Scand.J.Gastro-enterol. 7 : 177	Synthetic human 15-leucine gastrin	^{125}I-synthetic human 15-leucine gastrin 300 μCi/μg	Used McGuigan's method (1968)			Amberlite CG 400 II	6 pg/ml	25-125 pg/ml (n=11)
Walsh	1974 In: Nuclear Medicine in Vitro ed. Rothfeld Lippincott : 231	Porcine gastrin II SHG I	^{125}I-natural human gastrin 17 I 900 μCi/μg	Rabbit Guinea Pig	SHG I (1-17) or (2-17) conjugated to BSA with CDI	24-48 hrs x 4°C, or 5 days x 4°C	Amberlite resin IRP 58-M		20-80 pg/ml
Dockray & Taylor	1976 Gastroenterology 71 : 971	Human gastrin I	^{125}I-gastrin I	Rabbit Intradermal	Mixture natural porcine gastrin 17 I and II conjugated to BSA	Method of Yalow & Berson (1970) and Walsh (1974)			

occurs through the single amino group of gastrin (2-17) (Walsh, 1974). Young, Byrnes et al. (1969) immunised chickens with pentagastrin conjugated to rabbit serum albumen. Yalow and Berson (1970a) obtained antisera upon immunising guinea pigs with unconjugated crude porcine gastrin, as did Ganguli and Hunter (1972). Chan Yip and Jordan (1970) used a mixture of partially purified porcine gastrins I and II conjugated to BSA with CDI to obtain their antiserum in rabbits, and in 1976 Dockray and Taylor described measurement of heptadecapeptide gastrin with a specific antiserum raised by immunising rabbit with natural porcine gastrin conjugated to BSA (Dockray and Taylor, 1976).

Although McGuigan (1968a) isolated the gamma globulin fraction from his antiserum by ammonium sulphate precipitation, the general practice is to use the straight serum as collected after centrifugation of the blood, without further purification (Rehfeld, Stadil and Rubin, 1972). Dilutions quoted refer to the dilution of such antisera with assay diluent.

Gastrin Standard

Synthetic human heptadecapeptide gastrin I (SHG-17 I) prepared by Imperial Chemical Industries (I.C.I.) was the preparation used most often as the assay standard (Stadil and Rehfeld, 1971; Schrumpf and Sand, 1972; Ganguli and Hunter, 1972; Hansky and Cain, 1969). McGuigan (1968a) used the hexadecapeptide molecule (2-17) as standard, while Feurle, Ketterer, Becker and Creutzfeldt (1972) used synthetic human 15-leucine gastrin as standard and for iodination. Yalow and Berson (1970a) found a discrepancy in immunochemical potency between two different batches of synthetic human gastrin obtained from I.C.I. and they chose to use porcine gastrin I as standard, since it showed the greatest immunochemical potency, and porcine

gastrins I and II were equipotent immunologically in their assay system. Stadil and Rehfeld (1972) also reported that different ampoules of SHG obtained from I.C.I. contained impurities and differed in immunoreactivity. Porcine gastrin I was used as standard by Ganguli and Hunter (1972); Walsh (1974) reported using both porcine gastrin 17 II and human gastrin 17 I as standards. According to Walsh (1974), newly synthesised batches of SHG are available from I.C.I., and differences in potency among various vials have not been reported with this material.

Iodination of Gastrin

Earlier assays were carried out using synthetic human gastrin labelled with ^{131}Iodine (McGuigan, 1968a; Young, Byrnes et al., 1969; Chan Yip and Jordan, 1970), whereas more recently ^{125}Iodine has been the isotope of choice (Schrumpf and Sand, 1972; Ganguli and Hunter, 1972; Walsh, 1974). To obtain maximal sensitivity in a radioimmunoassay it is necessary to have a satisfactory counting rate with a very small concentration of labelled hormone. The ratio of counting rate to tracer concentration is influenced by the specific activity of the tracer and the efficiency of counting (Yalow and Berson, 1968).

The specific activity attainable depends on the individual hormone and on the isotopic abundance of the iodine itself (Yalow and Berson, 1968). ^{131}Iodine has a half-life of 8 days and ^{125}Iodine has a half-life of 57 days, so it would appear that ^{131}Iodine would be better for obtaining a high count rate because of its shorter half-life. However, fission-produced ^{131}Iodine only has an abundance of approximately 30%, whereas ^{125}Iodine is available virtually in a carrier-free state. In addition, the efficiency of detection of ^{125}Iodine may be two to three times that of ^{131}Iodine (Yalow and Berson,

1968). Thus ^{125}Iodine is the more suitable isotope for use in a radioimmunoassay sensitive enough to detect hormone concentrations in the picogram range (Yalow and Berson, 1968).

The specific activity of carrier-free ^{131}Iodine is approximately 125 millicuries per microgram (mCi/μg) and of ^{125}Iodine is approximately 18 mCi/μg (Yalow and Berson, 1968). This value refers to the ratio of radioactive iodine atoms to cold iodine atoms and should not be confused with the specific activity of the labelled hormone, which refers to the ratio of radioactive iodine atoms incorporated per unit weight of hormone. Increasing the number of radio iodine substitutions in the tyrosine ring increases the specific activity of the labelled hormone, but over-iodination may decrease the immunoreactivity of the labelled preparation (Yalow and Berson, 1968). In the case of monoiodinated gastrin, with a substitution of 1 atom of iodine per molecule of gastrin, the specific activity of the labelled product is approximately 900 μCi/μg according to Walsh (1974) and approximately 1 000 μCi/μg according to Ganguli and Hunter (1971), assuming an isotopic abundance of 100% for ^{125}Iodine.

Iodinations have been performed as modification of the chloramine-T technique described by Hunter and Greenwood (1962) for the iodination of human growth hormone with ^{131}Iodine (Ganguli and Hunter, 1972; Yalow and Berson, 1970a; Stadil and Rehfeld, 1971), or according to the method described by Ganguli and Hunter (1971) for the iodination of gastrin with ^{125}Iodine. Specific activities obtained in assays described ranged from 200 - 500 μCi/μg for ^{131}I-gastrin labelled by Young, Byrnes et al. (1969) to 900 μCi/μg for ^{125}I-gastrin described by Walsh (1974).

Standard gastrin preparations that have been used for iodination include SHG-17 I (Stadil and Rehfeld, 1971), synthetic human 15-leucine gastrin (Feurle, Ketterer et al., 1972) and highly purified porcine gastrin (Yalow and Berson, 1970a; Ganguli and Hunter, 1972).

Assay Procedure

Assay diluent buffers that have been used include 0,15M-NaCl-0,01M-potassium phosphate pH 7,4 (McGuigan, 1968a; Chan Yip and Jordan, 1970), phosphate buffer pH 7,5, 0,15M (Young, Byrnes et al., 1969) or 0,05M (Ganguli and Hunter, 1972), 0,02M-veronal pH 8,4 (Stadil and Rehfeld, 1971; Yalow and Berson, 1970a), or straight charcoal-adsorbed serum (Hansky and Cain, 1969). All buffers contained low concentrations (usually 0,25%) of ovalbumen, bovine serum albumen (BSA), or human serum albumen (HSA), presumably to minimise adsorption of hormone to the assay vessel. Schrumpf and Sand (1972) obtained a steeper standard curve with 0,1M-sodium phosphate buffer pH 7,5 compared to 0,1M-veronal buffer pH 8,4, and 0,1M-Tris-HCl buffer pH 7,5, both of which gave similar results.

Total volumes incubated ranged from 0,5 ml (McGuigan, 1968a) to 2,5 ml (Yalow and Berson, 1970a) and incubation times varied from 24 hours at 4°C (McGuigan, 1968a) to 4-6 days at 4°C (Stadil and Rehfeld, 1971). The length of time of incubation depends on the nature of the antibody and the range of gastrin concentrations in unknown samples (Walsh, 1974), so that longer incubation periods are used when low concentrations of antibody and tracer are used to measure very low concentrations of gastrin (Walsh, 1974).

In some cases the use of preservatives in the assay diluent has been described. Young, Byrnes et al. (1969) used 0,005% mercuric chloride in their buffer, while Schrumpf and Sand (1972) added 0,02% sodium azide to

their phosphate buffer.

Stadil and Rehfeld (1973a) found that sodium chloride and heparin in the incubation mixture interfered with the binding of antigen, and when separation of antibody-bound and free labelled hormone was carried out using Amberlite anion exchange resin, heparin had to be excluded altogether.

Separation Techniques

Early methods for separation of antibody-bound and free labelled hormone involved the technique of high voltage paper electrophoresis using Whatman no. 3 or 3MM paper, or chromatoelectrophoresis using Whatman WB-2 resin paper (Yalow and Berson, 1970a). Yalow and Berson (1970a) reported that free labelled gastrin added to plasma does not adsorb to Whatman no. 3 or 3MM paper but migrates immediately in front of serum albumen, whereas antibody-bound gastrin migrates only very slightly from the site of application. This paradoxical behaviour of gastrin on paper strips rules out the technique of chromatoelectrophoresis for separation of antibody-bound and free labelled gastrin. Yalow and Berson (1960) developed the technique of immunoassay of insulin in plasma using Whatman 3MM paper to separate antibody-bound and free labelled insulin in the presence of increasing concentrations of insulin. However, the use of separate paper strips for each standard and unknown is laborious and this separation technique has been superceded by more suitable techniques described below.

Separation of antibody-bound and free labelled hormone has been described using Amberlite anion exchange resin (Young, Byrnes et al., 1969; Yalow and Berson, 1970a; Stadil and Rehfeld, 1971; Feurle, Ketterer et al., 1972; Walsh, 1974) and dextran-coated charcoal (Hansky and Cain, 1969; Schrumpf and Sand, 1972; Hayes, Ardill, Kennedy, Shanks and Buchanan, 1972) to

adsorb and precipitate the free labelled hormone. Precipitation of antibody-bound labelled hormone has been effected using a double antibody precipitating technique such as goat anti-rabbit gamma globulin (McGuigan, 1968a; Chan Yip and Jordan, 1970; Ganguli and Hunter, 1972) or 50% polyethylene glycol (Carbowax 6000) in water (Stadil and Rehfeld, 1973a).

Stadil and Rehfeld (1973a) obtained identical curves of displacement of antibody-bound labelled gastrin with increasing concentrations of unlabelled gastrin when separation was effected using charcoal, polyethylene glycol, and two types of Amberlite resin, CG 4B and IRP 58. They showed no advantage in using dextran-coated charcoal over untreated charcoal and they found that it was essential to have a uniform protein concentration in all samples when separation was carried out using polyethylene glycol and uncoated charcoal, whereas this was not critical when Amberlite was used (Stadil and Rehfeld, 1973a). The presence of heparin influenced separation using Amberlite resin CG 4B, making it necessary to avoid using this combination (Stadil and Rehfeld, 1973a).

Rehfeld and Stadil (1973b) described a solid phase gastrin radioimmunoassay employing the immunosorbent bromoacetylcellulose (BAC) to which they covalently coupled their antiserum 2604-7. This technique allows a very simple preparation procedure whereby the antibody-bound free labelled hormone is precipitated by centrifugation of the BAC-antibody conjugate. Apart from a small decrease in sensitivity and the fact that more antiserum is used than with conventional methods, the assay parameters using this method compare favourably with those obtained when separation of free and bound hormone is carried out using charcoal, polyethylene glycol, and anion exchange resin (Rehfeld and Stadil, 1973b).

Detection Limits of the Assay

The lowest concentration of hormone measurable that differs significantly from zero is known as the detection limit or sensitivity of the assay. The most sensitive assays for gastrin reported sensitivities of 5 picograms per millilitre (pg/ml) or less (Hansky and Cain, 1969; Yalow and Berson, 1970a; Stadil and Rehfeld, 1971). Ganguli and Hunter (1972) reported an assay sensitivity of 25 pg/ml, while Young, Byrnes et al. (1969) and Hayes, Ardill et al. (1972) reported a sensitivity of 50 pg/ml.

Serum Gastrin Levels in Control Subjects

McGuigan and Trudeau (1968) reported a mean fasting serum gastrin level of 425 pg/ml in 24 normal adult subjects. In 1970 the same workers found a mean fasting serum gastrin concentration of 165 pg/ml in 102 subjects (McGuigan and Trudeau, 1970a), while in 1971 they reported an even lower normal fasting serum gastrin level in 35 controls of 85 pg/ml (Trudeau and McGuigan, 1971). A similar finding was described by Stadil and Rehfeld (1971), who reported a mean normal serum gastrin level of 93 pg/ml, whereas in 1973(a) they found a mean fasting level of 52 pg/ml $\pm$ 4,6 pg/ml ($\pm$ S.E.M.) in 120 control subjects. This decrease in gastrin levels is attributable to an improvement in the quality of the labelled preparation in more recent years (Stadil and Rehfeld, 1973a) or to improvements in the radioimmunoassay technique (McGuigan and Trudeau, 1970a; Berson and Yalow, 1972).

Most other groups reported mean fasting serum gastrin levels in normal subjects of below 125 pg/ml, which was the highest value found by Feurle, Ketterer et al. (1972), with the lowest values reported being 20 pg/ml (Walsh, 1974). In general, normal levels appear to be below 100 pg/ml, as shown in table (2.III.1). Two exceptions to this are the assays reported

by Young, Byrnes et al. (1969), who reported a mean fasting level of 400 pg/ml and Chan Yip and Jordan (1970) who found a normal level of 391 pg/ml. Since Young, Byrnes et al. (1969) could only obtain an assay sensitivity of 50 pg/ml it is hardly surprising that their normal levels were so high.

McGuigan and Trudeau (1970a) showed a direct relation between increasing age and increasing fasting serum gastrin concentrations. They found mean fasting serum gastrin levels of below 100 pg/ml in subjects from the age group 20–39 years, with an increase up to between 500 and 600 pg/ml in subjects over the age of 80 years. They suggested that the higher serum gastrin concentrations in older patients were due to gastric mucosal atrophy or reduced rates of acid secretion (or both) (McGuigan and Trudeau, 1970a). In contrast to these findings no increase in serum gastrin concentrations with age was found by Ganguli and Hunter (1972) or Stadil and Rehfeld (1973a). These two groups of workers did not find any differences in serum gastrin levels between normal males and females (Ganguli and Hunter, 1972; Stadil and Rehfeld, 1973a).

An increase in serum gastrin levels in response to feeding was first reported by Hansky and Cain (1969). This has been confirmed by several workers and in every case ingestion of proteins has been shown to elicit the greatest response. McGuigan and Trudeau (1970a) showed an increase in serum gastrin levels from 165 pg/ml in fasting subjects to 209 pg/ml postprandially. Young, Byrnes et al. (1969) reported an increase from a basal level of 400 pg/ml to 2 700 pg/ml following ingestion of protein. Ganguli (1970) examined the effect of ingestion of protein, carbohydrate and fat on serum gastrin levels and found the greatest response was to a

55% protein meal. Serum gastrin levels rose 4 to 5 times the fasting level, with the peak response occurring 30 to 60 minutes after eating. Korman, Soveny and Hansky (1971) also reported an increase in gastrin levels of up to 5 times basal 45 minutes after eating protein, with levels increasing from 15 $\pm$ 2,3 pg/ml ($\pm$ S.E.M.) to 78 $\pm$ 16 pg/ml. These authors showed a lower gastrin releasing potency with alcohol, fat and glucose, decreasing in that order, whereas Ganguli (1970) found that no significant effect on serum gastrin levels was produced by a 93% carbohydrate or a 93% fat meal. Normal fasting serum gastrin levels and postprandial levels in controls are shown in table (2.III.2).

A circadian pattern of gastrin release has been described by Feurle, Ketterer et al. (1972) and by Moore and Wolfe (1974). Both groups found higher gastrin levels during the day and the lowest levels during the hours following midnight. Moore and Wolfe (1974) found the highest mean 2-hourly secretion rate of gastrin at 8 p.m. (75 pg/ml) and the lowest at 4 a.m. (33 pg/ml). Feurle, Ketterer et al. (1972) reported that the major gastrin peaks during the day seemed to be in response to food intake.

In addition to ingested protein producing elevated gastrin levels, gastrin release is stimulated by a number of other factors. The mechanical effect of food in the stomach causing distension has been shown to stimulate release of gastrin in the dog (Grossman, Robertson and Ivy, 1948) and Feurle, Ketterer et al. (1972) reported very high gastrin levels in patients with prepyloric ulcer and pyloric stenosis, which leads to gastric distension. Insulin hypoglycaemia stimulates gastrin release in man (Stadil, 1972) and this may operate via vagal excitation, which is known to cause release of gastrin as determined by bioassay (Fyrø, 1967). Catecholamines stimulate release of gastrin in man (Stadil and Rehfeld, 1973b) and infusion of epi-

nephrine into dogs produced elevated gastrin levels (Hayes, Ardill et al., 1972).

Serum Gastrin Levels in Disease States

In keeping with the numerous radioimmunoassays published for gastrin which are outlined in table (2.III.1) there have been many reports on serum gastrin levels measured in various disease conditions. Some of this information has been extracted from the literature and is presented in table (2.III.2). From the table it appears that serum gastrin levels in duodenal ulcer (D.U.) patients in the fasting state do not differ notably from those in fasting controls (Stadil and Rehfeld, 1971, 1973a; Schrumpf and Sand, 1972; Feurle, Ketterer et al., 1972) or may even tend to be lower than controls (Trudeau and McGuigan, 1970; Hansky and Cain, 1969; Ganguli and Hunter, 1972). Higher levels in D.U. patients as opposed to normals were found by Byrnes, Young et al. (1970) and Reeder, Jackson et al. (1970), but the serum gastrin levels reported by Byrnes, Young et al. (1970) were particularly high, even in fasting controls. Generally gastrin levels in patients with gastric ulcer (G.U.) were higher than in controls and levels in D.U. patients (Hansky and Cain, 1969; Schrumpf and Sand, 1972; Ganguli and Hunter, 1972). Young, Byrnes et al. (1969) reported levels of 400 pg/ml in G.U. and normal fasting subjects, whereas in D.U. patients they detected levels as high as 1 300 pg/ml (Byrnes, Young et al., 1970). Stadil and Rehfeld (1973a) found no difference between gastrin levels in patients with pre-pyloric or duodenal ulcers and normals.

On the basis of their findings Trudeau and McGuigan (1970) suggested that although the mean fasting gastrin levels were not elevated in patients with peptic ulcer disease, these patients may be more sensitive than normal

TABLE (2.III.2)

Gastrin Levels in Various Clinical Conditions

Abbreviations

D.U.	:	Duodenal ulcer
G.U.	:	Gastric ulcer
Z.E.S.	:	Zollinger-Ellison syndrome
P.A.	:	Pernicious anaemia
pg/ml	:	picograms per millilitre
ng/ml	:	nanograms per millilitre

TABLE (2.III.2)

Authors	Date Published and Reference	Normal Fasting Levels	D.U. Fasting	G.U. Fasting	'Z.E.S. Fasting	P.A. Fasting	Peak Response in Normals after Eating	Peak Response in D.U.'s after Eating
McGuigan & Trudeau	1968 New Engl.J.Med. 278 : 1308	425 pg/ml (n=24)			3550 pg/ml - 21 ng/ml (n=3)			
McGuigan & Trudeau	1970 (a) New Engl.J.Med. 282 : 358	165 pg/ml (n=102)				997 pg/ml	209 pg/ml (n=30)	
Trudeau & McGuigan	1970 Gastroenterology 59 : 6	165 pg/ml (n=102)	82 pg/ml	126 pg/ml				
Young, Byrnes et al.	1969 J.Nucl.Med. 10 : 746	400 pg/ml (n=41)			200 ng/ml (n=1)		2700 pg/ml (protein)	
Byrnes, Young et al.	1970 Br.Med.J. 2 : 626		1300 pg/ml (n=27)	400 pg/ml (n=12)			4700 pg/ml (protein)	14900 pg/ml
Hansky & Cain	1969 Lancet II : 1388	113 pg/ml (5-290 pg/ml)	53 pg/ml	165 pg/ml	2800 pg/ml, 5000 pg/ml (n=2)	5000 pg/ml (n=1)		
Korman, Soveny & Hansky	1971 Gut 12 : 899	15 + 2,3 pg/ml (± S.E.M.)					78 ± 16 pg/ml (± S.E.M.) (protein)	
Yalow & Berson	1970 Gastroenterology 58 : 1	<75 pg/ml (n=30)			1500-10000 pg/ml (n=4)	300-9000 pg/ml (n=17)		
Reeder, Jackson et al.	1970 Surg. Forum 21 : 290	63 pg/ml (n=5)	106 pg/ml (n=6)				148 pg/ml	250 pg/ml
Stadil & Rehfeld	1971 Scand.J.Gastro-enterol. Suppl. 9 : 61	93 pg/ml (0-240 pg/ml)	76 pg/ml (0-166 pg/ml)					
Stadil & Rehfeld	1973 (a) Scand.J.Gastro-enterol. 8 : 101	52 pg/ml (2,9-95 pg/ml)	50 pg/ml (16-93 pg/ml)		10,97 ng/ml (290 pg/ml-13,5 ng/ml)	913 pg/ml (30-3750 pg/ml)		
Schrumpf & Sand	1972 Scand.J.Gastro-enterol. 7 : 683	62 pg/ml	62 pg/ml	114 pg/ml	630-2380 pg/ml (n=3)			
Ganguli & Hunter	1972 J.Physiol. 220 : 499	105 pg/ml (n=113)	91 pg/ml (n=27)	285 pg/ml (n=14)	1212 pg/ml-94 ng/ml (n=9)	1167 pg/ml (n=51)		
Feurle, Ketterer et al.	1972 Scand.J.Gastro-enterol. 7 : 177	25-125 pg/ml (n=11)	50-130 pg/ml (n=9)					

subjects to gastrin-provoking stimuli, resulting in responses of greater magnitude and longer duration.

Postprandial serum gastrin levels in D.U. patients are higher than those in normals (Byrnes, Young et al., 1970; Reeder, Jackson et al., 1970); the first group showed a greater integrated gastrin response from 0 to 20 minutes after ingestion of protein in D.U. patients as compared with normal subjects.

In their study on circadian gastrin concentrations Feurle, Ketterer et al. (1972) observed gastrin levels somewhat but not significantly higher in D.U. patients compared with controls during the day, whereas during the night gastrin levels in D.U. patients were consistently higher than in controls. Gastrin levels in patients with pyloric ulcer were higher than both controls and D.U. patients throughout the 24 hours.

Grossly elevated levels of circulating gastrin have been well documented in patients with Zollinger-Ellison syndrome (Z.E.S.) (McGuigan and Trudeau, 1968; Young, Byrnes et al., 1969; Hansky and Cain, 1969; Yalow and Berson, 1970a; Schrumpf and Sand, 1972; Ganguli and Hunter, 1972; Stadil and Rehfeld, 1973a). This condition, also known as gastrinoma, was first described by Zollinger and Ellison in 1955 (Zollinger and Ellison, 1955) and involves massive gastric acid hypersecretion caused by a non-beta islet cell tumour arising usually in the pancreas, which releases massive amounts of gastrin, producing severe and intractable peptic ulceration (Gregory, 1970). Primary tumours of this type have also been found in the submucosa of the duodenum (Creutzfeldt, Arnold, Creutzfeldt and Track, 1975), although they are uncommon (Isenberg, Walsh and Grossman, 1973). Gastrinoma was the first clinical condition described involving a tumour of gastrointestinal endocrine cells (Gregory, 1970). Serum gastrin levels reported in this condition

range from approximately 1 nanogram per millilitre (ng/ml) up to as high as 94 ng/ml (Ganguli and Hunter, 1972; Schrumpf and Sand, 1972; Yalow and Berson, 1970a) as can be seen in table 2.III.2). The importance of serum gastrin measurements in the pre-operative diagnosis of this condition is obvious.

Pernicious anaemia (P.A.) is also associated with very high gastrin levels (Hansky and Cain, 1969; Yalow and Berson, 1970a; McGuigan and Trudeau, 1970a; Ganguli and Hunter, 1972; Stadil and Rehfeld, 1973a). Reported serum gastrin levels appear in table (2.III.2). This condition is characterised by gastric mucosal atrophy with extensive destruction of oxyntic (parietal) cells and zymogen (chief) cells, resulting in the absence of gastric secretion of both hydrochloric acid and intrinsic factor, which in man are both produced by the parietal cells (McGuigan and Trudeau, 1970a). It is thought that the resultant achlorhydria in this condition causes withdrawal of the effect of inhibition of gastrin release exerted by antral acidification, permitting uninhibited release of gastrin in response to stimuli acting on the gastrin-containing cells, thus producing elevated serum gastrin levels (McGuigan and Trudeau, 1970a). Yalow and Berson (1970a) produced an acute fall in plasma gastrin concentration in five patients with pernicious anaemia by oral administration of hydrochloric acid, an observation lending support to the proposal of McGuigan and Trudeau (1970a).

The two conditions involving hypergastrinaemia are thus very different. Zollinger-Ellison syndrome involves hypergastrinaemia associated with acid hypersecretion, whereas pernicious anaemia presents as hypergastrinaemia occurring with achlorhydria. In P.A. patients an increase in serum gastrin levels in response to feeding has been reported (McGuigan and Trudeau,

1970a; Yalow and Berson, 1972) whereas in Z.E.S. Berson and Yalow (1972) reported no detectable increase in serum gastrin in response to a protein meal in 5 patients. Patients with Z.E.S. respond to infusions of calcium with secretion of acid and release of gastrin from tumour tissue (Creutzfeldt, Arnold et al., 1975; Passaro, Basso and Walsh, 1972). Intravenous secretin infused into Z.E.S. patients produces a paradoxical rise in gastrin secretion in about 75% of patients (Dockray, 1975; Creutzfeldt, Arnold et al., 1975), whereas in normal subjects secretin infusion produces a decrease in gastrin levels (Hansky, Soveny and Korman, 1971) or has no effect (Creutzfeldt, Arnold et al., 1975).

Hypergastrinaemia has been described in two cases of phaeochromocytoma, where the serum gastrin levels were 400 pg/ml and 450 pg/ml (Hayes, Ardill et al., 1972). These workers reported a normal serum gastrin level of 96 pg/ml. After successful removal of the tumour basal gastrin levels returned to normal in both patients. This situation, as well as the elevated gastrin levels obtained on infusion of epinephrine into dogs by the same workers, supports the notion that catecholamines stimulate gastrin release.

The fact that serum gastrin measurements obtained with various gastrin immunoassays vary considerably among different groups emphasises the need to characterise the antiserum used in the assay with regard to its specificity for the different gastrin types. Antisera raised to gastrin may recognise sulphated, non-sulphated or both types of gastrin, of small or large molecular forms, and only when the specificity of the antiserum is defined are the gastrin levels quoted really meaningful. Some of the better known antisera have been well characterised and are discussed in the next section.

Section IV Specificity of Gastrin Antisera

Since gastrin does not circulate only in the heptadecapeptide form described in the previous sections, but exists in several different forms of various molecular size, it is important to characterise the specificity of the antiserum used in the assay so that one knows what type of gastrin is being measured.

The various gastrin forms are described in detail in the following two sections and will be outlined here for the purposes of this discussion. Yalow and Berson (1970b, 1971a, 1972) and Yalow and Wu (1973) have described the existence of three types of gastrin, namely "big big" gastrin which elutes in the void volume on Sephadex G-50 gel filtration, big gastrin which elutes between molecular weight markers proinsulin and insulin, and heptadecapeptide gastrin. Four gastrin types have been found by Rehfeld, Stadil and Vikelsøe (1974). They did not find the "big big" gastrin of Yalow and Berson in serum, although they did find a very large molecular weight heterogeneous component in gastrinoma and antral mucosa tissue extracts. They have identified component I which elutes immediately before proinsulin, component II which corresponds to big gastrin, component III or heptadecapeptide gastrin, and component IV, which they called minigastrin. Both groups of workers showed that the major form of gastrin occurring in serum is big gastrin and not the heptadecapeptide form as was previously thought.

Big gastrin has been purified from human gastrinoma extracts (Gregory and Tracy, 1972) and from hog antral mucosa (Gregory and Tracy, 1973, 1975) in two forms corresponding to the sulphated and non-sulphated species. Their amino acid sequences were determined by Gregory and Tracy (1972, 1973, 1975), who showed that each consisted of 34 amino acids of which the

C-terminal 17 amino acids were identical with the sequence of heptadecapeptide gastrin, and the presence or absence of a sulphate group on the tyrosine residue in position 12 of the heptadecapeptide determined whether they were big gastrins type II or I respectively. The N-terminal (1-17) sequence of big gastrin is linked to the glutamyl group of heptadecapeptide gastrin via a lysine residue, this being the peptide bond which is cleaved on incubation of big gastrin with trypsin to release heptadecapeptide gastrin (Yalow and Berson, 1971; Rehfeld and Stadil, 1973). Component I of Rehfeld and Stadil and "big big" gastrin of Yalow and Berson have not yet been chemically or biologically characterised (Dockray, 1975; Gregory, 1976).

Minigastrins type I and II have been isolated from gastrinoma tissue extract and have been shown to correspond to the (5-17) tridecapeptide sequence of heptadecapeptide gastrin (Gregory and Tracy, 1974) in the non-sulphated and sulphated forms respectively. The amino acid sequences of human big, heptadecapeptide and minigastrin are shown in figure (3.1.2.) in the next chapter.

Walsh, Trout, Debas and Grossman (1974) formulated a convenient nomenclature for each gastrin type referring to its source, amino acid content and presence or absence of sulphated tyrosine in position 12 of the heptadecapeptide. The source of gastrin is designated "P" for porcine, "H" for human, and other species are spelled out. The designation "G" refers to gastrin, followed by a number indicating the number of amino acid residues. There is uncertainty as to whether big gastrin consists of 33 or 34 amino acids, so it could be abbreviated as G-33 or G-34. By the same token, minigastrin is thought to comprise either 13 or 14 amino acids and is abbreviated as G-13 or G-14. Thus the abbreviation for human heptadecapeptide gastrin II is

HG-17 II. This convention has been adopted for the purposes of this thesis. If an abbreviation appears in which there is no letter before the "G" to indicate the source, it should be assumed that the peptide is human in origin.

Peptides bearing a structural resemblance to heptadecapeptide gastrin include caerulein and cholecystokinin-pancreozymin (CCK-PZ) both of which bear the identical C-terminal pentapeptide sequence as does heptadecapeptide gastrin and both of which contain a sulphated tyrosine residue one position removed from the pentagastrin sequence. Caerulein is a decapeptide which has been isolated from the skin of the Australian bullfrog, Hyla caerulia (Anastasi, Erspamer and Endean, 1967). CCK-PZ is a peptide hormone consisting of 33 amino acids which is secreted by the duodenal mucosa in response to stimulation by the products of fat and protein digestion and causes the secretion of pancreatic juice rich in enzymes, as well as stimulating contraction of the gall bladder (Ganong, 1977). The primary structures of caerulein and the C-terminal octapeptide of CCK-PZ (Ondetti, Squibb) are shown in figure (3.1.1.). Commercially prepared pentagastrin (Peptavlon, I.C.I.) as used for acid-stimulatory tests has the glycine residue in position 13 of the heptadecapeptide replaced by alanine, which is blocked by a butyloxycarbonyl group (BOC), a remnant of the synthesis of this molecule.

From the structural homology displayed by these gut and gastrin-related peptides it is apparent that an antiserum produced by immunizing an animal with one of these peptides will not necessarily be specific for that peptide only, but may show a degree of recognition for one or more of the related peptides as well. Thus it is essential to determine the degree of cross-reaction in the assay of all the gastrin types, of CCK-PZ, of caerulein and of pentagastrin with each antiserum used. Only then can one be sure of what is

being measured by the particular antiserum. If the specificity of an antiserum is not stated then the gastrin levels reported should be viewed with caution.

A further consideration is whether the antiserum is specific for sulphated or non-sulphated types of gastrin or whether it recognises both types equally well. It may happen that an antiserum raised using synthetic human heptadecapeptide gastrin I measures only the non-sulphated form of gastrin. Hansky, Soveny and Korman (1973) described two antisera raised in rabbits to synthetic human gastrin I. One antiserum (4) only recognised gastrin I and showed minimal reactivity with gastrin II, whereas the other one (35) cross-reacted equally with both gastrins I and II. Using these antisera to measure serum gastrin levels in normal subjects and ulcer patients they found significantly higher levels measured with antiserum 35 than measured with antiserum 4, emphasising the importance of the specificity of the antiserum.

Rehfeld, Stadil and Rubin (1972) immunized 41 guinea pigs and 72 rabbits according to 15 different immunisation schemes. They obtained antibodies to gastrin in 23 animals, of which only one was a guinea pig. Only three antisera had antibodies of sufficient affinity for measurement of low physiological concentrations, with a value of K^{o}, the equilibrium constant, greater than or equal to 10^{11} litres/mole (l/mol). This gives one an idea of how difficult it is to obtain a good antiserum to gastrin. Antiserum 2484, raised in guinea pig to unconjugated crude porcine gastrin showed only a minimal degree (1:0,001) of cross-reactivity with porcine CCK-PZ prepared by Jorpes and Mutt. The specificity of the antisera is expressed by the molar ratio between the inhibition dose 50 (ID_{50}) for synthetic human gastrin I and the ID_{50} of the peptide with which it is being compared (Stadil and Rehfeld, 1973a). The inhibition dose 50 refers to the molar concentration of peptide that reduces the binding of labelled gastrin by antibody to 50%

of its initial value. Antiserum 2604 raised to SHG(2-17) conjugated to bovine serum albumen reacted poorly with porcine CCK-PZ (1:0,006) and with the synthetic octapeptide of CCK-PZ prepared by Ondetti of Squibb (1:0,001) (Rehfeld, Stadil et al., 1972). An aliquot of the seventh bleed obtained from this animal, designated 2604-7, was kindly donated by Dr. J. Rehfeld and was used in studies in this thesis. The third antiserum, 2720, which was raised to SHG(1-17) coupled to bovine serum albumen, showed a high degree of cross-reactivity with porcine cholecystokinin (1:0,2) making it unsuitable for gastrin determinations in biological fluids (Rehfeld, Stadil et al., 1972). These three antisera did not cross-react with human growth hormone, human insulin, porcine glucagon or synthetic secretin, nor with pentagastrin (I.C.I.) or tetragastrin (Leo Pharmaceutical Products, Copenhagen) except in the case of antiserum 2720, which cross-reacted negligibly with the latter two peptides (Rehfeld, Stadil et al., 1972). Antisera 2604 and 2484 were used in studies which showed the presence of three components of immunoreactive gastrin in serum (Rehfeld and Stadil, 1973a).

Dockray (1975) and Walsh, Trout et al. (1974) have described two antisera, 1296 and 1295, with different specificities for different regions of the heptadecapeptide gastrin molecule. These antisera were raised in rabbits immunized with G-17 conjugated to bovine serum albumen (Dockray, 1975). Antibody 1296 gave parallel inhibition curves with G-17 I and G-34 I; other peptides with the same carboxy-terminal portion as G-17, such as the (2-17) sequence of G-17, and G-13, also cross-reacted with this antibody. Amino-terminal fragments of G-17, the (1-13) sequence of G-17 and desamido G-17 did not cross-react, indicating that antibody 1296 was specific for the C-terminal region of G-17 (Dockray, 1975). On the other hand, antibody 1295 was found to be specific for the N-terminal region of G-17, since it cross-reacted

with G-17, the (1-13) sequence of G-17 and desamido G-17, but not with G-34, the (2-17) sequence of G-17, or G-13 (Dockray, 1975). With both antibodies serum from a patient with gastrinoma gave parallel displacement curves, indicating that they were both suitable for estimating serum gastrin components (Dockray, 1975).

Confirmation of the specificity of these antisera was obtained by measurement of column eluates obtained on fractionation of serum from a gastrinoma patient on Sephadex G-50. Antibody 1296 revealed peaks of immunoreactivity which emerged in the same regions as standard G-34 and G-17, whereas antibody 1295 detected the G-17 peak but not the G-34 peak. However, this antibody also detected a peak which had the pattern of immunoreactivity of an N-terminal fragment of G-17 and the same elution volume as the (1-13) fragment of G-17 (Dockray, 1975). Since this fragment is not biologically active (Dockray and Walsh, 1975), Dockray (1975) continued to use antibody 1296 for measurement of biologically active gastrin in blood and tissues.

Dockray and Taylor (1976) described another antibody, L 6, which is virtually absolutely specific for heptadecapeptide gastrin. It was obtained by immunizing a New Zealand white rabbit with approximately equal amounts of natural porcine G-17 I and G-17 II conjugated to bovine serum albumen. Parallel curves showing inhibition of binding of ^{125}I-G-17 to antiserum L 6 were obtained with human G-17 and serum from a patient with Zollinger-Ellison syndrome, while no inhibition of binding was shown by G-34, the (2-17) sequence of G-17 or the (1-13) sequence of G-17 (Dockray and Taylor, 1976). The antiserum cross-reacted with both sulphated and non-sulphated human and porcine G-17 as well as with feline G-17 I and 15-leucine G-17 I (Dockray and Taylor, 1976). No significant inhibition of

binding of ^{125}I-G-17 was obtained with a combination of equimolar amounts of the sequences (1-13) and (14-17) of G-17, confirming that the intact peptide backbone was required for recognition by the antibody (Dockray and Taylor, 1976).

Walsh, Trout et al. (1974) compared the specificity of five different antisera raised in rabbits. Antibody 1296 recognises all forms of gastrin as already described, and shows little cross-reactivity with CCK-PZ and its octapeptide. Antibodies 1295 and 1294 are highly specific for G-17 but do not distinguish between G-17 I and G-17 II. Antibody 1292 distinguishes between sulphated and non-sulphated G-17 (Walsh, Trout et al., 1974). Antibody SF-10, raised by immunizing a rabbit with the conjugated C-terminal tetrapeptide amide of gastrin, was inhibited similarly by G-33, G-17, G-13, CCK-PZ octapeptide and to a lesser extent by equimolar amounts of whole CCK-PZ (Walsh, Trout et al., 1974).

McGuigan and Herbst (1974) obtained region-specific antibodies by immunizing rabbits with either SHG I (2-17) or SHG I (1-13) covalently conjugated to bovine serum albumen. Their antibodies raised using the (2-17) sequence of heptadecapeptide gastrin exhibited nearly equivalent immunological reactivity with big gastrin as with equimolar amounts of heptadecapeptide gastrin, whereas the antibodies raised to SHG (1-13) did not react with big gastrin or "big big" gastrin, but did recognise heptadecapeptide gastrin. Using these two antibody types McGuigan and Herbst (1974) demonstrated that fasting immunoreactive gastrin in serum comprised more than 80% big gastrins, and that gastrin released into the circulation in response to feeding was the heptadecapeptide form.

Antisera may also vary in their specificity for gastrins of different species. Generally antibodies raised in rabbits with conjugated gastrin do not

distinguish among gastrins from different species but may react differently with different sizes of gastrin and may distinguish sulphated and non-sulphated gastrins (Walsh, Trout et al., 1974). As a rule gastrin antibodies prepared in guinea pigs against crude porcine gastrin are often specific for substitutions in the 8 and 10 positions of G-17 but do not differ in reactivity with G-17 and G-13 and do not discriminate between sulphated and non-sulphated gastrins. Thus sheep gastrin, which bears a substitution of alanine for glutamic acid in position 10 of the heptadecapeptide, as well as valine in place of leucine in position 5, reacted with a potency of less than 10% of that of porcine gastrin with a guinea pig antibody, while with a rabbit antibody the potency was more than 50% (Walsh, Trout et al., 1974). Porcine gastrins I and II were more reactive than synthetic human gastrin I with Rehfeld's antiserum 2484 which was raised in guinea pig to crude porcine gastrin, while in the case of antiserum 2604, raised in rabbit to SHG (2-17), the reaction with porcine gastrin was less than that with human gastrin (Rehfeld, Stadil et al., 1972).

Antibodies can be regarded as the "tools" for measurement of hormone levels by radioimmunoassay. Once they have been characterised with regard to their specificity, they can be used to investigate hormonal heterogeneity in serum and tissue extracts.

Section V Heterogeneity of Gastrin in Human Serum

By combining the techniques of radioimmunoassay and Sephadex column chromatography it was shown that gastrin occurs in several forms of different molecular size. Gastrin heterogeneity in serum and tissues should be considered together, since the findings of various types of gastrin in antral mucosa and gastrinoma tissues were confirmed by the discovery of similar gastrin forms in the circulation. For simplicity, gastrin heterogeneity in serum and tissues will be dealt with separately.

Yalow and Berson (1970b) were first to show that the major fraction of immunoreactive plasma gastrin was not identical with the heptadecapeptide gastrin isolated by Gregory and Tracy from antral mucosa in 1964. Using plasma samples obtained from hypergastrinaemic patients with pernicious anaemia and Zollinger-Ellison syndrome, and one normal subject whose gastrin secretion was stimulated by feeding, they compared these with plasma containing negligible amounts of endogenous gastrin to which was added purified antral porcine gastrin I or synthetic human gastrin I. Endogenous plasma gastrin exhibited less acidic behaviour on starch gel and paper electrophoresis than did the heptadecapeptide. On Sephadex chromatography using a mixture of G-25 and G-50, or G-50 alone, most of the endogenous plasma gastrin emerged between the proinsulin (molecular weight 9 000) and insulin (molecular weight 5 700) markers, whereas the heptadecapeptide gastrin standards eluted after the insulin marker. A small fraction of plasma gastrin was found in the same region as heptadecapeptide gastrin. The behaviour of endogenous plasma gastrin on Sephadex chromatography led Yalow and Berson (1970b) to conclude that it had an approximate molecular weight of 7 000.

The electrophoretic and filtration characteristics of endogenous plasma gastrin were not altered by boiling, and incubation of plasma with gastrin-free aqueous extracts of porcine antrum did not convert endogenous gastrin to the heptadecapeptide form, confirming that heptadecapeptide gastrin is not released by enzymic cleavage of the larger gastrin form in the antrum. From these findings and the fact that endogenous plasma gastrin showed the same immunoreactivity as the heptadecapeptide, Yalow and Berson (1970b) proposed that plasma gastrin was composed of the heptadecapeptide sequence firmly bound or possibly covalently linked to a larger basic peptide of molecular weight about 5 000.

Further studies on gastrin in plasma from hypergastrinaemic patients again showed the presence of the larger basic big gastrin and a heptadecapeptide-like component, and that the ratio between the two gastrins varied in different subjects (Yalow and Berson, 1971a), with the heptadecapeptide-like component comprising up to 50% of the total immunoreactive gastrin in some instances, especially after feeding. The secretion of both types of gastrin was stimulated by feeding (Yalow and Berson, 1971a). Incubation of plasma from a patient with Zollinger-Ellison syndrome in 8M-urea and fractionation of this plasma in 8M-urea buffer did not alter the distribution of the two gastrin components (Yalow and Berson, 1971a). However, incubation of the same plasma with trypsin converted all the big gastrin component into heptadecapeptide-like gastrin and the total concentration of immunoreactive gastrin did not change, suggesting that big gastrin consists of a basic peptide linked via a C-terminal lysine or arginine residue to the N-terminal glutamyl residue of heptadecapeptide gastrin (Yalow and Berson, 1971a).

A further gastrin component was described by Yalow and Berson (1972) when they found a large molecular weight gastrin component in plasma from Zollinger-Ellison patients and extracts of human jejunum. This component, which they called "big big" gastrin (BBG), eluted in the albumen region on Sephadex G-50 chromatography and maintained its integrity on refractionation. Following incubation with trypsin most of this fraction was converted to the heptadecapeptide-like component as shown by chromatography, while none appeared to be converted to the big gastrin form (Yalow and Berson, 1972). This component comprised less that 2% of the total immunoreactive gastrin in plasma of patients who were gastrin hypersecretors. It forms a major fraction of circulating gastrin in the basal state and was not stimulated by feeding (Yalow and Wu, 1973). "Big big" gastrin in eluates from the void volume of a Sephadex G-50 superfine column behaved immunologically identically with porcine gastrin I, suggesting that BBG contains the heptadecapeptide sequence within its structure (Yalow and Wu, 1973). The molecular weight of BBG as determined by ultracentrifugation was similar to that of human growth hormone, which has a molecular weight of 20 000 (Yalow and Wu, 1973). This suggests that BBG is not globular in conformation, since if it were, its elution in the albumen region would suggest a molecular weight closer to 65 000.

Not long after the discovery of gastrin heterogeneity by Yalow and Berson, similar reports of more than one type of gastrin appeared from another group of workers. Rehfeld (1972) found three components of immunoreactive gastrin in human serum from normal subjects and patients with pernicious anaemia on Sephadex G-50 fine gel filtration. He described a component which eluted immediately before proinsulin and comprised 18,4% of the

total immunoreactive gastrin, which he named component I. The bulk of the immunoreactive gastrin (72,6% of the total) in his subjects eluted between proinsulin and insulin, in the region corresponding to the big or basic gastrin described by Yalow and Berson (1970b). He named this component II. A third component which consisted of 9,1% of the total immunoreactivity eluted after insulin (Rehfeld, 1972). It was shown that these components were not artefacts due to in vitro conformational changes due to fractionation using veronal buffer, since the same elution profile was obtained using human plasma as the mobile phase. This was also shown by the fact that incubation of sera with the protein denaturing agents, urea or dithiothreitol, produced no change in the elution pattern. However, after incubation of serum with trypsin the first two components disappeared and all the gastrin immunoreactivity was eluted in a single peak corresponding to a smaller molecular size (Rehfeld, 1972).

The following year four immunoreactive gastrin components were described by this group. Using serum from patients with Zollinger-Ellison syndrome and Sephadex G-50 superfine columns of dimensions 1 cm x 2 metres, Rehfeld and Stadil (1973a) showed the existence of components I and II as previously described and confirmed that component III, which eluted as a biphasic peak, corresponded to heptadecapeptide gastrins II and I respectively. In addition they found a smaller component which they called component IV (minigastrin) which eluted immediately before the salt peak. In this study the percentages of total gastrin immunoreactivity contributed by components I to IV were 9,7%, 57,8%, 26,9% and 9,5% respectively. Incubation of components I and II with trypsin converted all the immunoreactive gastrin to a form which eluted in the region of heptadecapeptide-like gastrins (Rehfeld and Stadil, 1973a). This confirmed the

findings of Yalow and Berson that incubation of serum with trypsin converted big gastrin into heptadecapeptide-like gastrin (Yalow and Berson, 1971a). However, Rehfeld and Stadil (1973a) did not find the "big big" gastrin described by Yalow and Berson (1972) in any of the 15 serum samples they studied.

Extending these studies, Rehfeld, Stadil and Vikelsøe (1974) examined immunoreactive gastrin components in serum from patients with pernicious anaemia and Zollinger-Ellison syndrome by Sephadex gel filtration and aminoethylcellulose chromatography. They showed that component I was monophasic whereas components II, III and IV each consisted of two peaks, corresponding to big gastrins I and II, heptadecapeptide gastrins I and II and minigastrins I and II respectively. Fractionation of serum collected from porcine antral vein revealed a similar distribution of gastrin components with more of the total immunoreactivity in the smaller components (Rehfeld, Stadil and Vikelsøe, 1974). No component in serum corresponding to "big big" gastrin (Yalow and Berson, 1972) could be found, although a component in gastrinoma and antral mucosa extracts corresponding in size to "big big" gastrin was detected. It was heterogeneous, with components of apparent molecular weight between 30 000 and 100 000 (Rehfeld, Stadil and Vikelsøe, 1974).

Further gel filtration studies followed by ion exchange chromatography and disc gel electrophoresis of serum resolved both components I and II into six different gastrins (Rehfeld, Stadil, Malmstrøm and Miyata, 1975); at the time of publication of this study components III and IV were under similar investigation. These studies subsequently revealed 4 heptadecapeptide gastrins and 4 minigastrins, giving a total of 20 different gastrin components in all (Rehfeld, cited by Gregory, 1976, p. 260). By reproducing the technique

whereby Yalow and Wu (1973) showed the existence of "big big" gastrin in the circulation, Rehfeld, Stadil et al. (1975) found a large proportion of immunoreactive gastrin eluting in the void volume. However, by increasing the ionic strength of the elution buffer the amount of gastrin eluted in this region was significantly reduced and subsequent treatment with urea caused all the gastrin in the void volume to disappear. This led them to conclude that at least a significant part of the "big big" gastrin described in circulation is due to non-specific binding of smaller gastrins to plasma proteins. This finding is supported by Dockray (1975) who showed that purified bovine and human serum albumen, in concentrations comparable to those of proteins in serum, inhibited binding of labelled gastrin to antiserum 1296. They too ascribed at least some of the "big big" gastrin peak found with this antiserum on chromatography of dog serum to the action of serum proteins which elute in the void volume of Sephadex G-50 columns and cause non-specific inhibition of antibody binding of labelled gastrin.

Since basal plasma gastrin concentrations are usually below 100 pg/ml, the recovery of gastrin in serum from normal subjects is very low, making it impractical to use basal serum for fractionation studies in most cases. Thus chromatographic separation is usually performed using serum from hypergastrinaemic patients, which may or may not be a true reflection of the normal situation. The demonstration by Rehfeld, Stadil, Malmstrøm and Miyata (1975) that sera from normal subjects and patients with pernicious anaemia showed a similar heterogeneity to that observed in Zollinger-Ellison sera would seem to indicate that this is an acceptable extrapolation. However, it should be borne in mind that cases of the Zollinger-Ellison syndrome have circulating tumour gastrin, whereas in normal subjects and patients

with pernicious anaemia the circulating gastrin originates from the antral mucosa.

Increased serum gastrin levels following ingestion of food, especially protein, has been well documented (Hansky and Cain, 1969; McGuigan and Trudeau, 1970a; Young, Byrnes et al., 1969; Ganguli, 1970). This increase is due chiefly to increased levels of the smaller gastrin components. Stadil, Rehfeld, Christiansen and Malmstrøm (1975) showed that the increased serum gastrin levels in response to eating were due to changes in components II and III, the increase in component III, or heptadecapeptide-like gastrin, being greater and of earlier onset. Patients with duodenal ulcer had a larger total gastrin response to eating, which was due mainly to component III. This may be a reason for the gastric hypersecretion characteristic of these patients, since heptadecapeptide gastrin is more potent than big gastrin (Walsh, Debas and Grossman, 1974).

Yet another gastrin fragment was found in the circulation by Dockray and Walsh (1975), who described a fragment which behaved identically to the natural amino-terminal (1-13) sequence of G-17 isolated from gastrinoma tissue and the synthetic (1-13) sequence on chromatography and in radioimmunoassay using antiserum 1295, which is specific for the amino-terminal region of heptadecapeptide gastrin. This tridecapeptide was present in these sera in high concentrations, and its concentration increased in response to intravenous injections of secretin, as is the case for G-17 gastrin. The same (1-13) sequence had been isolated and purified from hog antral mucosa in 1967 and was found to be biologically inactive (Gregory, 1976).

Hansky, Korman, Soveny and Cain (1973) also reported heterogeneity of gastrin in hypergastrinaemic patients as determined by column chromatography. They found most of the gastrin in serum of patients with pernicious anaemia in two fractions of estimated molecular weight 7 000 and 9 500, which they called gastrins type I and II respectively and an additional component with a molecular weight of 4 000 - 5 000. Patients with Zollinger-Ellison syndrome had mainly the gastrin type I component in their serum. These findings are confusing in the light of the findings of Yalow and Berson (1970b, 1971a, 1972), Rehfeld's group (1972, 1973a, 1974) and Vinik and co-workers (1973, 1975). The discrepancy may be partly due to the fact that Hansky, Korman et al. (1973) chromatographed their samples on a Sephadex column that was only 40 cm in length, affording poorer resolution than the larger columns used by other groups investigating this field.

Studies on gastrin heterogeneity in tissues can be broadly divided into studies on extracts of porcine or human antral and duodenal mucosa, which reflect the normal situation, and extracts of gastrinoma tissue, in the case of patients with the Zollinger-Ellison syndrome.

Section VI Heterogeneity and Distribution of Gastrin in Tissues

2 VI(i) Normal Tissues

Although the phenomenon of hormonal heterogeneity may be detected by fractionation of serum into different immunoreactive components, actual chemical characterisation of these hormonal forms is usually performed on extracts prepared from the tissue of origin of the hormone. Hormones may be extracted from serum for chemical purification, but this requires large volumes of serum to yield an amount of hormone equivalent to that present in glandular tissue. In the case of gastrin, studies of heterogeneity have been carried out chiefly on extracts of porcine and human antral mucosa, as well as on extracts of mucosa from the duodenum and lower gastro-intestinal tract.

Gregory and Tracy (1964) were first to describe isolation of two heptadecapeptide gastrin molecules, types I and II, from extracts of hog antral mucosa. These gastrin molecules were purified and their heptadecapeptide sequence was determined by Gregory, Hardy et al. (1964). Heptadecapeptide gastrins I and II were extracted and purified from human antral mucosa and sequenced by Gregory, Tracy and Grossman (1966). Human heptadecapeptide gastrin was found to differ from the porcine heptadecapeptide in the substitution of the methionine residue in position 5 with leucine (Bentley, Kenner and Sheppard, 1966).

The presence of a gastrin form larger than the heptadecapeptide in extracts of porcine antral mucosa was noticed by Gregory and Tracy in 1968. Encouraged by the findings of Yalow and Berson of a larger form of immunoreactive gastrin in serum (1970b), Gregory and Tracy (1972) succeeded in isolating a pair of big gastrin peptides from porcine antrum,

which occurred in the sulphated and non-sulphated forms. The total amount of big gastrin in the antral mucosa comprised less than 20% of the total gastrin in this tissue (Gregory and Tracy, 1972). The sequences of big gastrins I and II were determined and it was found that each comprised 34 amino acids, of which the C-terminal 17 correspond to the sequence of heptadecapeptide gastrin (Gregory and Tracy, 1975). The sequence of human big gastrin was determined using a purified extract of gastrinoma tissue as detailed in the next section. As was the case for heptadecapeptide gastrin, the proportion of sulphated to non-sulphated big gastrin in the pig was 2:1, whereas in the human the same ratio was approximately 1:2 (Gregory and Tracy, 1975).

As early as 1967 Gregory and Tracy isolated from hog antral mucosa a pair of tridecapeptides which corresponded to the amino-terminal (1-13) sequence of heptadecapeptide gastrin. These peptides occurred in the ratio 2:1 in favour of the sulphated form, as was the case for the two larger forms of porcine gastrin that have been identified (Gregory and Tracy, 1975). At the time the reason for the existence of this peptide was not understood. It was biologically inactive, which is not surprising since it comprises the heptadecapeptide sequence, except for the C-terminal tetrapeptide, which is responsible for the biological activity of gastrin (Tracy and Gregory, 1964). However, this fragment was subsequently identified in the serum of patients with Zollinger-Ellison syndrome by Dockray and Walsh (1975). Such a component has not yet been detected in extracts of human gastrinoma tissue (Gregory and Tracy, 1975).

Material corresponding to component I found in serum has been identified in extracts of porcine antral mucosa and partially purified but it has not yet been chemically characterised (Gregory, 1976).

"Big big" gastrin was extracted from human jejunal mucosa obtained post mortem and was shown to comprise 6-24% of the total gastrin immunoreactivity in extracts of this tissue (Yalow and Berson, 1972). This form of gastrin was undetectable in the antrum and its proportion increased distally on descending the gastrointestinal tract. The chemical nature and physiological role of BBG have not yet been established (Gregory, 1976).

Berson and Yalow (1971) investigated the relative proportions of big and heptadecapeptide gastrin in human antral, duodenal and proximal jejunal mucosa. Extracts of mucosal tissue obtained at post mortem examination were prepared by boiling in water followed by centrifugation and collection of the supernatant for fractionation on starch gel electrophoresis and Sephadex G-50 chromatography. The heptadecapeptide-like gastrin component was found to predominate in antral mucosal extracts and the relative abundance of big gastrin increased distally along the gastrointestinal tract. Although these components were not purified and sequenced in this study, they were shown to behave immunologically identically with plasma big and heptadecapeptide gastrin and porcine heptadecapeptide gastrin (Berson and Yalow, 1971).

In a study on extracts of gastrointestinal tissues obtained at 4 autopsies and in 6 surgical cases, Yalow and Berson (1973) found that big gastrin comprised more than 50% of the total extracted gastrin in 2 antra obtained post mortem, in 5 surgical cases big gastrin ranged up to 37% of the total antral gastrin and in the other 3 cases almost all of the gastrin was in the heptadecapeptide form. In the 4 post mortem cases 45-80% of the duodenal gastrin was in the big form and in 2 surgical specimens big gastrin comprised 52-60% of the total (Yalow and Berson, 1973). Thus there seems to be considerable variation in the proportion of the different types of gastrin in

the gut of different individuals. Generally the ratio of big gastrin to heptadecapeptide gastrin is much higher in the plasma than in antral extracts, possibly due to preferential secretion of big gastrin, more rapid removal of heptadecapeptide gastrin from plasma, or perhaps to significant duodenal-jejunal contribution to plasma gastrin (Yalow and Berson, 1973).

The yield of extracted gastrin obtained by Berson and Yalow (1971) on ice-water extraction (maceration of quick-frozen tissue in ice-water) was 7 µg/g frozen mucosal tissue, as opposed to 30 µg/g on extraction in boiling water. However, the relative amounts of big gastrin and heptadecapeptide gastrin were approximately the same. A lower yield was obtained from tissues that had been kept on ice for 30-60 minutes prior to extraction compared with tissues that were quick-frozen on dry ice. However, material obtained 5 to 6 hours post mortem did not contain significantly smaller amounts of gastrin than surgical specimens refrigerated for 30-60 minutes. This could be due to an initial rapid loss of gastrin in excised unfrozen tissues or to random variation among a small number of samples (Berson and Yalow, 1971). Malmstrøm and Stadil (1975) confirmed these findings by showing that the gastrin content of untreated tissue specimens decreased rapidly to 50% within 50 minutes. This degradation was stopped by immediate boiling, or freezing followed by boiling at a later stage. They found no decrease in gastrin activity during storage of the tissue for 6 months in the frozen state (Malmstrøm and Stadil, 1975).

A higher proportion of the heptadecapeptide gastrin component in antral as opposed to duodenal tissue extracts of biopsies from normal subjects was also found by Vinik, Grant et al. (1975). In keeping with the findings of Berson and Yalow (1971), the pattern of gastrin immunoreactivity obtained on gel filtration of duodenal extract resembled that of gastrin in the circulation

of a fasting normal individual, with a minor fraction in the heptadecapeptide form. The tissue extracts in this study were also prepared by boiling in water followed by centrifugation of the solid matter and collection of the supernatant for fractionation studies.

Using refined chromatographic separation of tissue extracts prepared from normal subjects and duodenal ulcer patients with various sized columns of Sephadex G-50 superfine, Rehfeld, Stadil et al. (1975) showed that heptadecapeptide gastrin (G-17) constituted 95% of total gastrin immunoreactivity in antral extracts, and in duodenum and jejunum the small gastrins constituted approximately 50% of the total, with components I and II making up the other 50%. In some normal subjects, however, most of the duodenal and jejunal gastrin consisted of components III and IV, the heptadecapeptide and minigastrin species (Rehfeld, Stadil et al., 1975). This variation is similar to that observed by Yalow and Berson (1973), although the latter group found a relatively larger proportion of duodenal gastrin in the big form. A marked gradient of gastrin concentration in the mucosa down the gastrointestinal tract was observed, with concentrations of 12,9 n mol/g (28,38 µg/g) mucosa in the antrum, 1,8 n mol/g (3,96 µg/g) in the duodenal bulb, 0,1 n mol/g (0,22 µg/g) in the distal duodenum and 20 p mol/g (0,044 µg/g) mucosa in the proximal jejunum (Rehfeld, Stadil et al., 1975).

In a detailed study of the concentrations and components of gastrin in gastric, duodenal and jejunal mucosa in normal subjects and patients with duodenal ulcer, Malmstrøm, Stadil and Rehfeld (1976) again found that the gastrin concentration in proximal duodenum was 10%, in distal duodenum 1 to 2%, and in the jejunum and corpus of the stomach 0,5-1% of the gastrin concentration in antral mucosa. The concentration of gastrin was higher in the antrum of normal subjects (12,1 n mol/g mucosa; 26,62 µg/g)

than in patients with duodenal ulcer (9,0 n mol/g; 19,8 µg/g). There was a gradient in total gastrin concentration along the duodenum, with approximately one tenth of the bulbar content at the ligament of Treitz, and the mean duodenal concentration of gastrin was higher in the patients with duodenal ulcer and prepyloric ulcer than in normal subjects (Malmstrøm, Stadil et al., 1976). A small amount of gastrin, 150 to 200 times less than in the antral mucosa, was detected in the corpus mucosa and this value was almost identical in normal subjects and duodenal ulcer patients.

Fractionation of the corpus mucosal extract on Sephadex G-50 superfine revealed that most of the gastrin occurred in the component III or heptadecapeptide form (Malmstrøm, Stadil et al., 1976). No difference was found between the pattern of gastrin components in the duodenal ulcer and control group. Antral extracts contained more than 91% component III gastrin immunoreactivity, although components I and II were also detected when large amounts of gastrin from antral homogenates were fractionated. Duodenal extracts contained approximately equal amounts of components II and III with a higher proportion of component II in the bulbar area; the possibility that duodenal extracts were contaminated with CCK-PZ was excluded since the same component pattern was obtained using antisera with high and low degrees of cross-reaction with CCK-PZ. Surprisingly, 92% of the gastrin immunoreactivity in the jejunal extract was eluted as component III, a finding which is at variance with Berson and Yalow's (1971) reports of larger gastrin components in this region of the gut. No immunoreactive gastrin was found in the void volume after gel filtration of any of these extracts (Malmstrøm, Stadil et al., 1976).

In contrast to the findings of Malmstrøm, Stadil et al. (1976), Creutzfeldt, Arnold, Creutzfeldt and Track (1976) showed a significantly higher antral

gastrin content in duodenal ulcer patients than in controls (35,9 µg/g as opposed to 15,9 µg/g). The proximal duodenal mucosal gastrin content of 15 duodenal ulcer patients was 3,2 µg/g whereas in 10 control subjects the value was 1,8 µg/g (Creutzfeldt, Arnold et al., 1976). The discrepancy in findings of antral gastrin content in normal subjects and duodenal ulcer patients found by this group and by Malmstrøm, Stadil et al. (1976) is difficult to explain. The actual concentrations found in the extracts by both groups were of the same order of magnitude and the extraction procedures used by both groups were almost identical. In both cases mucosal biopsies were frozen and weighed and stored frozen until extraction by boiling for 10 minutes in water, followed by homogenisation of the tissue. Creutzfeldt, Arnold et al. (1976) centrifuged the tissues after boiling whereas Malmstrøm, Stadil et al. (1976) did not find this necessary. The difference may be due to the lower number of normal subjects investigated by Malmstrøm's group (8) as opposed to those studied by Creutzfeldt and co-workers (21).

Close agreement on the distribution of gastrin components in the tissue extracts was obtained in both these studies. Creutzfeldt, Arnold et al. (1976) found that fractionation of antral mucosal homogenates on Sephadex G-50 produced two major immunoreactive components corresponding to the elution patterns of G-17 and G-34, while component I and minigastrin were detected in a few cases. G-17 comprised 92% of the total immunoreactive gastrin in the antral mucosa of controls and 93,3% in the case of duodenal ulcer patients. G-34 constituted 5% and 4% respectively in the antral mucosa of controls and duodenal ulcer patients; thus the component pattern in antral mucosa was identical for normal subjects and duodenal ulcer patients. In the duodenal mucosa G-34 amounted to 35,8% and 50,1% of the total immunoreactive gastrin in controls and duodenal ulcer patients respectively.

The corresponding values for G-17 were 59,0% and 47,2%. The concentration of G-34 in duodenal mucosa of patients with duodenal ulcer was not significantly higher than that in controls (Creutzfeldt, Arnold et al., 1976).

Early studies on the distribution of gastrin along the gut using the technique of bioassay were carried out by Lai (1964b), who prepared extracts of human tissues obtained from various sites along the gut at operation and measured the gastrin in the extracts using a rat preparation (Lai, 1964a). He found the highest concentration of gastrin-like activity in the antral mucosa with a gradient of concentration of gastrin activity down the gut. Tissue samples were collected from antrum, duodenum, jejunum, ileum, colon and pancreas. The unit activity of gastrin in the duodenum was less than in the antral mucosa although the total amount of extractable gastrin activity was greater in the duodenum. No activity was found in the pancreas at any time.

Elwin and Uvnäs (1966) found the gastrin activity in duodenal mucosa of cats to be one tenth that of the antral activity, whereas Emås, Borg and Fyrö (1971) found that duodenal activity in humans was about one third that of the antral activity as determined by bioassay and expressed relative to the weight of mucosa extracted. Such discrepancies may in part be due to differences in extraction methods, bioassay methods and species differences. The bioassay method of Lai (1964a) involved measurement of gastrin activity using a crude gastrin extract as standard in a rat preparation, whereas Emås, Borg et al. (1971) used histamine as standard, and cats with gastric fistulae as the test animals. Measurement of the gastrin content of duodenal extracts by radioimmunoassay has shown marked differences in duodenal gastrin between man and other species (Nilsson, Yalow and Berson, 1973). The total gastrin

content in the duodenum of man was found to be 97% of that of the antrum in man, whereas in the dog, cat and hog the corresponding values compared to the antral gastrin content were 1,5%, 1,9% and 0,1%.

Nilsson, Yalow and Berson (1973) carried out an extensive quantitative study on the gastrin content of tissues in the gastrointestinal tract and at other sites in man, dog, cat and hog. They found no detectable gastrin in liver, kidney, heart and lung. Minute concentrations were detected in the buccal mucosa, tongue and oesophagus but they emphasised that these results were only tentative, awaiting further characterisation of this material. The highest gastrin concentrations were found in the antral mucosa of all species, with a yield of 22 µg gastrin/g mucosa from hog antrum and approximately 2 µg/g in the case of human, dog and cat antrum. Within each species the antral content of gastrin varied from one individual to another. The extra-antral gastrin content of the stomach was between 1 and 4% of that of the antrum. A concentration gradient of gastrin was found along the intestines of all species examined, with the highest intestinal gastrin content in the proximal duodenum. As mentioned above, the duodenal content of gastrin in man was much higher than in the other species. The gastrin found in extracts of jejunum and ileum agrees with the findings of Lai (1964b), and the jejunal gastrin in the extracts of Nilsson, Yalow et al. (1973) was shown to be distinct from CCK-PZ by starch gel electrophoresis. The gastrin content of the small intestine excluding the duodenum ranged between 0,1 and 1,4% of the gastrin content of the antrum in man, dog, cat and hog. Immunoreactive gastrin was detected in extracts of human pancreas at very low concentrations (0,4 ng/g tissue) but not in the pancreas of the other species investigated. Starch gel electrophoresis revealed that human pancreatic gastrin was almost all in the heptadecapeptide form (Nilsson,

Yalow et al., 1973).

The remarkably greater concentration of gastrin in human duodenum compared with the other species suggests species differences in the distribution of extra-antral gastrin. Duodenal gastrin was found to resemble plasma gastrin more closely than did antral gastrin with regard to the distribution of big and heptadecapeptide components (Yalow and Berson, 1973; Berson and Yalow, 1971), since big gastrin constituted a large proportion of the total immunoreactivity. This suggested that duodenal gastrin may be physiologically important in stimulating gastric acid secretion (Nilsson, Yalow et al., 1973). Stern and Walsh (1973) showed that duodenal gastrin was released by food entering the duodenum in patients who had had a Billroth I gastrectomy (vagotomy, partial gastrectomy and gastroduodenal anastomosis), providing strong evidence for a physiological role of duodenal gastrin. The same study showed that Billroth II subjects who had undergone vagotomy, partial gastrectomy and gastrojejunal anastomosis had basal and postprandial gastrin levels significantly lower than control subjects or subjects with duodenal ulcer who had not been operated on, again suggesting the importance of duodenal gastrin. Korman, Soveny and Hansky (1972b) have also presented evidence for the release of gastrin from extra-gastric sites, since they observed gastrin responses to a protein meal in patients who had been totally gastrectomised.

The findings of Vinik, Grant et al. (1975) that the heptadecapeptide form of gastrin originates chiefly in the antrum, and that antrectomy abolished the release of gastrin stimulated by intravenous arginine (Vinik, Kalk et al., 1975) confirm the earlier report that smaller gastrin forms are released from the antrum in response to arginine stimulation (Kalk, Vinik et al., 1973). Release of the big gastrin species, found largely in the duodenal extracts (Vinik, Grant et al., 1975), following arginine stimulation in antrectomised subjects was not observed. This suggests that duodenal gastrin is not released by arginine, although it may

be released by food ingestion, but does not exclude a role for duodenal gastrin in hydrochloric acid secretion.

In another study of the distribution of gastrin in gastric mucosa, Malmstrøm and Stadil (1975) investigated mucosal extracts prepared from pig, dog, cat and rabbit. They found small but measurable amounts of gastrin in the corpus (body) of all species, with highest gastrin levels in the antrum in each case. They showed that the borderline between body and antrum was narrow, with an abrupt change in gastrin content of the mucosa, and that the gastrin activity in each area was evenly distributed. Like Nilsson, Yalow et al. (1973) they noticed a great variation in gastrin content of tissues between different individuals of the same species. In contrast to Nilsson, Yalow et al. (1973), Malmstrøm and Stadil (1975) found the highest antral gastrin content in man. They suggested that the higher levels in pig antrum detected by Nilsson, Yalow et al. (1973) may be due to the fact that the human tissues in this study were either autopsy specimens or were not processed immediately, thus reducing the antral gastrin content.

The gastrin producing cells or G-cells have been shown to occur predominantly in the mid-portion of the pyloric glands in the antrum of the stomach, interspersed between adjacent mucous epithelial cells, in the human and the hog (McGuigan, 1968b; McGuigan and Greider, 1971; McGuigan, Greider and Grawe, 1972; Pearse and Bussolati, 1972). G-cells have also been demonstrated in the duodenal mucosa (Polak, Stagg and Pearse, 1972). Localisation of G-cells was achieved using the immunocytochemical techniques of immunofluorescence or peroxidase anti-peroxidase (P.A.P.), which involve reacting cryostat or paraffin sections with anti-gastrin antiserum raised in rabbit for example, followed by labelling of the positive cells with anti-rabbit immunoglobulin to which is attached either a fluorescent dye or a

peroxidase marker, which is developed using hydrogen peroxide (Greider, Steinberg and McGuigan, 1972). By coupling immunohistochemical techniques with electron microscopy the typical appearance of a G-cell was determined (Greider, Steinberg et al., 1972). The G-cell is flask-shaped with a broad base and a narrow neck which reaches the lumen of the mucosal gland and projects microvilli into the lumen. In the apical pole of the cell are situated the synthesising organelles which include lamellar endoplasmic reticulum with numerous ribosomes and the Golgi apparatus. The base of the cell, which is often adjacent to a capillary, contains numerous granules of varying electron density (Creutzfeldt, Arnold, Creutzfeldt, Feurle and Ketterer, 1971). Greider, Steinberg et al. (1972) confirmed that this cell contains gastrin by staining secretory granules with peroxidase-labelled antibodies. Different functional states of G-cells observed on ultrastructural analysis by Creutzfeldt, Arnold et al. (1971) led them to suggest that gastrin was not secreted by emeiocytosis (granule extrusion after fusing with the cell membrane) but rather by dissolution of the granule within the membranous sac followed by transfer of the hormone through the granule membrane to the cell membrane via the cytoplasm.

According to Creutzfeldt, Creutzfeldt and Arnold (1974) the distribution of G-cells in the antral mucosa is patchy and uneven. Extra-antral G-cells were found in the upper duodenum in this study, whereas no G-cells were found immunohistologically in the fundic mucosa of animals and man. The ultrastructure of the G-cell is relatively constant in different species which have been investigated, which include the guinea pig, rat, mouse, rabbit, cat, dog, pig and man (Creutzfeldt, Creutzfeldt et al., 1974).

The question of whether gastrin is present in the pancreas has not been resolved. Some investigators have been able to extract small amounts of immunoreactive gastrin from normal pancreas or have detected gastrin-containing cells by immunohistochemistry, while others have not. Greider and McGuigan (1971) demonstrated positive immunofluorescent cells stained with gastrin antiserum in cryostat sections of normal human pancreas obtained at autopsy, which was confirmed by finding immunoreactive gastrin in extracts of 4 human pancreatic tissue samples, with a mean content of 75 ng/g. Lomsky, Langr et al. (1969) also demonstrated positive gastrin cells by indirect immunofluorescence in normal human pancreas obtained at autopsy. Like Greider and McGuigan (1971) they found these cells to be located at the border and in the inner portions of the islets. In contrast, Lotstra, van der Loo and Gepts (1974) failed to demonstrate gastrin cells immunohistologically in the pancreas of man, dog, rabbit, rat and pig, and their pancreatic extracts did not contain any immunochemically detectable gastrin.

Nilsson, Yalow et al. (1973) demonstrated small amounts of gastrin in human pancreatic extracts when measured by radioimmunoassay (0,4 ng/g) but could not detect gastrin in extracts of cat, dog or hog pancreas. Minimal amounts of gastrin were detected in human pancreatic extracts by Rehfeld and Iversen (1973), who found 1,5 ng/g and Gepts (1973), who measured 2-10 ng/g (both cited by Creutzfeldt et al., 1974, p. 50).

Emås and Fyrö (1968) failed to demonstrate any gastrin activity in extracts of cat pancreas by bioassay. Gepts (1973) (cited by Creutzfeldt et al., 1974, p. 50) failed to extracts gastrin from isolated rat islets when measured by radioimmunoassay. Creutzfeldt, Arnold et al. (1971) could not demonstrate the presence of G-cells in human pancreatic islets using indirect

immunofluorescence, and they did not detect any immunoreactive gastrin in human pancreatic extracts. According to Creutzfeldt, Creutzfeldt et al. (1974), G-cells have never been found ultrastructurally in the pancreatic islets of adult animals or man.

Erlandsen, Hegre, Parsons, McEvoy and Elde (1976) suggested that these inconsistencies in staining of gastrin in pancreatic islets reported by different investigators may in part be due to the use of antisera with differing specificities for different gastrin forms. In addition, different methods of tissue preparation or fixation may also give rise to discrepancies. To add to the confusion, Erlandsen, Hegre et al. (1976) very elaborately demonstrated the presence of gastrin and somatostatin (growth hormone release inhibiting hormone) in the same pancreatic D or delta cells (Wiesbaden nomenclature) of man. The pancreatic D cell has been demonstrated to contain somatostatin independently by other workers (Polak, Pearse, Grimelius and Bloom, 1975).

Turning to the question of pancreatic gastrin in the foetus and the neonate, Larsson, Rehfeld, Sundler and Håkanson (1976) demonstrated pancreatic gastrin cells at the 18th day of foetal life in the rat, and observed an increase in the number of these cells until day 4 after birth, after which they decreased. Gastrin cells were never observed in the pancreas of rats older than 20 days when stained by the immunoperoxidase method. Gastrin cells in the duodenum followed a similar pattern, increasing in number until a few days after birth and then decreasing. Antral mucosal gastrin cells could only be detected after birth. Extracts prepared from the pancreas and antrum of 4-day old rats eluted in the same chromatographic pattern as antral gastrin from adult rats. Duodenal extracts prepared from 4-day old rats contained a greater proportion of component II (big gastrin) than did the

antral and pancreatic extracts, as was the case in the adult human, as discussed earlier in this section. This study suggested that gastrin cells appear first in the duodenum and pancreas and not in the antral mucosa.

In keeping with these findings, Braaten, Greider, McGuigan and Mintz (1976) found pancreatic gastrin for the first time in the 16-day old rat foetus, but in this case the gastrin levels increased until day 5 after birth, and then a relatively constant level was maintained until day 35 of neonatal life, as measured by radioimmunoassay of gastrin in the cell culture medium and in pancreatic extracts. Antral gastrin was also detected in foetal rats and the concentration increased rapidly and continuously in the neonatal period, as determined by radioimmunoassay of antral extracts. Monolayer cultures of pancreatic cells studied by immunofluorescence revealed that the gastrin-containing cells were located at the periphery of the endocrine cell clusters, which were thought to represent whole islets. This distribution of gastrin cells is in agreement with that reported in intact pancreatic islets by Greider and McGuigan (1971) and Lomsky, Langr et al. (1969). However, the finding of increased pancreatic gastrin content in the neonatal rat is at variance with the findings of Larsson, Rehfeld et al. (1976).

Two studies of the development of antral gastrin activity in the newborn rat which suggest that gastrin levels increase at the time of weaning have been described. Zelenkova and Gregor (1971) studied the development of antral gastrin activity in newborn rats using the bioassay method involving continuous perfusion of the rat stomach as described by Ghosh and Schild (1958). No gastrin activity was detected at birth or during the first 21 days of life, during which time the animals were fed exclusively on mother's milk. Gastrin activity appeared during the weaning period when solid food was eaten and was found to increase steadily in the juvenile period. During

sexual maturation gastrin activity declined, after which it continued to increase.

Similar results were obtained by Lichtenberger and Johnson (1974) who measured antral gastrin concentrations in the rat by radioimmunoassay. Gastrin was present in low concentrations in the antrum of newborn rats and remained at this level until 21 days, when the levels rose to those found in the adult. This increase was coincidental with the time of weaning. At the same time major developmental changes in the structure of rat gut were found to occur. By comparing gut development in weaned rats with that in rats which were prevented from weaning Lichtenberger and Johnson (1974) showed that gastrin levels remained low and gut development was suppressed in the group prevented from weaning, suggesting that some aspects of gut development are dependent on weaning and that gastrin may be the mediator between these two events.

In the case of the human, it appears that the gastrin measured in the plasma of neonates is of neonatal origin, and that the newborn baby can secrete gastrin independently. Rogers, Davidson, Lawrence, Ardill and Buchanan (1974) found higher gastrin levels in cord blood than in the maternal circulation in mothers undergoing spontaneous labour, and on the fourth day after birth the gastrin levels in the neonate were higher than the cord level. Von Berger, Henrichs, Raptis, Heinze, Jonatha, Teller and Pfeiffer (1976) also found higher gastrin levels in cord plasma than in the peripheral venous plasma of the mothers, and an increase in gastrin levels in the neonate was observed after the first feeding. In keeping with the suggestion that the neonate produces gastrin independently, this group demonstrated that ^{125}I-labelled gastrin injected into female rats did not cross the placenta.

A spate of recent reports showing the occurrence of several gut peptides in neural tissue and brain as well as in gut tissue, as demonstrated by immunohistochemical and extraction procedures, has reinforced the theory that endocrine cells in gut and neural tissue may be related, in that the endocrine polypeptide producing cells of the intestine may have a common neuroectodermal origin. Pearse and Polak (1971) have proposed that the ancestors of gut endocrine cells originate in the neural crest, on the basis of their observations that a group of cells displaying APUD-FIF characteristics were found to migrate ventrally from the neural crest to colonize the developing foregut and its derivatives, including the pharynx, stomach, duodenum, ultimobranchial body and pancreas in the mouse embryo. The abbreviation APUD-FIF stands for Amine Content and/or Amine Precursor Uptake and Decarboxylation - Formaldehyde - Induced Fluorescence (Pearse, 1974), staining characteristics which are peculiar to this type of cell. These cells take up injected amine precursors such as dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan, and contain the fluorogenic amines catecholamine and 5-hydroxytryptamine (Pearse and Welbourn, 1973). Exposure of such tissues to formaldehyde vapour at 60°C or 80°C for four hours induces these cells to fluoresce by virtue of their amine content.

The discovery of a gastrin-like peptide in extracts of human and animal brain by Vanderhaegen, Signeau and Gepts (1975), which reacted with anti-gastrin antibodies is in keeping with the theory of a neural origin of polypeptide hormone producing cells. The highest levels of this brain gastrin peptide (BGP) were found in the cortical grey matter of the cerebral lobes, with concentrations of 200 pg/mg dry weight of tissue, whereas lower concentrations were found in the hypothalamus, brain stem and spinal chord. High levels of this peptide were also found in the hemispheres of the brain

of the dog, pigeon, trout and frog. No BGP was detected in the peripheral nervous system, the cerebellum or in the molluscan or crustacean nervous tissue studied. Extracts of human cortical grey matter did not behave immunologically identically with gastrin (2-17) on dilution in the assay, suggesting that this peptide is not identical with gastrin. Preliminary results of Sephadex G-25 chromatography showed that BGP eluted between gastrin (2-17) and ^{131}Iodine, suggesting a molecular weight of less than 2 020 (Vanderhaegen, Signeau et al., 1975).

Dockray (1976) showed that extracts of entire rat brain and of the cerebral cortex of hog and dog contained immunoreactive material that differed from normal gastrin and CCK-PZ. From the elution profile obtained on Sephadex G-25 fractionation of these extracts measured with antiserum 1296, which is specific for the C-terminus of gastrin, it appeared that these brain factors resembled CCK-PZ-like peptides more closely than they did gastrin.

These findings were substantiated by the demonstration of Straus, Muller, Choi, Paronetto and Yalow (1977) of a peptide resembling the carboxyl-terminal octapeptide of CCK-PZ in neurons of rat cerebral cortex. The presence of this material was demonstrated using the immunohistochemical peroxidase-antiperoxidase system and an antiserum raised in rabbit which cross-reacted equally with CCK-PZ octapeptide and porcine gastrin I. Extracts of pig cerebral cortex were found to contain two peptides on Sephadex G-25 chromatography, one resembling intact CCK-PZ and the other resembling the C-terminal octapeptide of CCK-PZ (Muller, Straus and Yalow, 1977).

Somatostatin and vasoactive intestinal polypeptide (VIP) have also been found to occur in neural and intestinal tissues. Somatostatin has been

localised immunocytochemically in the neurons of the nucleus periventricularis of the hypothalamus, in nerve fibres in the neurohypophysis, and in some nerve fibres in the wall of the intestine (Hökfelt, Efendic, Hellerström, Johansson, Luft and Arimura, 1975). Somatostatin-like immunoreactivity has also been demonstrated in the D cells of the pancreatic islets and the D cells of the upper gastrointestinal tract (Wiesbaden classification) by Polak, Pearse, Grimelius, Bloom and Arimura (1975). VIP immunoreactivity has been demonstrated in brain tissue by Bryant, Bloom, Polak, Albuquerque, Modlin and Pearse (1976) and by Said and Rosenberg (1976). Bryant, Bloom et al. (1976) showed that 94% of the VIP extractable from porcine and human brain eluted in the same position as the pure intestinal peptide on gel filtration. In addition they found that VIP in the human and in the pig occurred not only in the endocrine VIP cells of the intestine but also in some fine nerve fibres in the lamina propria, in the cell bodies of the myenteric plexus, and in the adrenal medulla.

The significance of these findings of gut peptides in brain and nervous tissue awaits further investigation. It seems likely that these peptides may have a role as neurotransmitter substances or releasing factors, as has been suggested by Dockray (1976) and Muller, Straus et al. (1977). This seems feasible in the light of demonstrations that somatostatin, which has been localised in many neural and gastrointestinal tissues, appears to play an important role in regulation of secretion of several hormones and has been suggested to have a neurotransmitter function (Polak, Pearse et al. 1975).

2 VI(ii) Abnormal Tissues

Studies on gastrin heterogeneity in abnormal tissues involve preparation of extracts of gastrinoma tissue collected from patients with Zollinger-Ellison syndrome at operation. These tumours, which arise from the pancreas or gastrointestinal mucosa, usually contain concentrations of gastrin as great as or greater than those found in normal antral mucosa (Walsh and Grossman, 1975b). High concentrations of gastrin in these tumours have been demonstrated by extraction of the tumour by boiling (Creutzfeldt, Arnold, Creutzfeldt and Track, 1975) and by immunohistochemical techniques (Polak, Stagg and Pearse, 1972; Creutzfeldt, Arnold et al., 1975).

Extraction of a pair of gastrin heptadecapeptides from human gastrinoma tissue and their subsequent purification and characterisation by Gregory, Tracy, Agarwal and Grossman (1969) revealed that the sequence of amino acids was identical to that of heptadecapeptide gastrin previously isolated from human antral mucosa (Bentley, Kenner et al., 1966). In 1972 Gregory and Tracy isolated two big gastrin peptides from gastrinoma tissue which had been collected from human subjects with Zollinger-Ellison syndrome over a period of four years. These big gastrin peptides were very similar to the big gastrins isolated from porcine antral mucosa by this group in amino acid composition, and were identical to porcine antral big gastrin and serum big gastrin in chromatographic and electrophoretic properties (Gregory and Tracy, 1972).

During the purification of big gastrin from gastrinoma tissue Gregory and Tracy (1974) identified a pair of smaller peptides in the heptadecapeptide fraction obtained on Sephadex G-50 gel filtration. These peptides corresponded in amino acid sequence to the (5-17) sequence of the heptadecapeptide, known as minigastrin, and were found in both the sulphated and non-sulphated forms

in the proportion 1:2, as was the case for the two larger forms of human gastrin previously purified. Any doubts that these C-terminal tridecapeptides were artefacts were dispelled by the demonstration of Rehfeld and Stadil (1973a) of an immunoreactive form of gastrin smaller than the heptadecapeptide in the circulation of gastrinoma patients. Both minigastrins were shown to be potent stimulants of gastric acid secretion when injected intravenously into conscious dogs with denervated fundic pouches (Gregory and Tracy, 1974).

Preparations of natural human big gastrin I (NHG-34 I) and natural human heptadecapeptide gastrins type I and II (NHG-17 I and II), extracted and purified from gastrinoma tissue by Gregory and Tracy, have been made available in small quantities and distributed by the Centre for Ulcer Research and Education by courtesy of Dr. M.Grossman. Such preparations have been used in various studies described in this thesis.

Recent studies have shown that the heterogeneity of gastrin in tumour extracts is more complicated than had been thought, since still more gastrin subtypes have been resolved on column chromatography by Gregory and Tracy (1975). During their isolation of big gastrins type I and II from gastrinoma tissue they found two additional small peaks in the region of the heptadecapeptide gastrins, which they called little or heptadecapeptide gastrins IIA and IIB. These peptides have the same amino acid composition and biological potency as heptadecapeptide gastrin and both contain sulphated tyrosine (Gregory and Tracy, 1975). Until their presence in serum has been demonstrated, the possibility that they are artefacts cannot be excluded. Likewise, a third big gastrin, intermediate in polarity between big gastrins I and II, has been identified on DEAE cellulose chromatography of the big gastrin II fraction from gastrinoma tissue (Gregory and Tracy, 1975).

Amino acid analysis revealed that its composition was the same as that of non-sulphated big gastrin, hence it was named big gastrin IA. Demonstration of the presence of this fragment in serum is awaited to rule out the possibility that this fragment is an artefact of the extraction procedure.

It seems likely that the findings of these multiple hormonal forms in tissue extracts are related to the multiple heterogeneity of serum gastrins observed by Rehfeld, Stadil et al. (1975), who have so far identified 20 different gastrin components in serum. However, it is possible that some of the multiple components of hormones found in tissue extracts are the result of nonspecific enzymic degradation and are thus not normally found under physiological conditions.

Component I has been identified chromatographically in an extract of gastrinoma tissue (Rehfeld, Stadil et al., 1974), and immunoreactive material eluting in the void volume of a Sephadex G-50 column, corresponding to "big big" gastrin has also been found in such extracts. BBG comprised less than 2% of the total gastrin immunoreactivity in a Zollinger-Ellison tumour investigated by Yalow and Wu (1973), and this fraction was found to comprise a series of immunoreactive components eluting between the positions of albumen and big gastrin on Sephadex G-50. The heterogeneous nature of BBG extracted from gastrinoma tissue was also reported by Rehfeld, Stadil et al. (1974), who estimated that the apparent molecular weight ranged from 30 000 to 100 000.

With regard to the relative proportions of the different gastrin types found in gastrinoma tissue, the predominant form of tumour gastrin is of the heptadecapeptide variety. According to Dockray (1975), big gastrin constitutes a fair proportion of tumour gastrin; in a few cases "big big" gastrin,

component I and minigastrin have together been found to comprise up to 15% of the total tumour immunoreactive gastrin. When serum and tumour extract from the same patients were examined chromatographically, G-34 usually constituted the major peak of immunoreactive gastrin in serum and G-17 I and II were the major peaks in tumour extract (Dockray, 1975). These measurements were made using antiserum 1296 which recognises the different gastrin components equally. Additional evidence that G-17 is the principal molecular form stored by gastrinoma tissue was provided by stimulation of gastrin release by intravenous secretin, which produced a marked increase in the levels of serum G-17 and G-34. The G-17 response was proportionally greater and its levels returned to basal values more rapidly than G-34, due to its shorter half-life in the circulation (Dockray, 1975).

Creutzfeldt, Arnold et al. (1975) also found that big gastrin was present in serum in larger amounts than in tumour extracts when they examined the distribution of immunoreactivity of gastrin components in 9 patients with gastrinoma. They could find no obvious relationship between the distribution of gastrin components in the tumour tissue and serum of individual patients. However, the antiserum used in these studies had a 70% lower affinity for G-34 compared to G-17 on a molar basis, so the percentages reported for big gastrin were underestimations of the real values (Creutzfeldt, Arnold et al., 1975).

A Zollinger-Ellison tumour examined by Gregory and Tracy was found to contain primarily big gastrin (Gregory, 1976), which suggests that something abnormal in a tumour might block the conversion of big gastrin to heptadecapeptide gastrin, so that big gastrin accumulates. This would only apply if it could be shown that one cell type produces both big and hepta-

decapeptide gastrin, and if the biosynthetic sequence BBG → component I → big gastrin → heptadecapeptide gastrin could be demonstrated. At present such information is not available (Gregory, 1976).

The first immunohistological demonstration of gastrin-containing cells in a Zollinger-Ellison tumour came from Creutzfeldt, Arnold et al. (1971), who showed that the cells of a Zollinger-Ellison tumour were ultrastructurally identical with the G-cells of human antral mucosa. In a later study (Creutzfeldt, Arnold et al., 1975) ten gastrinomas were investigated ultrastructurally and the appearance was found to vary from one tumour to another and sometimes in different areas of the same tumour. Differences involved the frequency of cells with or without secretory granules and the type of secretory granules found. Seven of the ten tumours contained cells with secretory granules identical to those of antral G-cells, showing a broad scale of varying electron density. Most tumours also contained cells with atypical granules which were electron dense, round and small. Some tumours contained only cells with atypical secretory granules, in which case extraction of gastrin from the tumour was necessary to demonstrate its presence. Other tumours were multihormone producing, containing, for example, cells with typical beta granules as well as G-cells; both gastrin and insulin producing cells in these tumours were demonstrated immunohistologically. No correlation was found between the relative number of cells with typical G-cell granules or cells with atypical granules and the gastrin content of the respective tumour.

It has become apparent that two distinct types or perhaps stages of Zollinger-Ellison syndrome exist. Berson and Yalow (1972) described a group of 9 patients with Zollinger-Ellison syndrome who could be divided into two distinct groups. Four of these patients had moderate hyperchlor-

hydria and gastrin levels in the range of Zollinger-Ellison syndrome, but they did not have detectable gastrin-producing tumours. They showed significant plasma gastrin responses to a test meal, of a magnitude 10-20 times greater than that found in normal subjects and patients with duodenal ulcer. The remaining five patients behaved predictably in that they showed no increase in serum gastrin levels in response to feeding.

These clinical findings can be correlated with the description of two types of Zollinger-Ellison syndrome based on immunofluorescent, cytochemical and ultrastructural studies by Polak, Stagg and Pearse (1972). According to these workers, Zollinger-Ellison syndrome type I is characterised by a short history, very high serum gastrin levels, extreme hyperplasia of the antral G-cells, normal or slightly hyperplastic pancreatic D-cells, and no tumour. This would correspond with the first group described by Berson and Yalow (1972), since the antral G-cell hyperplasia would account for the increase in gastrin levels in response to feeding. Zollinger-Ellison syndrome type II described by Polak, Stagg et al. (1972) manifests itself as a long history of high gastrin levels and ulceration, normal antral G-cells, hyperplasia of the pancreatic D-cells and the presence of a tumour or gastrinoma, which would correspond with the second group of Berson and Yalow (1972).

2 VI(iii) Significance of the Multiple Forms of Gastrin

With the discovery and characterisation of the different molecular forms of gastrin arises the question of how they are all inter-related. Chemical characterisation of heptadecapeptide gastrins isolated from human antral and gastrinoma tissue has shown that they are identical in structure (Gregory, Tracy et al., 1969; Bentley, Kenner et al., 1966) and this may prove to be true in the case of the big gastrins as well, once big gastrin from human antrum can be purified in sufficient quantities to allow sequencing studies (Gregory and Tracy, 1975). Characterisation of human duodenal big gastrin is difficult due to the impracticality of obtaining sufficient quantities of human material (Gregory, 1976). Thus gastrinoma tissue remains the most plentiful source of human gastrins, and extrapolations of findings relating to purified gastrins from this source to gastrins originating in normal human tissues should be made with caution until the chemical identity of gastrins from both these sites has been established. It seems likely that this will prove to be the case, since the gastrin producing cell in gastrinoma tissue is identical with the antral G-cell (Creutzfeldt, Arnold et al., 1971).

The hypothetical biosynthetic sequence in the gastrin series is "big big" gastrin → component I → big gastrin → heptadecapeptide gastrin → minigastrin, if one assumes that the larger forms represent precursor forms. Preparations of gastrin-forming cells in slices of gastrinoma tissue cultured in vitro would be useful in demonstrating such a sequence. However, this is not entirely practical since gastrinoma tissue is not readily available, and the preferred method of treatment of this condition is removal of the target organ by total gastrectomy rather than removal of the tumour (Gregory, 1976).

The conversion of big gastrin to heptadecapeptide gastrin by trypsin has been well documented, and the cleavage of the G-34 molecule at the

lys-gln sequence to yield G-17 suggests that G-34 may be the precursor of G-17 (Gregory and Tracy, 1975). The precursor forms of hormones are generally biologically inactive and the conversion from larger to smaller molecular forms is brought about by converting enzymes in the cell of origin (Grossman, 1975). If big gastrin is the precursor of heptadecapeptide gastrin, this constitutes a situation of a biologically active prohormone, since G-34 has definite biological activity (Walsh, Debas et al., 1974).

The amino acid sequence of big gastrin at its point of cleavage by trypsin is lys-lys-gln, and when heptadecapeptide gastrin is liberated the glutamine residue cyclizes spontaneously to the pyroglutamyl form, changing the molecule from ninhydrin-positive to ninhydrin-negative. The amino-terminal residue in big gastrin is also pyroglutamyl, which led Gregory (1976) to suggest that the same sequence of events might occur on liberation of big gastrin from a larger precursor, such as component I.

It may be that an even larger precursor form of gastrin, as yet undiscovered, exists. It has been suggested that a precursor peptide may be attached to the carboxyl terminal end of the gastrin molecule and is removed by transamidation at this point, resulting in the phenylalanine-amide residue found in the C-terminal position of all gastrin species which are biologically active (Gregory, 1976). Since removal of this carboxyl-terminal amide abolishes the biological activity of gastrin (Tracy and Gregory, 1964), such a precursor would almost certainly be physiologically inactive. Since many of the gastrin antisera currently in use recognise the C-terminal region of the gastrin molecule, which would be masked in a precursor of this nature, detection of such a precursor may prove difficult (Gregory, 1976). Relevant in this connection is the finding by Malagelada of a large gastrin component, eluting between the void volume and the position of G-34 on Sephadex

chromatography, which was found in the serum of some patients with Zollinger-Ellison syndrome and was biologically inactive (Malagelada, 1977).

Having discussed the possible significance of the various molecular forms of gastrin, it may be useful to consider what is known regarding the nature and significance of the different forms of other peptide hormones which have been found to be heterogeneous.

Section VII Heterogeneity of Other Peptide Hormones

It has become apparent that many, if not all, of the peptide hormones are found in more than one form, both in plasma and in glandular and other tissue extracts (Yalow, 1974b). The various peptide hormones can be separated into several groups on the basis of structural similarities in their amino acid sequences. These similarities, which are very striking in some cases, strongly suggest a common ancestral origin for the various hormones of each group (Tager and Steiner, 1974). Hormonal heterogeneity or polymorphism has been demonstrated in the case of hormones belonging to almost every group of peptide hormones, which have been classified by Tager and Steiner (1974) as follows:-

1. insulin, proinsulin, nerve growth factor
2. glucagon, secretin, vasoactive intestinal polypeptide (VIP), gastric inhibitory polypeptide (GIP)
3. gastrin, CCK-PZ, caerulein
4. adrenocorticotrophic hormone (ACTH), melanotropin, lipotropin
5. growth hormone, prolactin, placental lactogen or human chorionic somatomammotropin (HCS)
6. the glycoprotein hormones, which include luteinizing hormone (LH), follicle stimulating hormone (FSH), human chorionic gonadotrophin (HCG), thyroid stimulating hormone (TSH).

Hormonal polymorphism has been best characterised in the case of insulin, parathyroid hormone, growth hormone, glucagon, adrenocorticotrophic hormone, and melanocyte-stimulating hormone. Cholecystokinin-pancreozymin exists in at least two molecular forms (Mutt and Jorpes, 1968), whereas secretin has not yet been identified in heterogeneous forms (Grossman, 1975).

Multiple hormonal forms may be detected by fractionation of tissue extracts or plasma using Sephadex gel filtration, starch gel electrophoresis, or ultracentrifugation; the presence of hormonal activity in two or more separate fractions suggests heterogeneity. In addition, heterogeneity may be suspected when measurements of the hormonal content of a sample relative to a given hormonal standard change when different antisera are used in the assay.

According to Franchimont, Gaspard, Reuter and Heynen (1972) the causes of hormonal heterogeneity include:-

1. Precursors
2. Isohormones
3. Polymers
4. Protein-bound hormones (?)
5. Subunits
6. Metabolites
7. Artefacts of extraction and purification
8. Tumour or adenoma hormone analogues.

Isohormones are forms of a hormone which can be separated on electrophoresis or in polyacrylamide gel by ion focusing and which have identical or very similar molecular weight and the same biological activity (Franchimont, Gaspard et al., 1972). Three isohormones of bovine PTH, differing slightly in amino acid composition or sequence, have been isolated by Keutmann, Aurbach, Dawson, Niall, Deftos and Potts (1971). Preparations of bovine pituitary extracts yielded several distinct forms of TSH which were all biologically active (Bates and Condliffe, 1960), and a similar heterogeneity in TSH obtained from monkey pituitary extracts was reported by

Hummel, Webster and Brown (1970). As with the other glycoprotein hormones, which consist of two non-covalently bonded polypeptide subunits designated alpha and beta, linked to a carbohydrate moiety, the polymorphism of TSH could be due to micrcheterogeneity of the carbohydrate component as a result of its incomplete syntesis or degradation (Franchimont, Gaspard et al., 1972).

"Protein bound hormones" refers to the theoretical possibility of a peptide hormone bound to a serum carrier protein, a situation which has not yet been encountered (Franchimont, Gaspard et al., 1972). The possibility that the heterogeneity observed is due to artefacts of extraction and purification or due to the fact that metabolites are being measured must be excluded before the existence of a precursor can be established.

The use of trypsin and urea has been invaluable in elucidating the nature of some of the larger hormonal forms. Since urea is a protein denaturing agent which disrupts non-covalent bonds responsible for the native conformation of proteins, any alteration in the molecular weight of a large hormonal form following treatment with urea suggests the presence of an aggregate or the native hormonal form bound to a larger protein (Tager and Steiner, 1974). Liberation of the native hormonal form together with another peptide following trypsin cleavage of a larger hormonal form suggests the existence of a precursor or prohormone. The conversion of a large hormonal form into a smaller form by trypsin has been demonstrated in the case of insulin, glucagon, parathyroid hormone and ACTH as well as gastrin, and in each case, except for ACTH, the primary structure has been shown to contain at least two consecutive basic amino acids at the point of cleavage (Grossman, 1975). The most convincing evidence for the existence of a precursor is the demonstration that radioactively-labelled amino acids are transferred from the substance with higher molecular weight to the hormone itself <u>in vitro</u>

(Franchimont, Gaspard et al., 1972).

The classic example of a precursor-hormone relationship, and probably one of the most well defined, is that of insulin and its precursor, proinsulin. Steiner, Cunningham, Spigelman and Aten (1967) demonstrated that both the A and B chains of insulin are initially synthesised as part of a larger single-chain peptide, proinsulin, with the subsequent cleavage by a trypsin-like protease of a portion of the chain known as C-peptide, to produce the common form of insulin in which the A and B chains are linked by two disulphide bonds. Roth, Gorden and Pastan (1968) demonstrated the presence of a proinsulin-like component in plasma on Sephadex G-50 gel filtration and found that it constituted up to 50% of the total immunoreactive insulin in plasma. Each component maintained its integrity on refractionation, suggesting that the two components are not readily interconvertible in plasma. Proinsulin was shown to have low biological activity by Rubenstein, Cho and Steiner (1968). Conversion of proinsulin to insulin is apparently initiated within the Golgi apparatus of the pancreatic beta cell and continues within the secretory granules in the cytosol (Steiner, Kemmler, Tager and Peterson, 1974). Although only approximately 4% of the total proinsulin synthesised escapes conversion to insulin in the beta cell, the ratio of proinsulin to insulin in the circulation is considerably higher due to the more rapid turn-over of insulin than proinsulin in the circulation (Franchimont, Gaspard et al., 1972).

Immunochemical heterogeneity was first shown for human parathyroid hormone (hPTH) when it was observed that a plasma dilution curve was super-imposable on a curve of standards obtained from a normal parathyroid gland with two antisera but not with that obtained using a third antiserum (Berson

and Yalow, 1968). Fractionation of glandular and plasma PTH using Sephadex G-100 gel filtration yielded different elution profiles when measured with antisera 273 and C 329, with almost twice as much immunoreactivity detected by the former antiserum when a parathyroid adenoma extract was fractionated (Silverman and Yalow, 1973; Yalow, 1974a). The first component which eluted in the void volume, constituted a minor fraction of the total immunoreactivity. The second component corresponded to intact PTH, the third and predominant peak of immunoreactive hormone reacted only with antiserum 273, and the fourth component reacted only with antiserum C 329. All four components maintained their integrity on refractionation (Silverman and Yalow, 1973; Yalow, 1974a).

Biosynthetic experiments involving organ culture of bovine parathyroid glands which could be shown to secrete PTH in response to alterations in calcium and magnesium ion concentrations, revealed that the hormone synthesised in the glands was of a higher molecular weight than that released into the culture medium or that found in the bovine circulation (Sherwood, Rodman and Lundberg, 1970). This suggested that the parathyroid glands synthesise a precursor hormone, proparathyroid hormone, which is modified to the smaller molecule that circulates in blood. This was confirmed by Kemper, Habener, Potts and Rich (1972), who showed that the proparathyroid hormone synthesised by bovine parathyroid tissue in vitro is 15-20 amino acids larger than the native hormone and has a molecular weight of about 11 500 as determined by polyacrylamide gel electrophoresis. This group demonstrated the incorporation of ^{14}C-labelled amino acids into the precursor initially, followed by an increase of the labelled material in the native hormone and a decrease in radioactivity in the precursor. Peptides obtained on treatment of both the precursor and the hormone with trypsin were common

to both molecules, and both molecules reacted identically with an antiserum specific for PTH, again confirming the existence of a precursor form containing the PTH sequence within its structure.

Two components of human growth hormone were detected on gel filtration of large volumes of plasma using Sephadex G-75 by Bala, Ferguson and Beck (1970). More than 50% of the total immunoreactivity in all the plasmas investigated eluted in a molecular size region greater than that of extracted pituitary human growth hormone (ep-HGH), while significant HGH immunoreactivity was also found in areas corresponding to a smaller molecular size than ep-HGH. The larger HGH form was thought to consist of aggregated forms of native HGH or complexes of HGH and plasma proteins. The greatest total amount of biological activity, as measured using a sulphation factor assay in vitro, was found in the large molecular size fraction although the smaller HGH fraction displayed the most potent sulphation factor activity (Bala, Ferguson et al., 1970).

The studies of Goodman, Tanenbaum, Wright, Trimble and Rabinowitz (1974) showed that fractionation of plasma from normal subjects, acromegalic patients and a patient with the syndrome of dwarfism and high plasma HGH on Sephadex G-75 resulted in two peaks corresponding to 14 to 28% of the total immunoreactivity in the case of the big human growth hormone, and 67 to 86% of the total in the case of little HGH, which migrated almost identically with the major component of ^{125}I-HGH from the pituitary. Both components retained their integrity on refractionation, confirming that conversion from one form to the other did not occur during gel filtration. Freezing and thawing caused over half of the big HGH fraction isolated from plasma to convert to little HGH, a finding that was confirmed following treatment with urea.

Both these observations suggested that big HGH consists of little HGH bound non-covalently to another moiety. From its behaviour on Sephadex, Goodman, Tanenbaum et al. (1974) concluded that the molecular weight of big HGH was approximately double that of little HGH, which is about 22 000, suggesting the possibility of a dimer. Gel filtration of HGH extracted from the pituitary produced two peaks identical to those found for plasma HGH, suggesting that both the plasma components may be secreted by the pituitary.

In the case of ACTH in plasma and extracts of pituitary glands and an ACTH-producing tumour, Sephadex G-50 fractionation again revealed two major components of immunoreactivity, one corresponding in molecular size to the 39 amino acid little ACTH and the other eluting in or near the void volume (Yalow and Berson, 1971b). The relative proportions of the two components varied greatly among plasmas, and each component ran true on re-fractionation. The two components were immunochemically indistinguishable, and tryptic digestion of big ACTH resulted in quantitative conversion to a little ACTH-like peptide (Yalow, 1974a). Big ACTH showed very little biological activity, and conversion of this form to the little form resulted in biological activity equivalent to immunological activity. These findings suggested that the larger form of ACTH consists of native ACTH covalently linked to a larger peptide with the antigenic site recognised by the specific antiserum fully available but with a configuration that renders it biologically inactive (Yalow, 1974a). Since both components were found in plasma and in pituitary and tumour extracts Yalow and Berson (1971b) suggested that the hormone is produced at and secreted from its site of origin in at least two forms, in a similar manner to that suggested for growth hormone by Goodman, Tanenbaum et al. (1974).

Glucagon extracted from canine pancreas was also found to comprise two fractions when separated by gel filtration (Rigopoulou, Valverde, Marco, Faloona and Unger, 1970). Over 90% of the immunoreactivity corresponded in molecular size to the ^{131}I-glucagon marker, while the remaining immunoreactive fraction appeared to be at least twice as large. The same elution profile was obtained with human, duck, rat and bovine pancreatic glucagon. Urea treatment did not alter the molecular size of the large component, whereas trypsin converted the LGI (large glucagon immunoreactivity) to a molecular size close to that of ^{131}I-glucagon. Both LGI and its tryptic product behaved immunologically identically to crystalline glucagon but neither were biologically active in producing glycogenolysis in the perfused rat liver (Rigopoulou, Valverde et al., 1970). This emphasises the problem that plasma measurements of immunoreactive glucagon do not necessarily represent biologically active hormone, since it was not possible to differentiate immunochemically between intact glucagon and these biologically inactive substances.

The demonstration by Tung (1973) of a possible high molecular weight intermediate in the biosynthesis of avian glucagon using isolated islets of Langerhans from the pigeon provides additional evidence for the existence of a larger precursor form of glucagon. He isolated a component with an approximate molecular weight of 69 000 using Sephadex G-100 chromatography which was shown to possess glucagon immunoreactivity.

In addition, a minor component has been isolated from crystalline glucagon which consists of glucagon with eight additional residues attached at the carboxyl terminal end of the peptide (Steiner, Kemmler et al., 1974). It was assumed that this peptide represented a portion of the proglucagon molecule and it was tentatively identified as a synthetic intermediate.

From the foregoing discussion it would appear that most of the small peptide hormones are derived from larger precursor forms, and it seems likely that similar modes of cleavage are involved in each case. Since the immunoreactive site on the native hormone may be exposed for reaction on the precursor form of the molecule as well, measurements of total immunoreactivity in the circulation may include a contribution by a precursor with lower biological activity. Thus immunoreactivity and bioactivity measurements should ideally be coupled with fractionation data for absolute determination of hormone levels.

Section VIII Distribution of Gastrin in Vertebrates and Lower Species

2 VIII(i) Gastrin in Vertebrates

A handful of reports have appeared describing the occurrence of gastrin in vertebrate species other than man and the mammals. Gastrin or gastrin-like material has been detected in species of bird, reptile, frog and cartilaginous fish.

The tissue distribution of gastrin in mammals such as the hog, dog, cat and rabbit has been studied by Nilsson, Yalow et al. (1973) and Malmstrøm and Stadil (1975), and was described in detail together with distribution of gastrin along the human gastrointestinal tract, in section 2 VI(i). A further study on gastrin content of canine tissues by Thompson, Reeder, Davidson, Jackson and Clendinnen (1973) again showed the highest gastrin levels in extracts of antral mucosa. The only other tissue containing a notable amount of gastrin in this study was the gall bladder, which had a gastrin content of 12% of that of the antrum, but this may represent cross-reaction of CCK-PZ in the assay. Duodenal gastrin content in this study was only 2% of that in the antrum. These workers also investigated tissue extracts prepared from antrum, fundus, duodenum and pancreas as well as plasma of the frog, guinea pig, rat, rabbit, hog and cat. They found high concentrations of gastrin in the pancreas of the frog, rat and cat and in the duodenum of the guinea pig and rabbit, while the antral content in the frog and rat were roughly five to ten fold lower, with tissue concentrations of 500 ng/g and 475 ng/g respectively.

The amino acid sequence of heptadecapeptide gastrin isolated from the pig, cow, sheep, dog, cat and human was determined by Kenner and Sheppard (1973). Basically the structures resemble that of human heptadecapeptide gastrin with one or two amino acid substitutions. In position 5 methionine replaces leucine in

porcine and canine gastrin, while this position is occupied by valine in gastrin from cow and sheep, and feline gastrin bears the same residue as does human gastrin in this position. Alanine is substituted for glutamic acid in position 10 in gastrin of cow, sheep and cat, and in position 8 in canine gastrin. Cow and sheep gastrins appear to be identical in structure.

Investigations on the occurrence of gastrin in vertebrates and lower species can be carried out by various techniques. Extracts of the tissue being studied can be tested for gastrin content using a bioassay technique or by radioimmunoassay. The use of gel chromatography to separate the different gastrin components, combined with radioimmunoassay, is useful for determination of the nature of gastrin in the extract. The other line of investigation involves the immunohistological demonstration of gastrin-containing cells in the tissue under examination using the techniques of immunofluorescent or peroxidase anti-peroxidase labelling of gastrin-containing cells. Various workers have described their findings of gastrin in different species using one of these techniques. In a few instances refined investigations have shown the presence of gastrin by using a combination of two or more of these methods, and these are the studies upon which the greatest emphasis can be placed. The findings of gastrin in various animal species will be discussed in a sequence progressing down the evolutionary scale.

Olowo-Okorun (1975a) prepared extracts of gastrointestinal mucosa of cattle, goat, sheep and donkeys using the procedure described by Blair, Harper, Lake, Reed and Scratcherd (1961), which commenced with boiling the mucosa in water, followed by acetone precipitation. The extracts were assayed for gastrin using a modification of the continuous rat stomach perfusion technique of Ghosh and Schild (1958). When the gastrin activity detected in the extracts was expressed as SHG I equivalent, the pyloric mucosa of all the animals was found to contain significantly greater amounts than the duodenal mucosa. The histamine content

of the extracts was too low to produce an increase in acid secretion in the rat, confirming that the observed effects could not be attributed to histamine contamination (Olowo-Okorun, 1975a). The antral gastrin content was between 40 and 58 ng SHG I equivalent/mg crude extract for all species investigated.

Gastrin activity was found in the chicken proventriculus by Olowo-Okorun and Amure (1973). Extracts of the proventriculus and duodenum were prepared by the method of Blair, Harper et al. (1961) and tested for gastrin activity using the bioassay technique of Ghosh and Schild (1958). No increase in acid secretion was produced by duodenal extracts, whereas extracts of proventriculus were found to have gastrin activities approximately double that of pyloric antral extracts prepared from cattle, sheep and goat, with a content of 109,4 ng SHG I equivalent/mg crude extract.

In an excellent study on gastrin in the chicken Larsson, Sundler, Håkanson, Rehfeld and Stadil (1974) demonstrated gastrin-containing cells, using the technique of immunofluorescence, in a narrow zone joining the gizzard with the duodenum, where the mucosa resembled that of the mammalian pyloric antrum histologically. Gastrin cells were occasionally found in the duodenum and jejunum but were absent in the oesophagus, crop, proventriculus and gizzard. Extracts of tissue collected from the oesophagus, crop, proventriculus, gizzard, gizzard-duodenal junction, duodenum and jejunum prepared by boiling in water were tested for gastrin by radioimmunoassay. The gizzard-duodenal junction (antrum) was the only site where immunoreactive gastrin was detected, in keeping with the finding of gastrin cells at this site. Chicken gastrin did not behave identically with human heptadecapeptide gastrin on dilution in the assay. Sephadex G-50 gel filtration produced a major peak eluting in the region of heptadecapeptide gastrin, with smaller amounts in the position of minigastrin.

A third study in the chicken by Polak, Pearse, Adams and Garaud (1974) revealed cells showing gastrin immunoreactivity occurring throughout the digestive tract distal to the proventriculus, which is in close agreement with the findings in the above study, except for the finding of gastrin-containing cells in the gizzard by Polak, Pearse et al. (1974). Neither study could confirm the findings of gastrin activity in the proventriculus as described by Olowo-Okorun and Amure (1973), although all three studies were performed using the same species of chicken, the White Leghorn variety.

With regard to the reptiles, gastrin-like activity has been described in the pyloric antrum of the Indian python (Python molurus) by Olowo-Okorun (1975b). As with the previous studies by this worker, a crude extract of the antral mucosa was prepared by the method of Blair, Harper et al. (1961) and the gastrin activity was determined using a modification of the bioassay technique of Ghosh and Schild (1958). The gastrin-like activity was 52,1 ng SHG I equivalent/mg crude extract, which was about the same as that found in cattle, sheep and goat by the same worker (Olowo-Okorun, 1975a).

Using the same technique of extraction and the bioassay methods of Ghosh and Schild (1958) and Lai (1964a), Oyebola and Elegbe (1975) demonstrated the presence of gastrin activity in the stomach of the common African toad, Bufo regularis. Histamine contamination of the extract was minimal and did not interfere in the bioassay estimations, which yielded a gastrin content of 194,7 ng SHG I equivalent/mg crude stomach extract. This value is higher than the gastrin content found in extracts prepared from mammalian and avian species by the same group of workers.

A more convincing demonstration of amphibian gastrin has been provided by Gibson, Mihas, Colvin and Hirschowitz (1976) who prepared extracts of the oesophagus, fundus, antrum, duodenum, midgut, pancreas, liver and terminal

intestine of three species of amphibia, Rana catesbeiana, Rana pipiens and Necturus. Extracts were prepared by boiling in water followed by centrifugation, and the gastrin content of the supernatants was estimated by radioimmunoassay. Antibody 1296 which recognises the C-terminal portion of heptadecapeptide gastrin and cross-reacts equally with mini-, heptadecapeptide and big gastrin as discussed in section 2 IV, was used for these measurements. The highest tissue concentrations of gastrin in both species of Rana were found in the antrum, followed by the duodenum and pancreas. In the case of Necturus the highest tissue gastrin levels were detected in the area just distal to the pyloric sphincter, corresponding to the duodenum in mammals, followed in decreasing order by the antrum and pancreas.

Chromatography of amphibian serum and tissue extracts was performed using a Sephadex G-50 superfine column which had been calibrated with natural human G-13 I, G-17 I and G-34 I gastrins. Serum of R. catesbeiana was resolved in two peaks, one corresponding to G-34 and one in the void volume, which may correspond to "big big" gastrin. A component eluting in the region of G-34 predominated in extracts of antral mucosa of both R. catesbeiana and Necturus and a small fraction was found eluting in the regions of G-17 and G-13 (Gibson, Mihas et al., 1976). In the duodenal extracts much immunoreactive gastrin eluted in the void volume. R. catesbeiana duodenal extracts also yielded a peak in the region of G-34, while Necturus was found to possess a small amount of immunoreactivity corresponding to the G-34 and G-17 elution volumes. The pattern of an increasing fraction of gastrin immunoreactivity in the "big big" form as one proceeds down the gastrointestinal tract observed here in the amphibia parallels the findings of the distribution of "big big" gastrin in the human (Yalow and Berson, 1972; Yalow and Wu, 1973).

A gastrin-like substance has been extracted from the elasmobranch Rhinobatus productus, the shovelnose guitarfish, by Hansen (1975). Extracts of three sections of the gut, the upper stomach, the antrum and the spiral valve (including the duodenum and colon) were prepared according to the first stages of the method described by Gregory and Tracy (1964) for the extraction of porcine gastrin. Extracts of somatic muscle from the same fish were used as a negative control, and porcine antral mucosa was extracted at the same time to act as a positive control. Gastrin activity of the extracts was tested using a rat bioassay system, and any extract showing acid stimulatory activity was tested by paper chromatography to exclude the possibility of histamine contamination. Extracts of the ray antrum were found to be about 25% as potent as the porcine antral extract in this system, whereas no response was obtained with extracts from the upper stomach, spiral valve or somatic muscle. Although it is not known whether this gastrin factor is functional in gastric secretion, this work did establish that gastrin or a substance with gastrin-like activity was present in one of the lowest forms of vertebrate.

Proceeding to an even lower vertebrate species, the lampreys and hagfishes, there have been reports of immunofluorescent localisation of cells containing polypeptide hormones in the cyclostome or hagfish, Myxine glutinosa, and of secretin and CCK-PZ activity in intestinal extracts of both the river lamprey, Lampetra fluviatilis, and the marine lamprey, Petromyzon marinus. Östberg, Van Noorden, Pearse and Thomas (1976) identified cells in the epithelium of the hagfish intestine which reacted with antisera to synthetic human gastrin, pentagastrin, caerulein and porcine glucagon. These cells were argyrophil, contained spherical secretion granules, and were "open type" endocrine cells, extending from the basal lamina to the lumenal surface of the gut. Although they resembled mammalian gut endocrine cells cytochemically and ultrastructurally,

they did not display the APUD characteristics of uptake and decarboxylation of biogenic amine precursors. In this respect they differed from the insulin-producing beta cells of the hagfish, which had the appearance of "closed type" endocrine cells. These beta cells were found in the pancreatic islets and bile duct epithelium and displayed APUD characteristics (Östberg, Van Noorden and Pearse, 1975). The hagfish pancreas consists of exocrine zymogen cells scattered along the intestinal epithelium with islet tissue parenchyma located completely separately, in intimate association with the bile duct and intestine (Östberg, Van Noorden et al., 1975). These beta cells were demonstrated to contain insulin by immunofluorescence, whereas no cells in the bile duct mucosa or islet parenchyma showed immunoreactivity to gastrin, secretin or caerulein (Östberg, Van Noorden et al., 1975).

The demonstration of secretin and CCK-PZ activity in extracts of the intestine of the river and the marine lamprey by Barrington and Dockray (1970) was the first report on the occurrence of these hormones in lampreys. These extracts evoked an increased flow of pancreatic secretion and an increase in its protein concentration in a rat preparation, whereas no response was observed with extracts of control tissues, skin or liver. Although a substance or substances with secretin and CCK-PZ-like activity have been identified in these species, it was not established whether molecules similar to the secretin and CCK-PZ of mammals were involved, or whether they function physiologically in the hormonal control of secretory activity in the intestinal epithelium of lampreys. It was suggested that these substances may not be released into the blood stream but may act locally as tissue hormones, released for example by secretagogues in the food (Barrington and Dockray, 1970).

Having discussed the studies demonstrating the presence of gastrin in the vertebrates, the next section will cover what is known regarding the occurrence of this hormone in invertebrate species.

2 VIII(ii) Gastrin in Invertebrates

There is scant information on the occurrence of gastrin in invertebrate species and, according to Bentley (1976), its distribution in non-mammals has not yet been systematically explored. Straus, Yalow and Gainer (1975) have described the presence of immunoreactive gastrin in the blood and intestinal tissues of two species of mollusc, the sea hare Aplysia californica and the land snail Otala lactea. Moreover, this molluscan gastrin displayed molecular heterogeneity similar to that found in mammalian species. Gastrin concentrations in the blood ranged from 40-150 pg/ml, values similar to those found in mammals, and the intestinal tissues had immunoreactive gastrin contents of 1,5-5 ng/g wet weight of tissues. Blood and gut extracts of Otala contained two gastrin components, as determined using Sephadex G-50 chromatography, one eluting in the void volume and one with an elution volume greater than that of ^{125}I-porcine heptadecapeptide gastrin I. In the active as opposed to the aestivating snail the smaller molecular form was stimulated, presumably by feeding. The "big big" gastrin comprised 43% of the total immunoreactivity in the gut in contrast to a corresponding value of 0,1% of antral gastrin in man (Yalow and Berson, 1972). In the case of Aplysia three immunoreactive gastrin components were identified on Sephadex G-50 chromatography. A component corresponding to big gastrin as well as the "big big" and smaller forms found in Otala was identified. Each peak maintained its integrity on refractionation and the big gastrin component was converted to the small form on incubation with trypsin, as was the case for mammalian big gastrin (Yalow and Berson, 1971a). The prominence of a component resembling "big big" gastrin in chromatographic properties lends support to the theory that this molecule is a precursor form and suggests that the system for conversion to the smaller hormonal forms may not be as well developed in the lower phyla (Straus, Yalow et al., 1975).

Although Straus, Yalow et al. (1975) mentioned that their antiserum, which was raised in guinea pigs to crude porcine antral extracts, reacted poorly with CCK-PZ, the fact that their antiserum was raised to crude porcine gastrin may be reason to view this study with reservations. Further reports on investigation of the occurrence of gastrin in invertebrate species should either confirm or refute these observations. In the meantime it may be helpful to consider reports on the findings of two other gastrointestinal hormones, glucagon and insulin, in the invertebrates.

Assan, Tchobroutsky and Rosselin (1969) described the presence of immunoreactive glucagon-like material in acid alcoholic pancreatic extracts of various mammals, birds and amphibia, in mesenteric extracts prepared from two fish, in hepatopancreatic extracts from two molluscs and a crab, and in a total extract of one tunicate. In the vertebrates a glucagon-like immunoreactive material was found in gut extracts. None of this material was found in tissues other than those from the digestive system in any of the species studied. Curves of displacement of antibody-bound labelled glucagon by dilutions of these tissue extracts showed that the amphibian pancreatic extracts, the invertebrate hepatopancreatic extracts and the fish mesenteric extracts differed from those of mammalian pancreatic extracts. In addition, the glucagon immunoreactivity of gastric origin behaved differently to that extracted from the pancreas. Thus there is concrete evidence for the presence of glucagon or a glucagon-like substance in the higher vertebrates, whereas in the invertebrates evidence for the occurrence of a glucagon-like material resembling that of higher species is not as convincing.

Several reports on the occurrence of insulin, or an insulin-like substance, in lower species have appeared. Davidson, Falkmer, Mehrotra and Wilson (1971) identified insulin activity in protostomian and deuterostomian species by

testing acid alcohol extracts of the digestive tract using the mouse hemi-diaphragm insulin assay. These two main evolutionary lines are subdivided on the basis of embryological development; in the protostomes, which include the annelids, arthropods and molluscs, the blastopore gives rise to the mouth, whereas in the deuterostomes, including the echinoderms, tunicates and vertebrates, the blastopore gives rise to the anus (Falkmer and Patent, 1972). In the deuterostomes insulin was extracted and purified from the pyloric caeca of the starfish, Pisaster ochraceous and Asterias rubens (Wilson and Falkmer, 1965) and extracted from the stomach and intestine of the sea squirt, Ciona intestinalis (Davidson, Falkmer et al., 1971). Insulin activity was also found in the digestive tract of three molluscs, Buccinium undatum, Pecten maximus and Eledone cirrosa, and in the hepatopancreas of a crustacean, Carcinus meanus, both representatives of the protostomes. Neutralisation of the insulin activities of intestinal extracts from the sea squirt, C. intestinalis, the octopus, E. cirrosa and the whelk, B. undatum by antiserum to ox insulin provided strong evidence that immunoreactive insulin is present in the intestinal tissue of these species (Davidson, Falkmer et al., 1971).

This group had difficulty in demonstrating beta cells by light microscopical reactions although scattered dark epithelial cells showing beta cell staining characteristics were seen in the intestinal submucosa of the scallop Pecten maximus and in the hepatopancreas of two crustacean arthropods, Homarus gammarus and C. meanus investigated. Difficulty in demonstrating beta cells by light microscopy and histochemical analysis was also reported by Chan and Fontaine (1971), who were unable to demonstrate beta cells in the starfish Pisaster ochraceous, using paraldehyde-fuchsin stain. However, Wilson and Falkmer (1965) identified granular cells that gave histochemical reactions similar to those of vertebrate beta cells in the starfish P. ochraceous and A. rubens.

Davidson, Falkmer et al. (1971) failed to demonstrate cells which resembled mammalian beta cells in two coelenterate species, the jellyfish Aurelia aurita and the sea anemone Metridium senile, nor did they detect insulin activity in tissue extracts of these cnidarian species.

Insulin-like producing cells have been identified by immunofluorescence in the intestine in the area of the hepatopancreas of the bivalve mollusc Mytilus edulis (Fritsch, Van Noorden and Pearse, 1976). The cells were scattered unevenly in the epithelium and appeared to rest on the basal lamina of the single-layer epithelium and extend to the lumen of the gut, a typical arrangement for a primitive endocrine cell. No staining was obtained with antisera to glucagon, gastrin, pentagastrin or caerulein, either in the intestine or in the rectum. Like Chan and Fontaine (1971) they found that paraldehyde-fuchsin stain, commonly used to demonstrate insulin-containing cells, is insufficient when used alone. However, by coupling light microscopical staining with the technique of immunofluorescence the presence within these cells of a substance with insulin-like immunoreactivity was confirmed (Fritsch, Van Noorden et al., 1976).

Thus the information available on the distribution of gastrin or gastrin-like material in the invertebrate phyla appears to be limited to one report of immunoreactive gastrin in two species of mollusca (Straus, Yalow et al., 1975). Glucagon immunoreactivity has been detected in two invertebrate phyla, the mollusca and the crustacea (Assan, Tchobroutsky et al., 1969) but has not been well characterised. Insulin has been found in the echinodermata, mollusca and crustacea, as well as in the tunicates and many vertebrate species. According to Bentley (1976) beta cells are present in all vertebrates.

2 VIII(iii) Phylogenetic Considerations

The striking structural resemblance between the gastrointestinal hormones gastrin, cholecystokinin-pancreozymin and the frog skin peptide, caerulein, on the one hand, and secretin and glucagon on the other hand, suggests common ancestral origins. The structure of the C-terminal dodecapeptide of CCK-PZ was determined by Ondetti, Pluscec, Sabo, Sheehan and Williams (1970) following on the work of Mutt and Jorpes (1968), who deduced the partial structure of CCK-PZ and showed the last 8 amino acids of the C-terminal tryptic octapeptide to be identical with those in the same part of the molecule of caerulein, except for the replacement of threonine by methionine in the sixth position from the C-terminus. Anastasi, Erspamer and Endean (1967) described the isolation of caerulein in pure form from methanol extracts of the skin of Hyla caerulia, the Australian amphibian. Like CCK-PZ this molecule bears the identical C-terminal pentapeptide sequence to gastrin, and like both CCK-PZ and gastrin II it bears a sulphated tyrosine residue, which is situated in the 7th position from the C-terminus in the case of CCK-PZ (Ondetti, Plusec et al., 1970) and caerulein (Anastasi, Erspamer et al., 1967) and in the 6th position in gastrin II. Gastrin occurs naturally in both the sulphated and non-sulphated forms and both are biologically active (Gregory and Tracy, 1964), whereas CCK-PZ and caerulein require the presence of this sulphate group for biological activity (Erspamer, 1973). The chemical similarity of these molecules is accompanied by a close resemblance in the pharmacological effects displayed by caerulein and the gastroduodenal hormones; in fact the C-terminal heptapeptides of both caerulein and CCK-PZ are more potent than cholecystokinin itself, even on a molar basis (Erspamer, 1973). The structures of these molecules are shown in figure (3.1.1.) in the next chapter.

Glucagon and secretin share initial octapeptides which are identical except for one amino acid, and there are resemblances at other sites in the two molecules (Ganong, 1977, p. 365). Porcine secretin has two less amino acids than porcine glucagon but shares 15 amino acids at identical positions in the molecule (Bentley, 1976).

Studies on the occurrence of these hormones in vertebrates and lower species lend support to the theory of a common molecular ancestry. According to Barrington and Dockray (1970) insulin-secreting beta cells occur in both gnathostomes and agnathans, while glucagon-secreting alpha cells make their first appearance in the gnathostomes. Makhlouf (1974) is of the opinion that secretin probably antedates all the other gastrointestinal hormones, since it has been extracted from the intestine of the octopus, a mollusc (Ledrut and Unger, 1936, cited by Makhlouf, 1974, p. 172). Substances displaying secretin and CCK-PZ activity have been extracted from the intestine of the lowliest agnathan vertebrates (Barrington and Dockray, 1970). Since according to Makhlouf (1974) glucagon and gastrin appear later, coincidentally with the development of jaws and a stomach in gnathostomes, it is tempting to speculate that CCK-PZ and secretin are the molecular ancestors of gastrin and glucagon respectively (Makhlouf, 1974; Barrington and Dockray, 1970).

Track (1973) proposed an interesting theory in which he suggests that the ancestral protein for the gut peptide hormones was a proinsulin-like molecule. By determining the nucleotide sequence in the DNA molecule which codes for proinsulin and experimentally shifting the reading frame one nucleotide, he produced entirely new sequences of triplet codons in the DNA molecule. Frameshift mutations of the triplets coding for the distal 25 amino acids of proinsulin resulted in an amino acid sequence which resembled gastrin in 18% of its amino acids and glucagon in 25% of its sequence, and a 52% similarity

between glucagon and secretin was found.

From these findings Track (1973) proposed that the starting material for this family of polypeptide hormones was a DNA nucleotide sequence coding for a proinsulin-like protein, which underwent gene duplication to yield two pro-insulin-like molecules. One supposedly evolved into insulin while the other underwent a frameshift mutation followed by further gene duplication. One of these resultant DNA sequences gave rise to a protein which evolved into gastrin, while the other underwent yet another gene duplication and evolved into secretin and glucagon. Track (1973) assumed that the occurrence of insulin, glucagon and gastrin in lower species supports his theory of molecular evolution of the gastrointestinal hormones, although he admits that more comprehensive information on the presence of these hormones in lower phyla is needed to confirm his theory.

Another interesting theory on the evolutionary origin of peptide hormones was put forward by Adelson (1971), who considered the evolution of tissue function by examining the embryology of the protein-secreting glands and the biochemical characteristics of the proteins secreted by these tissues. His survey showed that many of the secreted proteins of vertebrates are related to each other biologically and chemically, and the proteins seem to have originated among the digestive enzymes of the gut of early pre-vertebrate species. Adelson (1971) suggested that many of the vertebrate peptide hormones arose by gene duplication and divergent evolution of a few primitive gene products.

The finding of gastrointestinal hormones similar to those of mammals in lower vertebrates and invertebrates does not necessarily indicate that they subserve the same function in these species as they do in mammals. Indeed, Steiner, Kemmler et al. (1974) have suggested that most peptide hormones evolved from previously existing gene products that originally served another role, perhaps as enzymes such as secreted hydrolases.

If gastrin is present in invertebrate species it may not have a role in stimulation of hydrochloric acid secretion, since the pH measured in the digestive organs of many invertebrates is predominantly neutral or slightly alkaline (Prosser and Brown, 1961). However, Straus, Yalow et al. (1975) emphasised that their finding of molluscan gastrin in the gastric portion of the gastrointestinal tract, which has the least alkaline pH, suggests that molluscan gastrin resembles mammalian gastrin in at least some of its functions. If gastrin or a gastrin-like molecule is found to be widely distributed in the invertebrates, it may subserve another function in connection with ionic regulation of the body fluids, or possibly may even act as a pheromone, or sexual attractant. These possibilities stem from the findings of large amounts of the gastrin-related peptide, caerulein, in the skin of amphibia (Anastasi, Erspamer et al., 1967). Since the amphibia inhabit an aquatic environment the suggested functions seem feasible in the species. Erspamer (1973) has suggested that caerulein may have something to do with local regulations in the skin, interfering in some way in the external secretion of the skin and/or in the passage through the skin of water and electrolytes.

By the same token, the mammalian hormone prolactin, which has a role in stimulating lactation in mammals, subserves an entirely unrelated role in different classes of vertebrates (Geschwind, 1967). During the course of evolution the functions of this hormone have included stimulation of the crop sac in pigeons, stimulation of melanogenesis in some teleosts, and it has been demonstrated to promote growth in the hypophysectomised tadpole. In addition it has been shown to act as a survival factor for certain hypophysectomised euryhaline fish transferred to fresh water (Geschwind, 1967), implicating a role in ionic regulation in this instance.

Another interesting feature in the gastrin story is the resemblance of the G-cell in the human pyloric antrum to the structure of the cells in which gastrin was localised by immunofluorescence in the epithelial lining of the hagfish by Östberg, Van Noorden et al. (1976). Both cells are of the primitive endocrine variety, flask-shaped or elongated, resting on the basement membrane of the epithelium with microvillous processes extending towards the lumen. This is in contrast to the morphology of other endocrine cells which in many cases have become more specialised, such as the alpha and beta cells of the pancreas which have become incorporated into specialised islets, and the cells of the thyroid, parathyroid and adrenal glands which have become incorporated into glandular structures. Since gastrin is released into the lumen of the human stomach in amounts 6-20 times greater than that into the blood stream (Knight, Fiddian-Green and Vinik, 1978), and a biological role for lumenal gastrin has not yet been found, this appears to be a wasteful process and suggests evolutionary immaturity. This suggestion is upheld by the appearance of the G-cell, which has not evolved to the same extent as other endocrine cells morphologically.

Measurements of the gastrin content of tissues discussed here represent static estimations; those described in the following section deal with kinetic measurements of circulating gastrin.

Section IX Gastrin Metabolism and Kinetics

2 IX(i) Where is gastrin inactivated?

The half-life of heptadecapeptide gastrin in the circulation is of the order of 3-10 minutes as determined by the rate of disappearance of injected or infused gastrin (Ganguli, Elder, Smith and Hunter, 1970; Schrumpf and Semb, 1973; Schrumpf, Semb and Vold, 1973; Straus and Yalow, 1974; Walsh, Debas and Grossman, 1974), the rate of disappearance of ^{125}Iodine-labelled gastrin (Jaffe and Newton, 1969) and inhibition of gastrin release by antral acidification (Yalow and Berson, 1970a). This short half-life is accompanied by a high metabolic clearance rate, indicating that some organs in the body have the capacity to remove heptadecapeptide gastrin rapidly from the circulation.

In an early study of the capacity of various tissues to inactivate gastrin, Thompson, Reeder, Davidson, Jackson and Clendinnen (1973) incubated fresh tissue slices in buffer in the presence of synthetic human heptadecapeptide gastrin I at 37°C for two hours, followed by measurement of gastrin content of the supernatant fluid by means of bioassay and radioimmunoassay. Their results suggested that the potential mechanisms for the inactivation of gastrin resided in many tissues, with the greatest inactivation occurring following incubation with liver, kidney and lung. The nature of the gastrin inactivation process was not clearly defined but it was thought that deamidation of the C-terminal phenylalanine amide residue could be involved, since this would alter the biologically active C-terminal pentapeptide sequence (Tracy and Gregory, 1964).

Laster and Walsh (1968) described an enzymatic activity that catalyses hydrolysis of the terminal amide group of the hormonally active acyl derivative

of gastrin, t-BOC-tetrapeptide amide, to produce an inactive product. This amidase activity was found to be greatest in tissue homogenates of rat liver and small intestinal mucosa. A similar enzymatic activity which catalysed hydrolysis of the peptide bond between the penultimate aspartyl and the C-terminal phenylalanine amide residues was found in kidney homogenates (Laster and Walsh, 1968). It is tempting to speculate that the gastrin inactivation by tissue slices described by Thompson, Reeder et al. (1973) could be due to amidase enzyme systems such as the one described by Laster and Walsh (1968). Walsh and Laster (1973) further characterised this amidase activity found in liver homogenates and showed that the pH optimum for the intracellular enzyme system was approximately 6,7. They identified the same amidase activity in liver, small intestine mucosa and stomach mucosa in the rat, hamster, cat and dog. They concluded that these enzymes may be an important mechanism for inactivation of gastrin peptides used in pharmacologic studies but that this system probably does not play an important role in the inactivation of endogenous gastrin.

More recent studies on the metabolism and inactivation of gastrin involved monitoring the arteriovenous difference in gastrin levels entering and leaving an isolated perfused organ or an organ or limb in situ in response to stimulated levels of endogenous gastrin or to infused exogenous gastrin. Endogenous gastrin levels were usually stimulated by instillation of, for example, 0,5% acetylcholine into the gastric antrum (Evans, Reeder, Becker and Thompson, 1974). Studies involving raised exogenous gastrin levels produced by infusion of synthetic gastrin preparations are subject to the criticism that the infused gastrin may be of a different species to that into which it is being infused, and that it may be infused to produce abnormally high or pharmacological levels which are not representative of the true physiological situation.

Furthermore, since basal circulating gastrin consists chiefly of the big or G-34 type gastrin (Yalow and Berson, 1970b) and the circulating gastrin is heterogeneous, as outlined in section 2 V, it is apparent that infusion of one gastrin type may not produce an entirely physiological situation. The same argument holds with regard to infusions of preparations of natural as opposed to synthetic gastrins, and extrapolations from one to the other should be made with reservations. As Booth, Reeder, Hjelmquist, Brandt and Thompson (1973) point out, the definition and discovery of a catabolic system in the body does not necessarily indicate a physiological role for that system.

Infusion of ^{125}Iodine-labelled hormones enables convenient monitoring of the organ distribution of the hormone but may produce very misleading information as a result of the ability of certain tissues to fix iodine. Newton and Jaffe (1971) observed that ^{125}Iodine-labelled immunoreactive gastrin disappeared rapidly from peripheral venous plasma in dogs after a single intravenous injection, due to the capacity of the gastric antrum, gastric fundus and skeletal muscle to concentrate more radioactivity in the form of ^{125}Iodine than ^{125}I-gastrin. On the other hand the renal cortex sequestered much more ^{125}I-gastrin than ^{125}Iodine alone, suggesting that the renal cortex plays a role in the inactivation of ^{125}I-gastrin.

The small intestine appears to be involved in gastrin metabolism. Becker, Reeder and Thompson (1973) found significantly higher gastrin concentrations in arterial blood than in mesenteric veins draining the lower intestine in dogs receiving antral stimulation by intralumenal acetylcholine. Samples collected from the abdominal aorta were taken as representative of the superior mesenteric artery, and the superior mesenteric vein was cannulated directly via the splenic vein. There was no arteriovenous gastrin difference

during the basal unstimulated period, whereas transit of stimulated levels of endogenous gastrin through the small bowel diminished the integrated gastrin response by 43%. These studies suggested that the small intestine may take up gastrin above a certain threshold level, and can be reconciled with the observation by Straus, Gerson and Yalow (1974) that patients who had undergone massive resection of the small bowel displayed gastrin and gastric acid hypersecretion, which could be due to loss of catabolic mechanisms for gastrin in these patients.

In a similar study the same group demonstrated that the gastric fundus extracts gastrin during periods of stimulated gastrin release from the antrum, whereas during basal conditions transit of the gastric fundus resulted in no change in gastrin levels (Evans, Reeder et al., 1974). Samples were collected simultaneously from the right femoral artery which was representative of the arterial supply to the fundus, and a fundic vein which was catheterised for sampling of venous outflow from the gastric fundus. In 9 out of 14 dogs studied there was a 30% arteriovenous difference in circulating gastrin values, while in the remaining 5 no difference was found. Since only the 9 dogs showing fundic extraction of gastrin responded by secreting gastric acid, and the remaining 5 did not exhibit an acid secretory response, probably attributable to the effect of anaesthesia on the parietal cell, Evans, Reeder et al. (1974) suggested that the rate of extraction of gastrin by the fundus is greater during active acid secretion.

The recovery of intravenously administered ^{131}I-gastrin in the gastric juice of dogs shown by Chan Yip and Jordan (1973) is in keeping with the proposed role of the gastric fundus in the extraction of endogenous gastrin. Chan Yip and Jordan measured the immunoreactive radioactivity recovered following injection of ^{131}I-gastrin with a continuous infusion of histamine

throughout the experiment. Four hours after injection of the labelled material 12,4% of the injected dose was recovered in the urine, whereas 50,7% was recovered in the gastric juice collected from innervated gastric fistulae in these dogs. In control experiments in which dogs were given Na ^{131}Iodide instead of ^{131}I-gastrin only 5% radioactivity was precipitated. This study suggested that the fundic gland area of the stomach is a significant site for gastrin clearance which is correlated with the rate of gastric acid secretion. It is difficult to reconcile these findings with the demonstration by Newton and Jaffe (1971) that the gastric fundus concentrated selectively ^{125}Iodine in preference to ^{125}I-gastrin.

Thompson, Becker, Evans, Hjelmquist, Brandt and Reeder (1974) demonstrated that vascular transit of the hind limb, lung and heart are not associated with changes in concentration of either basal or stimulated levels of gastrin. They catheterised the femoral vein, femoral artery, right heart and superior mesenteric vein in three dogs and found the basal gastrin concentrations to be all clustered around 85 pg/ml. After irrigating the isolated antrum with 0,1% acetylcholine for 50 minutes, the gastrin concentrations in the femoral artery, femoral vein and right heart were all about 400 pg/ml, whereas in the superior mesenteric vein the value was 230 pg/ml, confirming their previous demonstration of intestinal clearance of gastrin (Becker, Reeder et al., 1973). After antral acidification the differences in gastrin disappeared and all the levels fell towards basal. Failure to demonstrate any change in gastrin levels on vascular transit of the hind limb confirmed that the arteriovenous differences in stimulated endogenous gastrin observed for the small bowel and gastric fundus were not the result of non-specific tissue uptake of gastrin. The same was true for infusion of exogenous synthetic human heptadecapeptide gastrin in the dog (Thompson,

Reeder et al., 1973).

The lung was shown to have no effect on gastrin by Dent, Levine, James, Hirsch and Fischer (1973), thus confirming the findings of Thompson, Becker et al. (1974) on the role of this organ with respect to gastrin. Dent, Levine et al. (1973) infused synthetic human gastrin into isolated perfused canine lung preparations, since the lung is known to rapidly destroy or alter the polypeptide hormones bradykinin and angiotensin. Although the oedema-free lung had no effect on immunoreactive plasma gastrin, interstitial pulmonary oedema produced transudation of gastrin into the oedema fluid, but not catabolism.

Negligible amounts of gastrin are transported in the lymphatic system (Thompson, Reeder et al., 1973), so it is doubtful that this system plays an important role in gastrin sequestration or catabolism. McGuigan, Jaffe and Newton (1970) measured gastrin in serum and thoracic duct lymph before, during and after irrigation of the antrum with acetylcholine, and found lower concentrations of gastrin in thoracic duct lymph than in peripheral venous blood, again suggesting that thoracic duct lymph is not a major route of transport of gastrin. The small increase in thoracic duct lymph gastrin levels following antral stimulation actually amounted to less than 1% of the gastrin transported via the portal vein, and probably represented equilibration of gastrin molecules within the extracellular fluid compartment (McGuigan, Jaffe et al., 1970).

There is an overwhelming weight of evidence implicating a role of the kidneys in the catabolism of gastrin, and peptic ulcer disease complicating acute and chronic renal failure has been described many times. Elevated gastrin levels have been described in patients with acute (Dent, Hirsch, James and Fischer, 1972) and chronic (Korman, Laver and Hansky, 1972)

renal failure. Dent, Hirsch et al. (1972) showed that plasma from patients with renal failure bound twice as much ^{125}I-gastrin as did plasma from normal subjects, suggesting the possibility that the damaged kidney releases a protein involved in sequestration and inactivation of gastrin. Sullivan, Tustanoff, Slaughter, Linton, Lindsay and Watson (1976) demonstrated elevated fasting serum gastrin concentrations and prolonged circulation of stimulated gastrin levels in response to a test meal in patients with renal failure, suggesting that abnormalities in gastric function may be explained by fasting and stimulated hypergastrinaemia in these patients.

In dogs bilateral nephrectomy increased the half-life of infused exogenous synthetic human gastrin from 2,54 minutes to 5,15 minutes during infusion of a low dose of gastrin, and from 2,85 minutes to 7,88 minutes when gastrin was infused at a higher dose. The metabolic clearance rate was decreased approximately two-fold after nephrectomy, indicating the importance of the kidney in catabolism of exogenous gastrin (Clendinnen, Reeder, Brandt and Thompson, 1973).

Thompson, Reeder et al. (1973) infused synthetic human gastrin I into dogs to study the uptake and excretion by the kidney. They showed that 40% of the infused gastrin was taken up by the kidneys and only 0,4% of this extracted gastrin was excreted in the urine, so presumably the gastrin was metabolized within the kidney. The effect of the kidney on stimulated levels of endogenous gastrin following local irrigation of the excluded gastric antrum with 0,5% acetylcholine was studied by catheterising the aorta as representative of the renal artery, the renal vein and the ureter (Thompson, Becker et al., 1974). At all times after stimulation the gastrin levels were higher in the renal artery than in the renal vein. They demonstrated a 35% - 37% loss of stimulated levels of gastrin on renal transit, with less than 1% of the

total amount of extracted gastrin recoverable in the urine, confirming the earlier findings of this group.

Several other studies involving the role of the kidneys in relation to levels of stimulated endogenous gastrin have been reported. Hjelmquist, Reeder, Brandt and Thompson (1972), in one of the first studies of endogenous as opposed to exogenous gastrin, demonstrated 30% inactivation of endogenous gastrin during a single passage through the human kidney following acetylcholine stimulation. Booth, Reeder, Hjelmquist, Brandt and Thompson (1973) reported the same findings in dogs and suggested that the failure of the kidneys to inactivate basal gastrin levels may be due to the fact that a threshold gastrin concentration is needed to activate the catabolic mechanism. Davidson, Springberg and Falkinburg (1973) again found 30% of renal artery gastrin was extracted from the blood by the kidney in the anaesthetised dog. In keeping with the above reports these workers also found that the excretion of gastrin in the urine was inconsequential.

Dent, Levine et al. (1973) studied the effect of perfusion of isolated canine kidneys with synthetic human gastrin (1-17) and were able to demonstrate consistent arteriovenous differences in gastrin levels, confirming a role of the kidney in the catabolism of heptadecapeptide gastrin.

Intravenous injection of ^{125}I-human gastrin into dogs was followed by a biphasic disappearance of radioactivity from plasma, the initial rapid rate of disappearance lasting about 1 hour, with less than 2% of the immunoreactive labelled gastrin excreted in the urine within 2 hours (Jaffe and Newton, 1969). Significant sequestration of labelled gastrin in the renal cortex was observed 15 minutes after injection, suggesting inactivation at this site. The gastric antrum was found to concentrate extremely high levels of the ^{125}Iodine label alone in this study, illustrating the problem of iodine fixation

by some tissues, as mentioned previously.

The kidneys are well established as a major site of removal from the circulation and of metabolism of many peptide hormones (Walsh and Grossman, 1975a). Rabkin and Colwell (1969) demonstrated a linear relationship between arterial plasma insulin and the renal arteriovenous insulin difference in the dog, with a urinary clearance of about 10% of the insulin sequestered by the kidneys. They suggested that the remaining 90% was presumably degraded by the kidneys, which appeared to play an important role in the extrahepatic regulation of blood insulin concentrations.

In the light of the foregoing discussion it appears that gastrin is cleared from the circulation and inactivated by at least three organs in the body: the kidneys, small bowel and gastric fundus. However, the recent report of Strunz, Walsh and Grossman (1978) disagrees with these findings, in that they demonstrated removal of 21-30% of infused exogenous human heptadeca-peptide gastrin by a single passage through the vascular beds of the intestine and kidney, as well as the head and a hind limb in the dog. These findings suggest that gastrin is removed at multiple sites throughout the body, and that gastrin removal may be a general property of most if not all capillary beds. This is difficult to reconcile with the demonstration by Thompson, Becker et al. (1974) of failure of the hind limb to extract stimulated levels of endogenous gastrin, which suggested to them that removal of gastrin by the intestine and gastric fundus was not the result of non-specific tissue uptake of gastrin.

The discrepancy between the findings of Strunz, Walsh et al. (1978) and those of other workers described earlier may be related to the fact that Strunz, Walsh et al. (1978) used infused exogenous gastrin, whereas most of the earlier studies involved stimulation of endogenous gastrin levels by antral irrigation with acetylcholine (Becker, Reeder et al., 1973; Evans, Reeder

et al., 1974), or simply measurement of basal endogenous gastrin levels (Dent, Hirsch et al., 1972; Korman, Laver et al., 1972).

Using a separate group of 6 dogs, Strunz, Walsh et al. (1978) demonstrated removal of 40% of infused exogenous heptadecapeptide gastrin by both the intestine and the liver. After correction for intestinal removal alone and for hepatic arterial and portal contributions to hepatic blood flow, the calculated hepatic removal of gastrin was 23%, which was similar to that for the other organs investigated.

The question of whether gastrin is removed from the circulation and inactivated by the liver is the subject of the next section.

2 IX(ii) The role of the liver in relation to gastrin

Since all gastrin produced by the pyloric antrum and remainder of the gut must traverse the liver before entering the systemic circulation en route to its primary site of action, the gastric parietal cells, it is possible that the liver may play a part in the inactivation and rapid clearance of gastrin from the circulation. The liver has been shown to be capable, either *in vivo* or *in vitro*, of inactivating the gastrointestinal hormones insulin, glucagon and secretin. Samols and Ryder (1961) showed that the human liver can remove 20-50% of insulin passing through it for periods of over 1 hour, as measured by radioimmunoassay. Kaden, Harding and Field (1973) showed that the liver of anaesthetized dogs extracted 40% of the insulin presented to it during a single transhepatic passage during a control period. Following glucose administration in the duodenum the absolute amount of insulin extracted by the liver increased, demonstrating an important role for the liver in regulating peripheral insulin concentrations. It has been established that insulin is inactivated and degraded by an enzymatic process which is found in many tissues in the body, but predominantly in liver, kidney, pancreas, testes and placenta (Mirsky, 1964).

Inactivation of glucagon by tissue slices *in vitro* was shown in homogenates of rat, rabbit and dog liver by Kenny (1956), who measured glucagon activity by bioassay, using rabbit liver slices. Further studies on rabbit liver homogenates revealed that the inactivating system was enzymic in nature (Kenny, 1956).

Skillman, Silen and Harper (1962) showed that the volume of pancreatic juice obtained following a constant infusion of secretin via the systemic as opposed to the portal route in dogs was greater, indicating partial inactivation of secretin by the liver. A secretin inactivating enzyme was isolated

and partially purified from dog liver and was shown to be heat-labile, with an optimum pH range for activity of 3-5, by Bridgewater, Kuroyanagi, Chiles and Necheles (1962). They found the same enzyme in dog kidney. Way, Johnson and Grossman (1969) demonstrated a 25% reduction in pancreatic volume and bicarbonate output in dogs following portal administration of a low dose of synthetic secretin (0,06 µg/kg/hr) as opposed to systemic administration, although no effect was observed with a higher dose of secretin (0,24 µg/kg/hr). In the same study the pancreatic responses to portal administration of CCK-PZ and caerulein were shown to be unaltered by portal administration.

The liver appears to play a minor role in the inactivation of heptadecapeptide gastrin, whereas tetragastrin is almost completely inactivated by liver passage (Walsh, 1975). Very few reports have appeared showing inactivation of G-17 gastrin by the liver, and those that have are subject to a certain amount of criticism. Thompson, Reeder, Davidson, Charters, Brückner, Lemmi and Miller (1969) studied the effect of hepatic transit of histamine, pentagastrin and gastrin using two parallel experiments: firstly they observed the effect of alternate portal and caval injections of these substances in alert dogs in Pavlov stands, and secondly they injected histamine, ^{14}C-pentagastrin, synthetic human gastrin and inulin into the portal vein and measured the concentrations of these substances in hepatic vein blood. They showed a greater than 70% loss in acid stimulating potency following portal injection of histamine and pentagastrin, and a greater than 90% measured loss of each substance. The mean measured loss of immunoreactive human heptadecapeptide gastrin across the liver was 46%. However, since the radioimmunoassay system used was too insensitive to measure basal levels of circulating gastrin (Thompson, Reeder et al., 1969), the estimate of 46% inactivation of G-17 gastrin by the

liver could be erroneous. In addition, this study involved infusion of supra-physiologic doses of gastrin. Initially it was thought that this observation of partial hepatic inactivation of gastrin could be involved in gastric hyper-secretion following portacaval shunting (Thompson, 1969), but this theory had to be abandoned when Thompson, Reeder et al. (1973) demonstrated that serum gastrin concentrations actually decreased after portacaval shunting in dogs given a test meal.

McGuigan, Jaffe and Newton (1970) measured endogenous gastrin at various sites in anaesthetized dogs which had received antral irrigation with 1% acetylcholine for 10 minutes. Although this study appeared to indicate extraction of gastrin by the liver, the difference in gastrin levels observed between the hepatic vein and the portal vein was actually due to dilution of portal vein blood with hepatic arterial blood. McGuigan, Jaffe et al. (1970) emphasised that their findings do not support hepatic extraction of endogenous gastrin.

Beger, Meves, Witte and Kraas (1972, cited by Walsh, 1975, p. 92) found significantly less acid secretion in man when porcine G-17 II was infused into the portal vein as compared with peripheral administration, suggesting hepatic inactivation of the biological effect of heptadecapeptide gastrin. This again emphasises the fact that immunological measurements may not necessarily reflect measurements of biological activity. Gastrin activity may be substantially affected by relatively slight chemical modification of the physiologically active carboxyl-terminal portion of the molecule (Tracy and Gregory, 1964) such as deamidation of the carboxyl-terminal phenylalanine residue (Laster and Walsh, 1968). Such modifications in the structure of gastrin, if they occur at a site removed from the immunologically active site, may not be detected by radioimmunoassay, and can give rise to

immunochemical measurement of biologically inactive or degradation products. Such a situation has been reported by Lewin, Hunziker, Stagg and Wyllie (1971), who infused gastrin into the superior mesenteric artery of anaesthetized dogs and took samples simultaneously from the distal arterial tree and from the portal vein for analysis by bioassay and radioimmunoassay. Bioassay showed significantly lower venous than arterial plasma gastrin levels, whereas measurements using radioimmunoassay showed no difference between the arterial and venous values. Thus gastrin was partially inactivated as it passed through the small bowel vascular bed, but no corresponding fall in immunoreactivity was detected.

The findings of Gillespie and Grossman (1962) that gastrin was as effective in stimulating acid secretion when given to dogs via the portal or systemic route suggested that the liver is not a selective site for inactivation of gastrin. In the same system these workers were able to confirm the well documented observation that histamine is inactivated by the liver. Thompson, Reeder et al. (1969) proposed that the failure of Gillespie and Grossman (1962) to demonstrate hepatic inactivation of gastrin may be due to a protective action of contaminating protein and peptide material in preventing deamidation or other inactivation, or sequestration of pure gastrin, since crude gastrin was used in this study. This seems a reasonable suggestion, but in the light of several other reports showing that the liver has a negligible effect on heptadecapeptide gastrin metabolism, it is probably not of great importance.

Perfusion of the isolated rat liver in situ with synthetic human G-17 I gastrin in a plasma-free medium failed to produce hepatic removal of gastrin from the perfusate, both at physiological and supraphysiological perfusion levels of gastrin (Sacks, Grant and Vinik, 1978). Chromatography using a Sephadex G-25/G-50 mixture revealed the same elution patterns both before

and after perfusion through the rat liver, suggesting that the isolated rat liver has no function in removal from the circulation or metabolism of synthetic human heptadecapeptide gastrin I, measured by radioimmunoassay.

No hepatic removal of endogenous basal levels or stimulated levels of exogenous gastrin was observed in the pig on perfusion of the liver in vivo with synthetic human heptadecapeptide gastrin I (Vinik, Hickman and Grant, 1978). These measurements were made by radioimmunoassay, suggesting that the liver has no effect on basal or stimulated levels of immunoreactive gastrin. Sephadex G-25/G-50 chromatography suggested qualitative changes in the molecular size of gastrin following circulation in vivo in the pig (Vinik, Hickman et al., 1978).

Although G-17 gastrin does not appear to be affected by the liver, the smaller gastrin peptides pentagastrin and tetragastrin are rapidly removed by passage through the liver. Temperley, Stagg and Wyllie (1971) demonstrated that pentagastrin was rapidly inactivated by the liver in experiments involving measurement of acid secretion in dogs and rats following its administration via the jugular vein and the portal vein. Acid secretion was measured by the bioassay technique of Smith, Lawrence et al. (1970). By the same token, Temperley, Stagg et al. (1971) showed that synthetic human gastrin G-17 I was not inactivated in the liver, but measurements after infusion of gastrin into the jugular vein and superior mesenteric artery indicated inactivation of the heptadecapeptide molecule by the small bowel. These results were confirmed in vitro by incubation of gastrin and pentagastrin with homogenates of liver and small bowel mucosa.

Debas and Grossman (1974) reported that the degree of hepatic inactivation of the gastrointestinal hormones they studied was inversely related to the molecular size or chain length of the peptide. Using dogs with a complete

portacaval transposition, they investigated the effect of passage through the liver on the biological activity of the gastrin peptides G-17, G-13, the (8-17) and (11-17) sequences, and pentagastrin. Pentagastrin and the C-terminal heptapeptide were more than 90% inactivated by the liver, the gastrins with 10, 13 and 17 amino acid residues were progressively less inactivated by passage through the liver, and secretin (27 amino acids) and CCK-PZ (33 amino acids) were not significantly affected. It appears from this study that peptides with up to 7 amino acid residues are markedly inactivated by the liver.

Studies involving injection of pentagastrin into dogs by Stagg, Temperley and Wyllie (1971) showed that this peptide is excreted by the liver into the bile largely in an inactivated form. Unlabelled pentagastrin was measured by bioassay in the portal and hepatic veins, and ^{14}C-labelled pentagastrin was used to trace the fate of the injected compound. One hour after the end of a 30-minute infusion of labelled pentagastrin, 24% of the administered dose remained in the dogs, 47% was found in the bile and 20% was recovered from the liver at the end of the experiments. This represented isotope which had been excreted into the bile canaliculi as was demonstrated by autoradiography of these livers. According to Stagg, Temperley et al. (1971) pentagastrin appears to meet a similar fate in man. Since the biological activity of heptadecapeptide gastrin and pentagastrin resides in the C-terminal tetrapeptide sequence (Tracy and Gregory, 1964) which is common to both molecules, Stagg, Temperley et al. (1971) suggested that the (1-13) portion of heptadecapeptide gastrin may in some way protect the active tetrapeptide from excretion and metabolism by the liver.

The mode of inactivation of pentagastrin by the liver was clarified when Wyllie, Stagg and Temperley (1974) isolated high speed supernatant fractions

of rat, dog and human liver which deamidated pentagastrin to pentagastrin acid. This compound contained all the amino acids of the parent substance, with the C-terminal phenylalanine residue in the free acid form, and was biologically inactive. Parallel experiments in which ^{14}C-pentagastrin was infused into anaesthetized dogs revealed that much of the radioactivity was recovered in the bile within one hour, and thin layer chromatography showed the presence of pentagastrin acid as well as unchanged pentagastrin in the bile. These findings in vivo confirm the presence of an amidase enzyme system in liver tissue preparations, as described by Laster and Walsh (1968) and Walsh and Laster (1973).

Reeder, Brandt, Watson, Hjelmquist and Thompson (1972) studied the effect of hepatic transit on physiologic levels of endogenous gastrin, by stimulating antral gastrin release with 0,5% acetylcholine irrigation and measuring gastrin concentrations entering and leaving the liver in anaesthetized dogs. Gastrin levels were measured in the portal vein, hepatic artery and hepatic vein by radioimmunoassay. Blood flow measurements using an electromagnetic flowmeter were made in the portal vein and hepatic artery, which together gave the total hepatic inflow, and it was assumed that hepatic outflow was the same. These measurements allowed comparison of the total mass of gastrin entering and leaving the liver per minute. The ratio of portal venous blood flow to hepatic arterial flow was about 2,2 : 1, and the flow did not change significantly after initiation of antral stimulation. Plasma gastrin levels rose from approximately 190 pg/ml in the basal state to over 310 pg/ml during antral stimulation. The mass of gastrin entering and leaving the liver following acetylcholine stimulation was significantly increased above the same values under basal conditions, but there was no significant difference between the afferent and efferent hepatic gastrin mass either before or during

antral stimulation. The average mass of gastrin excreted in the bile was approximately 60 pg/min which amounted to less than 0,1% of the mass of gastrin passing through the liver. The results of this study indicate that hepatic transit does not affect physiologic levels of endogenous gastrin in the dog (Reeder, Brandt et al., 1972).

There is little direct information on the effect of hepatic transit on G-34 gastrin. Dencker, Håkanson, Liedberg, Norryd, Oscarson, Rehfeld and Stadil (1973) measured the gastrin concentrations in portal and peripheral serum taken from human subjects and at the same time they chromatographed the serum to see if there was any difference in gastrin components in portal and peripheral serum. No differences in fasting immunoreactive gastrin levels or postprandial stimulated gastrin levels between portal and peripheral blood were found, again suggesting that endogenous gastrin in man is not affected by passage through the liver. The distribution of immunoreactive gastrin components separated by gel filtration was similar in portal and peripheral serum, suggesting that the liver has no effect on the relative concentrations of G-17 and G-34 as well as the other gastrin components.

The kinetics of the different gastrin forms in the circulation have been determined chiefly on studies in the dog by infusion of exogenous gastrin preparations, as well as by following the fate of endogenous and exogenous gastrin in man. These studies are reviewed in the next section.

2 IX(iii) Disappearance Half Times, Space of Distribution and Metabolic Clearance Rates of Gastrins

Most of the studies on gastrin metabolism and kinetics in vivo have been performed in dogs or man, by following the fate of endogenous gastrin levels or elevated levels of exogenous gastrin produced by pulse injections or prolonged infusions of natural or synthetic gastrin preparations. Measurements have been made by bioassay, radioimmunoassay, or both. Table (2.IX.1) summarises various aspects of kinetic studies on gastrin which have been described.

The half-life of endogenous gastrin in man was found to be 7 minutes following oral or intragastric administration of hydrochloric acid to patients with pernicious anaemia and measurement of the fall off in serum gastrin levels after such treatment (Yalow and Berson, 1970a). This value represents the disappearance rate of big and heptadecapeptide gastrins, since the predominant form of circulating gastrin is the big or G-34 type (Yalow and Berson, 1970b; 1971). This is in reasonable agreement with the half-life for big gastrin of 9 minutes determined by Straus and Yalow (1974) in dogs, as detailed further on in this section.

A half-life of 3-4 minutes for endogenous plasma gastrin in man was determined following suppression of stimulated levels using somatostatin by Le Roith, Vinik, Epstein, Baron, Olkenitzky and Pimstone (1975). The fact that these estimations were made with an antiserum which recognises only non-sulphated gastrins, may explain why the measurement of endogenous gastrin half-life in this instance was less than the value of 7 minutes found by Yalow and Berson (1970a) in man. According to Le Roith, Vinik et al. (1975), somatostatin had no effect on basal serum gastrin levels in normal subjects, whereas it did inhibit raised serum gastrin levels in cases of

TABLE (2.IX.1)

Details of Gastrin Kinetics Studies Previously Published

Abbreviations

$T_{\frac{1}{2}}$	:	half-life time
V	:	volume of distribution
M.C.R.	:	metabolic clearance rate
RIA	:	radioimmunoassay
ECF	:	extracellular fluid
I.V.	:	intravenous
µg	:	microgram
kg	:	kilogram
p mol	:	picomole
min	:	minute
hr	:	hour
^{131}I	:	^{131}Iodine
^{125}I	:	^{125}Iodine

TABLE (2.IX.1)

Authors	Date published and Reference	$T\frac{1}{2}$	V	M.C.R.	Animals used	Method of Administration, Dose and Measurement.
Blair, Harper & Reed	1962 J.Physiol. 163 : 47-48P	less than 4 min			Anaesthetised cats	I.V. crude gastrin extracts. Bioassay.
Jaffe & Newton	1969 Surg.Forum 20 : 312-313	10,3 min	19% (ECF) 28% (iodide space)		10 Dogs	I.V. injected ^{125}I-human gastrin. Immunoprecipitation.
Yalow & Berson	1970a Gastroenterology 58 : 1-14	7 min or less			5 Humans	Pernicious Anaemia Oral HCl. RIA.
Ganguli, Elder, Smith & Hunter	1970 Brit.J.Surg. 57 : 848	5,5 - 10,5 min			3 Humans	Injected SHG 100-150 µg. RIA.
McGuigan, Isaza & Landor	1971 Gastroenterology 61 : 659-666	5,6 min for V = 19,2% of bodyweight 8,4 min for V = 28,5% of body weight	19,2% of body weight 28,5% of body weight	767 litres/day = 35,5 ml/kg/min	3 Dogs	I.V. infusions of SHG I : 0,125; 0,250; 0,50; 1,0; 2,0 µg/kg/hr. RIA and acid output.
Thompson, Reeder et al.	1973 Nobel Symposium 16 : 111-135	3,1 min in first component			18 Dogs	I.V. infusions SHG I 2 hours 8 or 16 µg/hr RIA.
Reeder, Jackson et al.	1972 Am.J.Physiol. 222 : 1571-1574	Biexponential 1,38 min in 1st phase 6,33 min in 2nd phase "Model" $T\frac{1}{2}$ = 2,07 min			10 Dogs	I.V. infusions SHG I 2 hours 8 µg loading dose RIA.
Schrumpf & Semb	1973 Scand.J.Gastroenterol. 8 : 203-207	3,7 min Single exponential		70 ml/min/kg	4 Dogs	I.V. infusions SHG 2 hours 1 µg/kg/hr. 5 times higher infusion rate during 1st 5 min. RIA + acid output.
Schrumpf, Semb & Vold	1973 Scand.J.Gastroenterol. 8 : 731-734	Biexponential 1st phase 7,5 min 2nd phase 12,8 min		9,1 ml/min/kg	5 Humans	I.V. infusions SHG 2 hours 1 µg/kg/hr. 4 times higher infusion rate during 1st 4 min. RIA + acid output.
Straus & Yalow	1974 Gastroenterology 66 : 936-943	PG I + II : 3 min ^{131}I-PG I : 4-5 min BG : 9 min ^{125}I-BG : 10 min BBG, early distribution phase : 8 min BBG, metabolic phase : 90 min ^{131}I-(1-13) : 3 min ^{131}I-(3-17) : 5 min	11 + 12% of body weight, or twice plasma volume 12-13% of body weight 10-12% of body weight 6% of body weight, or plasma volume equilibrium V = 4x plasma volume 12% of body weight		2 Dogs	Pulse injections of various natural or synthetic gastrins, administered with ^{131}I-PG I each time to monitor accuracy and reproducibility of the injection procedure. RIA.

TABLE (2.IX.1) (continued)

Authors	Date published and Reference	$T_{\frac{1}{2}}$	V	M.C.R.	Animals used	Method of Administration, Dose and Measurement.
Walsh, Debas & Grossman	1974 J.Clin.Invest. 54 : 477-485	G-17 : 3,2 min G-34 : 15,8 min	HG-17 I : 0,24 l/kg HG-34 I : 0,23 l/kg = 25% of body weight		4 Dogs	I.V. infusions of natural human and porcine G-17 and human G-34, 25-300 p mol/kg/hr. RIA and acid secretion.
Debas, Walsh & Grossman	1974 Gut 15 : 686-689	G-13 : 1,8 min	G-13 : 0,17 l/kg	HG-13 I : 79 ml/kg/min HG-17 I : 83 ml/kg/min	3 Dogs	Stepwise I.V. infusions of natural human G-13 I, 100; 200; 400; 800 p mol/kg/hr, 40 min/dose. RIA + acid secretion.
Le Roith, Vinik et al.	1975 S.Afr.Med.J. 49 : 1601-1604	3-4 min			Humans	Suppression of stimulated levels of endogenous gastrin using I.V. somatostatin. RIA.
Walsh, Maxwell & Isenberg	1975 Clin.Res. 23 : 259A	HG-17 I : 5 min HG-34 I : 42 min	G-17 : 0,11 l/kg G-34 : 0,12 l/kg	G-17 : 16,5 ml/min G-34 : 2,1 ml/min	4 Humans	I.V. infusions of NHG-17 and NHG-34 in 90-minute doses ranging from 6,25 to 200 p mol/kg/hr. Each dose given on separate days. RIA.
Strunz, Walsh & Grossman	1978 Gastroenterology 74 : 32-33	3 min	6,5 % of bodyweight	15,1 ml/kg/min	6 Dogs	I.V. infusion of NHG-17 400 p mol/kg/hr. RIA.
Vinik, Hickman & Grant	1978 S.Afr.Med.J. In press	3,2 min	0,216 l/kg or 21,6% of bodyweight	2,014 l/min = 67,8 ml/kg/min	6 Pigs	I.V. stepwise infusions of SHG-17 I in doses of 0,25; 0,5 and 1,0 µg/kg/hr for one hour at each dose level. RIA.

pernicious anaemia, chronic renal disease, and in patients with elevated gastrin levels produced by insulin hypoglycaemia.

The half-life of a crude gastrin extract prepared by the method of Blair, Harper et al. (1961) and injected intravenously into the anaesthetized cat was determined by bioassay (Blair, Harper et al., 1962) to be less than 4 minutes. Pentagastrin was cleared from the circulation with a similar half time. Although basal gastrin levels in humans could not be detected with this technique the report merits due consideration because it is one of the few instances when the disappearance half time of gastrin was measured by bioassay and not by radioimmunoassay.

The half-life of intravenously injected ^{125}I-labelled human gastrin in dogs was 10,3 minutes as determined by measurement of immunoprecipitable labelled gastrin in plasma using the double antibody technique (Jaffe and Newton, 1969). The spaces of distribution of the ^{125}I-gastrin were 19% (comparable to the extracellular fluid) and 28% (similar to the iodide space) respectively. However, the problems in interpreting distribution of radioactivity caused by fixation of iodine by certain tissues have already been alluded to in section 2 IX(i). The half-life value found in this study was longer than that found by most other workers for G-17 gastrin, a finding which may be due to subtle changes induced in the hormone by the labelling process. In keeping with this suggestion is the finding by Straus and Yalow (1974) of a longer half-life of ^{131}I-porcine G-17 I in the circulation (4-5 min) as opposed to unlabelled porcine G-17 I and II, which both had half-lives of 3 minutes.

The remaining studies outlined in table (2.IX.1) involved administration of exogenous natural or synthetic gastrin preparations of various molecular sizes, either by intravenous injection of a bolus, or by prolonged intravenous

infusion at constant or increasing dose levels. The half-life of these gastrin preparations in the circulation has been estimated by measurement of the decline in serum gastrin concentrations with time after such injection or infusion. The metabolic clearance rate is another index of degradation or elimination of the gastrin molecule, and for accurate estimation of both these parameters it is necessary to correct for endogenous gastrin production by subtracting the basal gastrin levels from the post-injection or -infusion concentrations (Schrumpf and Semb, 1973).

The volume of distribution, expressed as a percentage of the total body weight, gives an indication of the body compartments into which the infused gastrin distributes itself. For instance, a volume of distribution of gastrin of approximately 5% of total body weight indicates that gastrin is distributed in a volume equivalent to plasma volume, whereas a value approximating 20% for this parameter suggests distribution of gastrin throughout the extracellular fluid. The volume of distribution of infused gastrin is determined using the plateau principle as described by Goldstein, Aronow and Kalman (1974), which embraces the mean infusion rate of gastrin, the mean plateau level of gastrin and the mean elimination rate (Schrumpf and Semb, 1973).

Exogenous gastrin was administered as a bolus intravenous injection in the studies described by Jaffe and Newton (1969), Ganguli, Elder et al. (1970) and Straus and Yalow (1974). According to Reeder, Jackson et al. (1972), this procedure produces distortion in the disappearance curve caused by mixing, and these effects are minimized by infusion of gastrin at a constant rate over a period of two hours for example, so that the spaces of distribution become saturated with gastrin. In such experiments the initial component of disappearance of the hormone after injection of a bolus is generally attributed to mixing within the plasma and the later component to

metabolism of the hormone. In order to compute the model half-life of a hormone in the circulation from a biexponential disappearance curve, the first component, due to mixing, must be rapid relative to metabolism (Reeder, Jackson et al., 1972). For this reason the gastrin infusion is often commenced with administration of a loading dose, followed by a constant infusion, as described by Reeder, Jackson et al. (1972), Schrumpf and Semb (1973) and Schrumpf, Semb et al. (1973).

Actual values for $T\frac{1}{2}$ of G-17 gastrin in the circulation were all close to 3-5 minutes when natural or synthetic gastrin was injected or infused into dogs. The value in man was higher: 5,5-10,5 minutes, found by Ganguli, Elder et al. (1970) and 7,5 and 12,8 minutes in the first and second disappearance phases reported by Schrumpf, Semb et al. (1973). The longer half-life of human G-17 gastrin preparations in the human as opposed to the dog almost certainly reflects the more rapid clearance of heterologous as opposed to homologous gastrin as suggested by Schrumpf, Semb et al. (1973). This is borne out by the higher metabolic clearance rate of human gastrin infused into dogs, of 70 ml/kg/min (Schrumpf and Semb, 1973), compared with the metabolic clearance rate of 9,1 ml/kg/min following infusion of human gastrin into humans (Schrumpf, Semb et al., 1973).

Apart from the very low metabolic clearance rate of G-17 gastrin reported by Walsh, Maxwell et al. (1975) of 16,5 ml/min in the human, and the high value for clearance of human G-17 from the canine circulation found by Schrumpf and Semb (1973), there was reasonable agreement between the values found for this parameter by different groups. The range was from 35,5 ml/kg/min (McGuigan, Isaza et al., 1971) to 83 ml/kg/min (Debas, Walsh et al., 1974) for human G-17 in the dog.

Estimations of the space of distribution of human G-17 ranged from 6,5% of body weight in the case of natural human gastrin infused into dogs (Strunz, Walsh et al., 1978) to 25% of body weight when natural human and porcine G-17 were infused into dogs (Walsh, Debas et al., 1974). Straus and Yalow (1974) found a space of distribution of 11-13% of body weight following pulse injection of ^{131}I-labelled or unlabelled porcine G-17 into dogs. It is interesting that two entirely different values for this parameter were reported by Walsh in the two separate publications just mentioned, although in each case the same preparation, natural human G-17, was infused in approximately the same dose into dogs. Yet a further value of 0,11 litres/kg was found following infusion of NHG-17 into man (Walsh, Maxwell et al., 1975), although in this instance the dose infused was lower.

Turning to the kinetics of G-34 and G-13 gastrin in the circulation, studies have been described by only two or three groups who have had access to sufficient amounts of purified preparations of these peptides. The half-life of natural human G-34 following pulse injection in the dog was 9 minutes (Straus and Yalow, 1974) and following infusion into dogs it was 15,8 minutes (Walsh, Debas et al., 1974). In man a half-life of 42 minutes for G-34 after intravenous infusion was found (Walsh, Maxwell et al., 1975), again suggesting that homologous gastrin is broken down more slowly than heterologous gastrin. Only one estimation of the metabolic clearance rate of G-34 has been reported: 2,1 ml/min for the clearance of NHG-34 I from the human circulation following intravenous infusion (Walsh, Maxwell et al., 1975). This very low value amounts to only 0,03 ml/kg/min in a man weighing 70 kilograms. Estimations of the space of distribution of NHG-34 ranged from 10-12% of body weight in the dog (Straus and Yalow, 1974) and man (Walsh, Maxwell et al., 1975) to 23% of body weight in

the dog, found by Walsh, Debas et al. (1974).

One study on the kinetics of a "big big" gastrin fraction comprising pooled void volume eluates obtained on fractionation of a Zollinger-Ellison tumour extract has been reported. A half-life of 90 minutes for the metabolic phase of the disappearance curve of BBG following pulse injection into one dog was found by Straus and Yalow (1974), and the BBG preparation was found to distribute itself in a volume equivalent to approximately 24% of body weight.

The half-life of NHG-13 I following infusion into dogs was 1,8 minutes, with a metabolic clearance rate similar to that of G-17 gastrin, of 79 ml/kg/min (Debas, Walsh et al., 1974). The space of distribution of G-13 in this study was slightly less than that for G-17 and G-34, with a value of 0,17 litres/kg. Considering the limits of experimental error, this estimation suggests that G-13 was distributed throughout the extracellular fluid volume, as was the case for the larger gastrin peptides.

Since the half-life of G-17 in the circulation is approximately five times shorter than that of G-34 (3,2 minutes as opposed to 15,8 minutes) (Walsh, Debas et al., 1974), it follows that G-17 is cleared from the circulation five times more rapidly than G-34. Comparison of the exogenous potencies of G-17 and G-34, by measuring the acid secretory response to equimolar infusions of these peptides, gave similar values. However, measurement of the acid secretion in terms of circulating concentrations of G-17 and G-34 revealed that G-17 has an endogenous potency approximately five times greater than G-34 (Walsh, Debas et al., 1974). This is because the circulating concentrations of G-34 reached five to six times higher levels than G-17 due to the longer half-life and slower rate of elimination of G-34 from the blood.

Comparison of G-17 and G-13 revealed that the exogenous potency of minigastrin is less than half that of G-17, whereas the endogenous potency of the two preparations was similar (Debas, Walsh et al., 1974). If one takes into account the slightly shorter half-life of G-13 (1,8 min) as opposed to G-17 (3,2 min) the calculated endogenous potency of G-13 approximates 120% of that of G-17. However, since the metabolic clearance rates of G-13 and G-17 were so similar (79 ml/kg/min and 83 ml/kg/min), Debas, Walsh et al. (1974) felt that the differences in calculated half-times for G-13 and G-17 were probably not significant.

These findings emphasise the difference between endogenous and exogenous potencies of the different gastrin preparations and indicate the importance of distinction between potency estimations based on measurement of the dose of a hormone administered and the immunoreactive levels measured in the circulation.

In summary, this review of studies on gastrin kinetics has shown good agreement between different studies in some parameters, with considerable variations in others. Differences may be attributable to: (i) different modes of administration of the gastrin preparations, (ii) different origins of the peptides, such as whether they are porcine or human and whether they are natural or synthetic preparations, (iii) whether they are labelled with radioactive iodine or unlabelled, (iv) durations and doses of infusions administered, and (v) differences in sites of sampling, since higher gastrin levels would be measured in samples drawn from the portal site as opposed to samples drawn from a peripheral vein.

The differences in exogenous and endogenous potency may relate to the clearance of gastrin by tissues or the reaction of gastrins with their target organs or receptors. In the next section the interaction of gastrin with receptors will be reviewed.

Section X Interaction of Gastrin with Receptors

For a hormone to activate a target tissue it must first bind to some constituent of the cell. Binding of labelled polypeptide hormones to specific receptors on their target cells has been studied in the case of insulin, glucagon, growth hormone, adrenocorticotrophic hormone, calcitonin, oxytocin, prolactin and many others (Roth, 1973). Although a handful of studies have recently appeared describing binding of gastrin to receptors, the intimate interaction of gastrin with the parietal cell is still totally unknown.

Binding of tritiated synthetic human gastrin I to gastric plasma membranes purified from rat fundic mucosa was demonstrated by Lewin, Soumarmon, Bali, Bonfils, Girma, Morgat and Fromageot (1976). Using ^{3}H-SHG I, with a specific activity of 60 Ci/mmole, which was "immunologically and biologically as reactive as unlabelled native SHG I", binding to gastric plasma membranes was shown to increase with time until reaching equilibrium within 15 minutes, at which time 1 to 5% of the total radioactivity was bound per mg protein. Addition of unlabelled gastrin displaced the labelled gastrin from the binding sites. Increased adenyl cyclase activity in the gastric plasma membranes was noted following stimulation with tritiated gastrin, suggesting that cyclic 3', 5'-AMP is involved in the regulation of acid secretion following stimulation by gastrin (Lewin, Soumarmon et al., 1976).

Having demonstrated saturable and reversible binding of labelled gastrin to gastric plasma membranes, which included membranes derived from parietal, peptic and mucous cells, the next step was to isolate pure parietal cells from the gastric plasma membranes and to demonstrate binding specifically to these cells. Soumarmon, Cheret and Lewin (1977) prepared

fundic cell populations containing up to 70% parietal cells, and demonstrated binding of ^{3}H-labelled gastrin which was proportional to the cell concentration and temperature, and increased with time. The binding was reversed by ten-fold dilution of the incubation medium, and increased binding was obtained with increased purification of the large cells, which were identified as parietal cells on electron microscopy. Thus the presence of high affinity binding sites for ^{3}H-labelled gastrin on rat gastric parietal cells, which were shown to be specific by the criteria of saturability, reversibility and displacement, was confirmed.

The sequence of events following binding of gastrin to mucosal cells prepared from guinea pig fundus was investigated by Del Mazo and McGuigan (1976), who demonstrated specific binding of ^{125}I-labelled synthetic human gastrin to these cells. Maximum binding was demonstrated at pH 7,4 and at 4°C, under which conditions 2 to 4 picograms ^{125}I-gastrin were bound by 3×10^{5} gastric mucosal cells. As was the case with the studies of Soumarmon and Lewin, the binding of labelled gastrin to mucosal cells produced a characteristic saturation curve with increasing concentrations of ^{125}I-gastrin. The ^{125}I-gastrin was degraded following incubation with gastric mucosal cells, as shown by reduction in binding of these preparations to gastrin antibodies and by failure of ^{125}I-gastrin which had been preincubated with mucosal cell preparations to be bound by a second preparation of fresh gastric mucosal cells.

Hormone degrading enzyme systems exist within or on the surfaces of cells containing receptors for polypeptide hormones, although these enzyme systems appear to be functionally independent of the hormone receptor site (Del Mazo and McGuigan, 1976). The fact that the greatest degradation of ^{125}I-gastrin occurred at pH values 2 and 7,4 following incubation with

mucosal cells in this study suggested the presence of at least two enzyme-degrading systems in these cell preparations. It was shown that the mode of degradation did not simply involve removal of the iodine label, since the ^{125}I-gastrin following incubation with mucosal cells did not behave like Na ^{125}Iodide on paper chromatography when eluted with butanol, dioxane and ammonium hydroxide (Del Mazo and McGuigan, 1976). The precise nature of the degradation of labelled gastrin by these cells was not revealed in this study. However, this demonstration of degradation of gastrin by fundic mucosal cells confirms the role of the gastric fundus in the degradation of gastrin suggested by the in vivo studies of Evans, Reeder et al. (1974), as discussed in the previous section.

Prior to the appearance of these three reports of binding of gastrin to receptors, the uptake of gastrin by a cytosol protein, rat liver ligandin, in vitro was demonstrated by Kirsch, Vinik, Frith, Gordon, Grant and Saunders (1975). Binding of both ^{125}I-gastrin and unlabelled synthetic human G-17 gastrin to this basic protein was demonstrated on separate occasions, and the specificity of binding was confirmed by displacement of the bound labelled gastrin on addition of an excess of unlabelled gastrin. Failure of this protein to bind Na ^{125}Iodide alone confirmed that the binding was not to the iodine label, and the fact that binding to the gut hormones glucagon and insulin was not demonstrated further indicates the specificity of binding.

Ligandin, also known as Y-protein, has been purified from liver, kidney and intestinal homogenates of mammals and many vertebrates, where it constitutes approximately 4% of total supernatant protein in the case of liver and 2% of the total supernatant protein of kidney and intestinal homogenates. It is a basic protein with a molecular weight of approximately

46 000, consisting of 2 identical subunits (Fleischner, Mishkin, Reyes, Robbins, Levi, Gatmaitan and Arias, 1971; Litwack, Ketterer and Arias, 1971; Fleischner and Arias, 1976). It has been localised to the parenchymal liver cells, the proximal renal tubular cells and the non-goblet mucosal cells of the small intestine, and is known to bind certain organic anions including bilirubin, dyes, drugs, cholecystographic agents and steroids. Endogenous substances are bound non-covalently with varying affinities, whereas carcinogens differ from other compounds in that they are bound both covalently and non-covalently. Binding of different agents has been demonstrated both in vivo, on injection of the compounds, and following their addition to liver homogenates in vitro. The electrostatic binding of the ligand BSP to ligandin was used at each stage of the purification to locate the ligandin peak on gel filtration (Fleischner, Mishkin et al., 1971; Litwack, Ketterer et al., 1971; Fleischner and Arias, 1976).

The levels of ligandin increase to over 200% of control values following administration of phenobarbitone, DDT and other compounds which induce the drug and steroid-metabolising enzymes of the endoplasmic reticulum. The increased concentrations in response to phenobarbitone administration result from enhanced synthesis of ligandin rather than decreased degradation, and the increased levels of ligandin are associated with an increase in the disappearance rates from plasma and increased hepatic content of bromosulphthalein (BSP), indocyanine green (ICG) and bilirubin (Fleischner, Mishkin et al., 1971). This direct relationship between the hepatic concentration of ligandin and the net flux of bilirubin, BSP and other organic anions between the plasma and the liver cell suggests that ligandin is important in selective transfer of various organic anions from plasma into the liver

(Fleischner, Mishkin et al., 1971). More recently it has been shown that both liver and renal ligandin are identical with glutathione transferase B, confirming that ligandin is enzymic in nature (Fleischner and Arias, 1976).

If one regards a hormone receptor as an agent which binds a hormone, whether it be located in the plasma membrane of the target cell or in the actual cytosol, then the demonstration of binding of gastrin to rat liver ligandin by Kirsch, Vinik et al. (1975) must be regarded as one of the earliest reports of binding of gastrin to a receptor. It is certainly the first report of evidence for gastrin entering the cytosol. The gastrin molecule is strongly anionic at neutral pH, due to the presence of five glutamic acid residues in positions (6-10) of the heptadecapeptide. This suggests the possibility that the binding of gastrin to rat liver ligandin in this study could be merely an ionic effect due to the highly charged nature of the gastrin molecule, in the same way that ligandin binds many other organic anions. Ligandin has been shown to be an enzyme system (Fleischner and Arias, 1976), yet it does not appear to degrade gastrin. It may transpire that the liver cell is an additional target cell for gastrin, subserving a role yet to be clarified.

In the pages which follow, appear details of the experimental protocols of studies designed to fill a part of the hiatus in knowledge of the structure-antigenicity and physiological functions of gastrin and related peptides.

CHAPTER 3

MATERIALS AND METHODS

Section I Radioimmunoassay

3 I(i) Raising of Antisera

Synthetic human heptadecapeptide gastrin G-17 I, obtained from Imperial Chemical Industries Ltd. (I.C.I.), was coupled to B. cincta haemocyanin with carbodiimide according to a modification of the method described by Goodfriend, Levine and Fasman (1964). Nine hundred micrograms (µg) gastrin dissolved in 0,05M-aqueous ammonium hydrogen carbonate, 1 ml 0,05M-sodium phosphate buffer pH 7,5, 0,5 ml haemocyanin, and 40 mg 1-Ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDAP-CDI), (Sigma), were placed in a small beaker and mixed gently on a magnetic stirrer for 30 minutes at room temperature. The mixture was then dialysed against distilled water overnight at 4°C using 6 mm dialysis tubing with a pore size retaining molecules greater than 12 000 (A.H. Thomas). The granular pearly-grey conjugate was withdrawn from the dialysis tubing using a syringe and mixed with an equal volume of Freund's complete adjuvant (Difco) just prior to injection. Loss of gastrin through the dialysis tubing after conjugation was minimal as shown by measurement of the gastrin content of the dialysis fluid by radioimmunoassay. A gastrin concentration of 172 pg/ml in a volume of 1 074 ml gave a total of 0,185 µg gastrin in the dialysate, which is only 0,021% of the original dose of 900 µg gastrin used for the conjugation.

Three-month-old male albino rabbits were immunised with a priming dose of 100 µg coupled synthetic human heptadecapeptide gastrin in an emulsion with Freund's adjuvant, injected painlessly at multiple dorsal subcutaneous sites.

At the first immunisation each rabbit was also given 0,5 ml Bordetella pertussis (S.A.I.M.R.) (whooping cough vaccine) in the same manner to stimulate the immune system. Approximately 20 ml blood was collected from an ear vein from each animal 30 and 60 days after the first immunisation, and the sera were tested for antibody titre. Subsequent injections of 50 µg synthetic human gastrin I (SHG I) emulsified in Freund's complete adjuvant were given every six weeks, commencing 10 days after the 60-day bleed, and the sera collected 7-10 days after each booster injection for testing. Antiserum G 5 was raised using this method.

Antiserum G 1 was obtained commercially from CEA-IRE Sorin Pharmaceuticals Ltd., and was raised according to the method of McGuigan (1968a) in which synthetic human gastrin (2-17) was coupled to bovine serum albumen, using EDAP-carbodiimide. G 1 antiserum was used at a final dilution of 1:200 000 in the assay. McGuigan's (1968a) conjugation and immunisation procedures are outlined in the appendix.

Antiserum 2604-7 was donated by Dr. J.Rehfeld of Copenhagen. It was raised according to the method described by Rehfeld, Stadil and Rubin (1972), and could be used in the assay at a final dilution of 1:100 000. All three antisera were characterised and their cross-reactivity with related peptides was determined before use in the immunoassay.

The terms "antibody" and "antiserum" are used interchangeably in this thesis. Although strictly speaking incorrect, since an antiserum may contain more than one population of antibodies, the fact that these antisera have been characterised in terms of their predominant antigenic interaction(s) one feels is reason enough to permit this liberty. For the purist, where the term "antibody" appears in the text and figures one should read "antiserum".

3 I(ii) Iodination Procedure

Synthetic human heptadecapeptide gastrin I (I.C.I.) was labelled with ^{125}Iodine (Radiochemical Centre Ltd., Amersham, England) according to a modification of the method of Ganguli and Hunter (1971). The iodination was performed in a glass cone-shaped vessel to facilitate rapid mixing of reagents, which were blown into the vessel from glass micropipettes, with the exception of the radioactive iodine, which was added with a "hot" Hamilton syringe, reserved especially for this purpose. When aiming to incorporate 2 000 microcuries (μCi) ^{125}Iodine per microgram (μg) peptide, the reagents were added in the following volumes, in the sequence indicated:

0,2M-sodium phosphate buffer pH 7,5	20 μl
0,5 μg SHG-17 I	5 μl
1 mCi Na^{125}I	10 μl
Chloramine-T 16 μg	20 μl
Reaction volume	55 μl
Sodium metabisulphite 40 μg	50 μl
Potassium iodide 2 mg	200 μl
Normal human plasma	20 μl
	325 μl

(The potassium iodide was added to enable the high concentration of cold iodine to dilute out the ^{125}I atoms which were not incorporated into the hormone, and so reduce the damage they may cause by auto-irradiation of the labelled hormone. The human plasma was added to "mop up" the free radicals generated in the iodination reaction.)

The iodination mixture was immediately applied to a small Sephadex G-10 column (0,9 x 10 cm) which had been pre-saturated with 4% human serum albumen, and eluted with 0,05M-sodium phosphate buffer, pH 7,5, at room temperature. Fractions of 1 ml volume were collected and the elution profile of counts per second (c.p.s.) against fraction number was plotted. The percentage incorporation of ^{125}Iodine into the hormone was determined by charcoal adsorption of intact labelled peptide (Vinik, Deppe and Joubert, 1970; Binoux and Odell, 1973) using a 5 ul aliquot of the mother liquor before addition to the column, diluted appropriately. The integrity of the eluted fractions was tested by charcoal adsorption of intact labelled peptide (Vinik, Deppe et al., 1970; Binoux and Odell, 1973). The immunological integrity of eluted fractions in the ^{125}I-gastrin peak was determined by incubating an aliquot of the labelled hormone with an excess of antibody for 18 hours at 4°C. For each iodination the fraction displaying the best immunological integrity was used in the assay.

The purity and immunological integrity of the labelled hormone was not tested using high voltage paper electrophoresis as is customary for other hormones such as insulin (Yalow and Berson, 1960) because of the paradoxical reversed behaviour of gastrin on cellulose paper strips, due to the highly charged nature of the gastrin molecule. This finding has been reported by other workers as well (Yalow and Berson, 1970a).

The ratio of ^{125}Iodine to cold hormone aimed at for use in the radioimmunoassay was usually between 1 000 and 2 000 μCi ^{125}Iodine per μg protein. This term has been loosely referred to as "specific activity".

Alternative to this iodination procedure ^{125}Iodine-labelled synthetic human heptadecapeptide gastrin I was purchased from CEA-IRE Sorin and repurified on a Sephadex G-10 column when necessary. The integrity of the commercially labelled gastrin preparations was monitored by charcoal adsorption of intact labelled peptide in the assay. The immunological integrity of these preparations was assessed using antiserum G 5. Antibody dilution studies had shown that the maximal binding of labelled gastrin by this antiserum at 1:10 dilution was 63%; therefore the immunological integrity of the tracer was assessed as a proportion of this value.

3 I(iii) Assay Procedure and Validation Thereof.

All assays were performed in disposable plastic cuvettes 11 x 52 mm in size, obtained from LKB, Sweden, after testing a variety of materials which were unsuitable due to adsorption of gastrin and other small peptides to their surfaces. Furthermore the effects of albumen and gelatin on preventing adsorption of gastrin to glass and plastic was investigated in the following way: four tubes were set up to test each combination of glass or plastic and albumen, gelatin (Haemaccel, Hoechst) or buffer. Each tube contained 1 ml 0,05M-Barbital (Veronal) buffer, pH 8,5, plus 0,1 ml labelled hormone diluted in the same buffer. After 24 hours' incubation the tubes were counted, their contents decanted, and the empty tubes re-counted. The remaining counts were expressed as a percentage of the total counts.

Sample tubes contained 0,1 ml serum or standards, 0,1 ml ^{125}I-labelled synthetic human gastrin G-17 I (^{125}I-SHG-17 I), 0,1 ml antiserum, and the final volume was made up to 1 ml with 0,02M-Barbital (Veronal) buffer, pH 8,4. The barbital buffer was used to dilute serum, antibody, tracer and standards, and the standard reference preparation used each time was synthetic human gastrin G-17 I obtained from CEA-IRE Sorin. Standards covered the range of 0,625 - 80 pg synthetic human gastrin (SHG), equivalent to 6,25 - 800 pg/ml whole serum. Buffer, standards and unknowns were pipetted using a Micromedic Automatic pipetting station (Micromedic Systems, Inc.) and the antiserum and labelled gastrin were added with a Repette (Jencons, Hemel Hempstead, England) or a Hamilton Repeating Syringe.

Assay tubes were incubated at 4°C for 18-24 hours when G 1 antiserum was used. This incubation period was found to give the most sensitive standard curve compared with 90 minutes at 37°C followed by 30 minutes at 4°C, and

72 hours at 4°C. Assays performed with antisera G 5 or 2604-7 were incubated for 72-96 hours. After incubation, 0,1 ml charcoal-treated "gastrin-free" serum (Wellcome) was added to all standard tubes to equalise their serum content with that of the unknown serum samples. 0,1 ml water was added to the unknown tubes to correct for volume changes. To all tubes except the total count tubes, 0,5 ml of a constantly stirred 4% charcoal suspension in 0,02M-Barbital buffer was added and the mixture agitated on a whirli-mixer. The tubes were allowed to stand at 4°C for 5 minutes, then centrifuged at 2 000 revolutions per minute (rev./min) for 15 minutes at 16°C. The supernatant fluid was decanted by covering each polystyrene tray containing 100 cuvettes with surgical gauze secured by elastic bands, inverting the tray over a "hot sink", and draining the tubes for 20 seconds on a bed of cotton wool covered with tissues. This decanting method was found to give identical results to those obtained when tubes were decanted and the last drop wiped manually. The charcoal-adsorbed radioactivity in the precipitates and the total radioactivity were counted in a solid-state scintillation spectrometer (Packard Systems Inc.) for 10 minutes or 10 000 counts.

After correction for nonspecific adsorption of labelled gastrin to the charcoal, the free labelled hormone was estimated as the percentage of the total counts per minute (c.p.m.) in the charcoal precipitate of standard and unknowns. To calculate the antibody-bound labelled gastrin these values were subtracted from 100%. The value of % binding thus obtained for the tubes containing zero standard, or B_o, was ascribed the value of 100%, and other % binding (B) values were expressed as a fraction thereof ($B/B_o \times 100$). Standard curves were constructed by plotting either % B or $B/B_o \times 100$ values on semilogarithmic paper against the content of synthetic human gastrin or related peptides.

Blanks without antibody were assayed on each occasion to assess non-specific binding of labelled gastrin by plasma proteins or other plasma factors. Corrections were made for all samples where the non-specific binding was found to be greater than 5%. The gastrin content of unknowns was determined by reading the corresponding value of % binding or $B/B_o \times 100$ off the standard curve.

Mean standard curves were constructed from 16 consecutive assays using antiserum G 1 and 8 consecutive assays each with antisera 2604-7 and G 5. The lower limit of sensitivity, or the detection limit for the assay with each antiserum was determined as the concentration of added gastrin which depressed antibody binding of labelled hormone significantly ($p < 0,05$, t-test) compared with the binding in the absence of added hormone.

Samples of known gastrin content estimated to fall on three different parts of the standard curves were assayed each time to assess inter-assay variability. These three samples were aliquots prepared from large batches of Hyland Control Serum I, and Hyland Control Serum I with synthetic human gastrin G-17 I added to give final concentrations of 80 pg/ml and 160 pg/ml. Intra-assay variation, or precision, was assessed by measuring the serum gastrin content of a series of samples repeatedly in the same assay. For this purpose serum from a patient, or a Hyland control serum containing a known amount of gastrin was used. The coefficients of variation of inter- and intra-assay variability were calculated as the standard deviation x 100 divided by the mean gastrin concentration. The index of precision was determined as the coefficient of variation of the ID_{50} value of each standard curve, where ID_{50} refers to the concentration of gastrin required to displace 50% of ^{125}I-gastrin from the antibody.

The accuracy of the estimation, which reflects the approximation of the estimation to the true concentration of the peptide being measured, was assessed by regression analysis of observed versus expected values of added gastrin.

A Scatchard (1949) plot, as applied to radioimmunoassay by Berson and Yalow (1959), was constructed from the mean standard curve for each antiserum by converting % binding values to a bound/free ratio (B/F) and plotting this against the concentration of gastrin bound (B), including the mass of radioactive tracer added to each tube. The mass of tracer added to each tube was calculated to be approximately 5 pg. The equation of this regression line is:

$$B/F = K(x-B)$$

where B = concentration of bound antigen

F = concentration of free antigen

K =-slope (litres/mole)

x = molar concentration of total antibody combining sites.

The average affinity constants (K) (litres/mole) of the antisera were derived from the graphic plots. The total concentration of antibody combining sites was read from the intercept on the horizontal axis, since x=B when B/F=0. The affinity constant and molar concentration of binding sites were determined for antisera G 1, 2604-7 and G 5 in this manner.

3 I(iv) Immunoreactive Studies on Gastrin Fragments and Related Peptides Using Different Antisera

The ability of serum samples from fasted and intravenously infused arginine-stimulated individuals to displace ^{125}I-labelled gastrin from G 1 antiserum was studied to see if substitution of the gastrin standard with serial dilutions of serum gave parallel displacement curves to that obtained with synthetic human gastrin G-17 I.

The degree of cross-reaction of various gut and gastrin-related peptides in the assay system using antiserum G 1 was investigated by comparing the effects of increasing concentrations of these peptides with those of the gastrin standard. The peptides studied were: porcine crystalline glucagon (Lilly), highly purified natural porcine secretin (Mutt), crude cholecystokinin-pancreozymin (CCK-PZ) (Boots), the synthetic C-terminal octapeptide of CCK-PZ (Ondetti), pentagastrin (I.C.I.) and caerulein (Erspamer), the peptide isolated from the skin of the Australian bullfrog, Hyla caerulia. The immunoreactivity of the related peptides was expressed as the molar ratio between the inhibition dose 50 (ID_{50}) for synthetic human gastrin G-17 I and the ID_{50} value for the peptide, multiplied by 100 to give a percent. The ID_{50} refers to the molar concentration of peptide that reduces the binding of labelled gastrin by antibody to 50% of its initial value.

Using antisera G 1 and G 5 the cross-reactivity of synthetic gastrin peptide fragments derived from the parent heptadecapeptide molecule, as compared to that of SHG-17 I, was assessed by comparing the effects of increasing concentrations of the fragments with those of the gastrin standard. The synthetic peptide fragments were prepared and kindly donated by Dr. J. Morley of I.C.I. The structures of the fragments used and of the related

peptides caerulein and the C-terminal octapeptide of CCK-PZ are diagrammatized in figure (3.1.1.). Comparison of the relative abilities of the gastrin fragments to displace labelled gastrin allowed identification of the antigenic sites on the gastrin molecule which are recognised by antisera G 1 and G 5.

Figure (3.1.2.) shows the chemical structures of gastrins G-34, G-17 and G-13 as they occur in the human, and porcine heptadecapeptide gastrin. Heptadecapeptide gastrin constitutes the C-terminal (18-34) sequence of big gastrin or G-34, and minigastrin, G-13, constitutes the C-terminal (5-17) sequence of the heptadecapeptide molecule. Each gastrin species may occur with a sulphate group on the tyrosine in position 12 of the heptadecapeptide, known as type II, or without a sulphate group, in the case of type I gastrins. Porcine heptadecapeptide gastrin differs from that of the human in that leucine in position 5 is replaced by methionine.

The Centre for Ulcer Research and Education (C.U.R.E.), by courtesy of Dr. M.Grossman, kindly supplied preparations of natural human gastrin 17 I, 17 II and 34 I (NHG-17 I, NHG-17 II, NHG-34 I) isolated from tumour extract prepared by Drs. Gregory and Tracy. These were examined with three antisera as outlined for the gastrin fragments, along with synthetic human G-17 I obtained from CEA-IRE Sorin and from I.C.I.

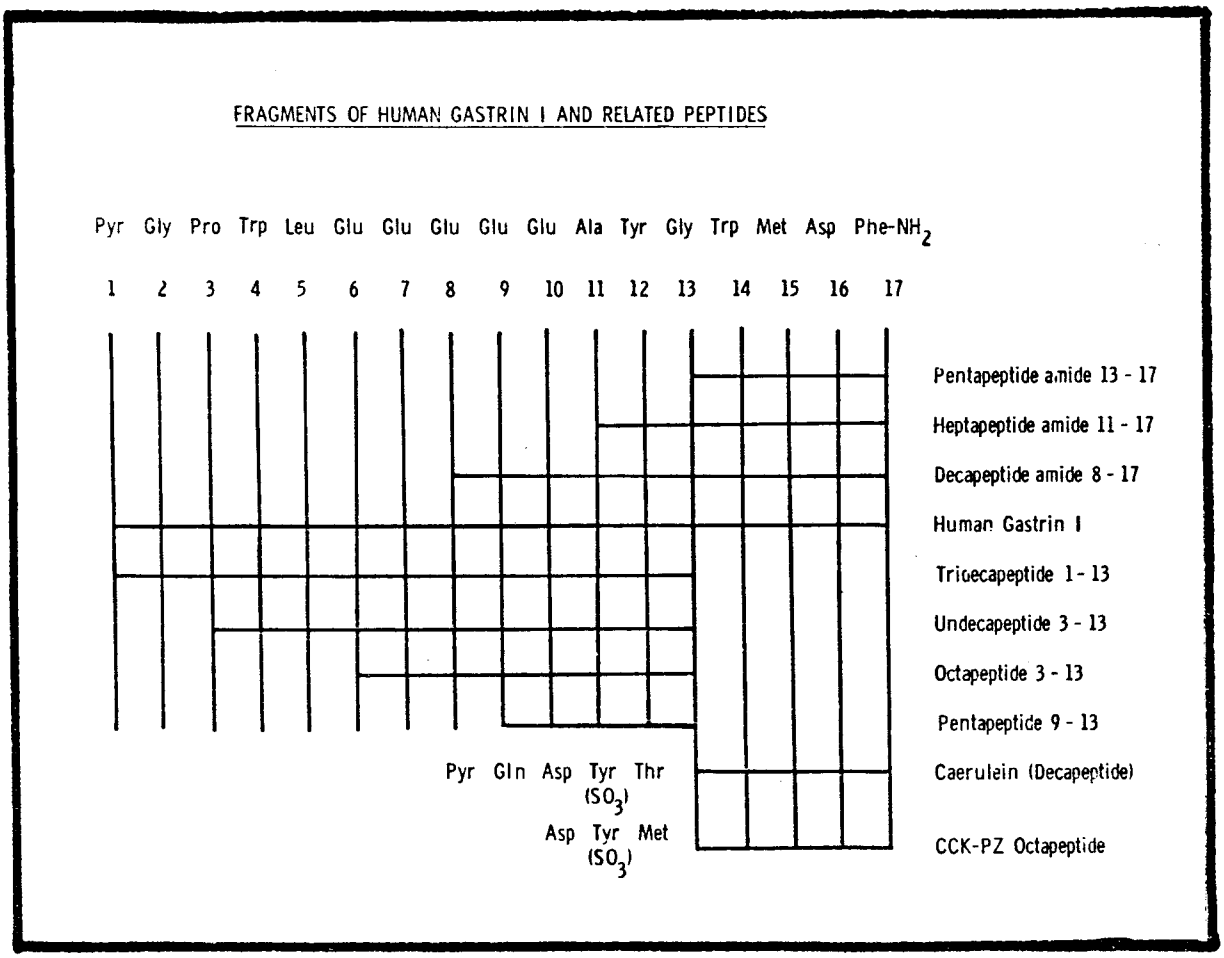


Fig. (3.1.1.) The schematic structure of heptadecapeptide gastrin, synthetic gastrin fragments, caerulein (the frog skin peptide) and the C-terminal octapeptide of cholecystokinin pancreozymin (CCK-PZ).

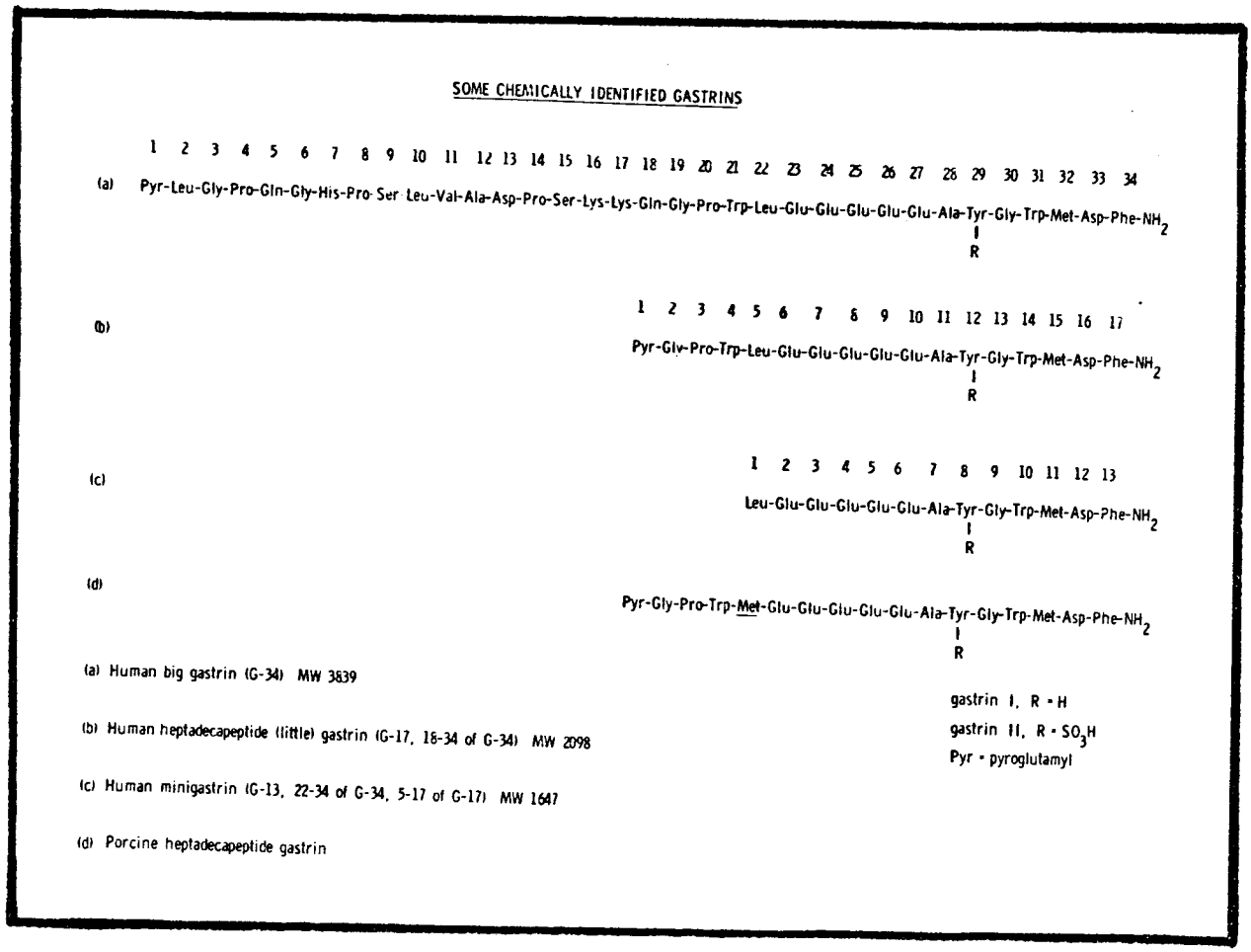


Fig. (3.1.2.) The chemical structures of human big gastrin, heptadecapeptide gastrin, mini gastrin* and porcine heptadecapeptide gastrin.

*Currently it is thought that mini gastrin consists of 14 amino acids.

3 I(v) Displacement Curves of Various Sera

In order to establish identity of the material in serum obtained from patients with various disease conditions with the synthetic human heptadecapeptide gastrin standard, displacement curves were set up to examine the ability of serial dilutions of these sera to displace radioactive gastrin from antisera G 1, 2604-7 and G 5. Serum from patients A.K. and C.K. suffering from the Zollinger-Ellison syndrome, from patient M.B. with isolated retained antrum, and from patient M.Y. with pernicious anaemia were studied.

Since many of the gastrin studies described in this thesis involve measurement of serum gastrin levels in pigs, it was important to confirm that pig serum did not interfere in the assay. Displacement curves were constructed using serial dilutions of pig serum in place of gastrin standard and the resultant curve was compared with that obtained using standard gastrin.

3 I(vi) Measurement of Gastrin in Tissues

Gastrin levels were measured in aqueous extracts prepared by boiling tissues collected from various animals at different sites along the gut, as described in section 3 III(ii). The effect of different treatments during the extraction procedure on the recovery of gastrin was investigated using rat thigh muscle to which was added a known concentration of gastrin. One ml containing 800 pg was added to a final volume of 5 ml extract each time to give an expected concentration of 160 pg/ml. Gastrin measurements were made in the extracts after the following treatments:

(a) gastrin added to water was measured to assess recovery from addition to glassware

(b) gastrin was added to water and boiled for 20 minutes to assess the effect of boiling on gastrin

(c) muscle was added to water, and after mixing the fluid was assayed for gastrin to see if there was any detectable gastrin in muscle

(d) muscle with added gastrin as prepared in (c) was boiled for 20 minutes to see if gastrin could be recovered from boiled muscle

(e) muscle extract was prepared by boiling muscle in water to inactivate any enzymes and the extracted fluid was tested for gastrin content

(f) gastrin was added to the extracted fluid in (e) and the mixture was boiled again to determine whether the extract from muscle contained any gastrin-inactivating enzymes

(g) to muscle in water, gastrin and 10% by volume of Trasylol (Aprotinin, 100 000 KIU/10 ml) was added to test the effect of an enzyme inhibitor on gastrin measurement

(h) the muscle with gastrin and Trasylol described in (g) was boiled and the

extract was assayed for gastrin to assess whether inactivation of the enzyme inhibitor had any effect on gastrin measurement.

3 I(vii) In Vivo Studies in Man and the Pig

Serum gastrin levels were measured in 27 control subjects in the basal state to establish the mean fasting gastrin level and range in normal people, using antiserum G I. Serum gastrin measurements done routinely on a large number of patients were divided into categories of age (357), ethnic group (369) and sex (372), to see if there were any marked differences in basal gastrin levels between these groups. The total numbers vary slightly because not all the information was available for every patient. Serum gastrin measurements in 285 patients from this group, in which the diagnoses had been confirmed, were separated into groups according to the various disease states shown by these patients. A study was made in a group of patients with rheumatoid arthritis to see if there was any correlation between serum gastrin levels and the degree of severity of the arthritis.

The response of normal subjects to a 30-minute intravenous infusion of arginine at a rate of 10 mg/kg/min, a 30 g dose of oral arginine, adjusted to pH 7,5 with sodium bicarbonate, and oral Oxo, 4 cubes in 150 ml warm water, was studied and the maximum increment of serum gastrin over the basal level was determined. In 3 patients who had been antrectomised, and in 6 patients who had previously had a vagotomy and partial antrectomy, the serum gastrin response to a 30-minute intravenous infusion of arginine was investigated in the same way.

The response of pigs to eating creep meal was tested by measuring the serum gastrin levels in a group of pigs at 30-minute intervals after eating creep meal, for up to 150 minutes.

All the measurements of serum gastrin levels in the human and the pig described above were made using antiserum G I, which detects primarily the non-sulphated heptadecapeptide gastrin species.

Section II Chromatography

3 II Column Procedure

Characterisation of the nature of gastrin in various serum and human gut extract samples, and studies on gastrin heterogeneity were performed using Sephadex column chromatography. Sephadex columns of dimensions 1,5 cm x 100 cm and 1,5 cm x 150 cm, containing a mixture of equal volumes of Sephadex G-25 fine and G-50 fine were poured. Using a combination of two Sephadex grades gave a larger fractionation range (molecular weight 1 000 - 30 000 for peptides and globular proteins), allowing separation of gastrin species both larger and smaller than the heptadecapeptide molecule.

Pouring a long column, such as 1,5 metres, with a mixture of two grades of Sephadex inevitably leads to a certain amount of uneven distribution of the Sephadex particles, as the G-25 beads would tend to settle faster than the G-50 beads, due to their larger size. To minimize the extent of this uneven settling the contents of the funnel attached to the top of the column were stirred continuously with an electric stirrer while the column was being poured. The packing of the column was found to be satisfactory as was evident from the behaviour of blue dextran and vitamin B_{12} on the column, which could be seen to move down the column in compact bands with regular fronts.

Each column was pre-coated with 4% human serum albumen to prevent non-specific adsorption to the column matrix. The columns were equilibrated and eluted with 0,05M-Barbital (Veronal) buffer, pH 8,5, containing 0,01% sodium azide, at a constant flow rate of between 13 and 16 ml/hour, controlled by an LKB Varioperpex pump, at room temperature. Elution was carried out by downward flow and buffer passed into the column from a Mariotte flask which

was kept at a constant height. Samples were applied to the columns in volumes of 1-2 ml, and fractions of 1,5-2 ml were collected using an LKB Ultrorac Fraction Collector, and assayed for gastrin content. The columns were washed with at least two column volumes of eluant between application of samples, and when the columns were not in use they were stored with veronal buffer containing 0,02% sodium azide to prevent growth of organisms, and re-equilibrated with the eluting buffer before use.

The molecular weight markers blue dextran, albumen (Behringwerke), ^{125}I-labelled monocomponent porcine insulin (gift of L.Heding, Ph.D., Novo Institute, Denmark), ^{125}I-glucagon, ^{125}I-synthetic human gastrin I, vitamin B_{12} (cyanocobalamin), and ^{22}Na were used in various combinations to calibrate the columns. Internal standards of blue dextran and vitamin B_{12} were run with each elution in later fractionations. The elution profile of gastrin species was plotted either directly as elution volume (ml) or as a percentage of the elution volume between blue dextran and vitamin B_{12} markers, when these were used as internal standards. The concentration of gastrin in the eluates was expressed as the relative ability to displace ^{125}I-synthetic human gastrin G-17 I from the antibody, or $B/B_o \times 100$, where B refers to % binding of ^{125}I-gastrin by antibody, and B_o denotes the value of % binding in the absence of added standard gastrin, as detailed in section I(iii) of this chapter. It was not possible to determine absolute levels for the concentration of the peptides obtained on fractionation because the standard preparation used in all assays was synthetic human gastrin G-17 I.

High gastrin-content serum from a patient with pernicious anaemia was chromatographed to investigate the heterogeneity of gastrin species found in this serum. The eluted fractions obtained on gel filtration of serum from

patient K.V. were assayed with antiserum G 5.

Standard reference preparations of natural human gastrin G-17 I, G-17 II, and pure human big gastrin, G-34 I, kindly donated by Dr. M.Grossman and prepared by Drs. Gregory and Tracy, were chromatographed to establish the exact elution patterns of the natural gastrins in our system. Comparison of these elution profiles with those obtained from serum and gut extract samples aided the identification of the gastrin types in the various preparations.

This chromatographic technique was applied to several other studies which are described in the relevant sections, and are merely outlined here:

(a) Tissue extracts prepared by boiling biopsies of human antrum and duodenum in water, as described in section III(i) of this chapter, were chromatographed to examine the gastrin species found in these extracts.

(b) An extract prepared by boiling the skin of the frog Xenopus laevis in water was passed over the column to investigate the behaviour of the extracted material as compared to that of the gastrin standard. Frog serum collected from several frogs of the same species was pooled and 1 ml of the pooled serum was fractionated in the same way, to see how the gastrin-like material in the skin extract and serum compared on chromatography.

(c) Serum samples were taken from different vascular sites in the pig following infusion of synthetic human gastrin G-17 I, and chromatographed to see if the same gastrin species could be recovered.

(d) Samples of perfusate collected during perfusion of the isolated rat liver in situ with synthetic human G-17 I were chromatographed.

Comments and Criticisms

The method of chromatographic separation described here was suitable for separation of gastrin into large and small molecular weight forms on an analytical basis. In order to obtain total refinement of a heterogeneous sample on chromatography several chromatographic steps are necessary; refractionation of peaks of immunoreactive material on a second Sephadex column or on an anion exchange column would allow further purification on the basis of molecular weight and charge respectively.

Although Sephadex columns permit resolution of gastrins on the basis of molecular weight, this is not the sole factor governing separation by this method. A weak ion exchange effect due to a concentrating mechanism within the Sephadex bead has been observed by several workers (Morris and Morris, 1976), which leads to non-ideal chromatographic behaviour on Sephadex columns. Thus for example it has been found that the aromatic amino acids tyrosine and tryptophan adsorb to the column matrix (Gelotte, 1960) so that peptides and proteins containing a large proportion of these residues are retarded in their passage through the column, and produce an elution profile which deviates from that expected on the basis of molecular weight alone. This phenomenon is well known in the case of small peptides such as LRF (Luteinizing hormone releasing factor) and ADH (Antidiuretic hormone), and has been observed in the case of the amino-terminal (1-13) fragment of gastrin by Dockray and Walsh (1975). They found that this fragment eluted between G-34 and G-17 on Sephadex columns and suggested that the faster migration of the (1-13) fragment compared with the G-17 molecule could be due to the fact that the former contains only one aromatic residue whereas G-17 contains three, and is thus retarded relative to the tridecapeptide molecule. By coating the Sephadex columns used in this

study with albumen these adsorption effects were reduced.

Since each sample that was chromatographed in this study was only fractionated once, only rough separation of the different gastrin components on the basis of molecular weight was obtained. However, by comparison of the elution profiles of unknown samples with those of pure natural gastrins and with the elution volumes of markers of known molecular weight, it was possible to identify heptadecapeptide and big gastrins on the basis of their chromatographic properties.

Section III Extractions

Introduction

Since gastrin exists in the circulation in several forms, it is of interest to determine the tissue of origin of these various gastrins. The pyloric antrum is the major site of gastrin production, and gastrin originating in this tissue is chiefly of the heptadecapeptide species. Gastrin is also produced at extra-antral sites, the most important of which is the duodenum in the normal individual. In some instances, such as in the case of the Zollinger-Ellison syndrome, gastrin originates from tumour sites and grossly elevated levels are found in the circulation. Although these tumours, or gastrinomas, arise chiefly in the pancreas they do not involve pancreatic delta cells, but rather cells which resemble the antral G-cell.

To investigate the forms of gastrin at several sites in the human stomach and duodenum, extracts of biopsies collected at these sites were prepared by boiling in water and the gastrin content of the extracted fluid was examined immunochemically and chromatographically.

The distribution of immunoreactive gastrin at various sites along the gastro-intestinal tract was investigated in five mammalian species by preparing extracts of tissues collected at these sites in the same manner as for human tissue. This allowed the question of pancreatic gastrin to be investigated in these species. To examine the occurrence of immunoreactive gastrin amongst vertebrates in general, serum samples were collected from nineteen vertebrate species and assayed for gastrin. A detailed study aimed at characterisation of a gastrin-like material found in frog serum and frog skin extracts was carried out.

Extracts were also prepared from tissues of several invertebrate species

and tested for the presence of gastrin in an attempt to trace the origin of gastrin down the evolutionary tree.

3 III(i) Human Tissue Extracts

Biopsy specimens were collected by Dr. B.Novis of the Gastrointestinal Clinic, Groote Schuur Hospital, from the mucosa of the cardia, body and distal antrum of the stomach, and from the duodenal cap and second part of the duodenum, in 4 patients who had undergone endoscopic examinations for suspected but disproved peptic ulcer disease. The biopsies were taken using an Olympus GF type D fibrescope and standard biopsy forceps (Vinik, Grant and Novis, 1975). Gastrin extracts were prepared by boiling the tissues for 5-10 minutes in 1 ml distilled deionized water, in test tubes covered with aluminium foil lids to prevent evaporation, followed by cooling and centrifugation of the solid matter at 3 000 rev./min for 10 minutes. The supernatant was frozen and stored for measurement of gastrin content by radioimmunoassay, using antiserum G 1.

The tissues were lyophilised in a Virtis Freeze Drier and weighed, so that the gastrin content of the extracts could be expressed as gastrin per mg dry weight. The lyophilised tissues were then treated with 1 ml dilute sodium hydroxide for 1 hour to dissolve them, and homogenised in a Potter Elvehjem tissue homogeniser. The protein content of each homogenate was estimated according to the Folin-Lowry method, which is detailed in the appendix. This allowed expression of the gastrin content per mg total protein. The mean values of gastrin content per mg dry weight and gastrin content per mg total protein for the four patients were calculated for each site biopsied.

To characterise the gastrin extracts prepared from the antrum and second part of the duodenum further, the extracts of these sites taken from patient R.E. were chromatographed on a Sephadex G-25/G-50 fine column of dimensions 1,5 m x 1,5 cm, according to the chromatographic procedure

described in section II of this Chapter. The elution profiles were plotted and the distribution of the gastrin species detected in these extracts with antiserum G 5 was compared with that found in serum. The effect of dilution of the extracts on the binding of radioactive gastrin to antibody was examined to determine whether or not the curve of displacement was similar to that found with standard synthetic human gastrin G-17 I.

Prior to separation of antibody-bound and free labelled gastrin in assays containing gut extract samples and column eluates, 0,1 ml charcoal-treated "gastrin-free" serum (Wellcome) was added to the unknown tubes as well as to the standards to equalize the serum content throughout.

It was critically important to ensure that the labelled hormone used in the radioimmunoassay was not being degraded by the tissue extracts and giving rise to erroneously high readings in the assay. Thus control tubes without antibody were run with each extract being measured, and in almost all cases the apparent binding to substances in the incubation medium other than antibody (non-specific binding) was less than 5%, indicating little non-specific adsorption of tracer to tissue extract, and little inactivation of the tracer during incubation. In the few cases where the non-specific binding was greater than 5% the appropriate corrections were made. This applied in the case of the vertebrate and invertebrate tissue extracts as well.

3 III(ii) Tissue Extracts in Mammals and Measurements in Vertebrates

Tissues were collected from mammalian species which included rat, rabbit, guinea pig, dog and pig. The animals were sacrificed by exsanguination or asphyxiation in nitrogen, and tissues were dissected out as soon as possible, within 30 minutes after death. Sites from which tissues were collected included fundus and antrum of stomach, duodenum, jejunum, ileum, colon and pancreas, as indicated in figure(3.III.1.), and a piece of muscle was taken from the thigh of the rat, rabbit and pig to act as controls, as one would expect the gastrin content of muscle to be zero. In the case of the dog and pig the fundic and antral mucosae were dissected free from the underlying muscle so that only the mucosa was used for extraction.

The tissues were cut into small pieces, approximately 5 mm x 5 mm, and boiled for 20-30 minutes in a small volume of distilled deionized water, 4 to 6 ml, with 10% by volume of Trasylol (Aprotinin, 100 000 KIU/10 ml) (Bayer) added to inactivate any proteases which may be present. As with the human biopsy extractions the tubes were covered with aluminium foil during boiling, and the supernatants obtained on centrifugation were stored frozen until assayed for gastrin using antisera G 1 and 2604-7. Samples containing very high levels of gastrin were re-assayed in several dilutions until they fell within the range of the sensitive portion of the standard curve. The tissues were lyophilised and weighed and then treated with a convenient volume of dilute sodium hydroxide for 1 hour to dissolve them, and homogenised in the same manner as the human tissue extracts. The gastrin content of each tissue was expressed per mg dry weight of tissue and per mg total protein, after correction for dilution in the assay and for the volumes of water and sodium hydroxide used in the extraction and protein estimation procedures. A table and a composite figure showing the

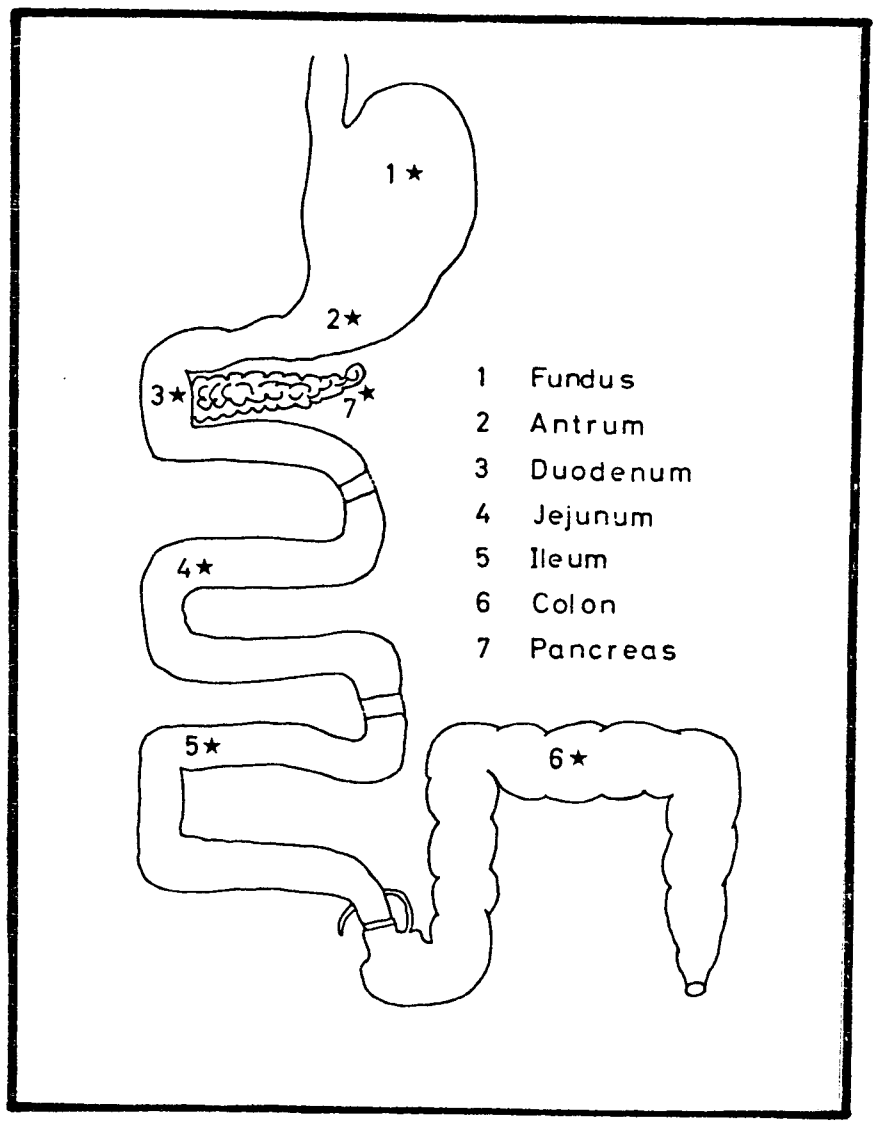


Fig. (3.III.1.) Location of sampling sites along the mammalian gut where tissues were obtained for extraction of gastrin.

gastrin content of the tissues and species studied, including the human, was constructed. Values used for the figure were gastrin content per mg total protein except in the case of the rat antrum, where the gastrin content per mg dry weight was substituted. The gastrin extracted from these species was not characterised further.

In order to study the distribution of gastrin in other vertebrate species, the gastrin content of serum collected from various species was measured by radioimmunoassay. Serum samples were received from Dr. B.Shapiro of the Isotope Unit, Department of Medicine, University of Cape Town, as well as other people mentioned in the legend to table (4.III.2). Extracts from the gut were not prepared for these species, which included the rat, rabbit, dassie, guinea pig, fowl, frog, snake, monkey, baboon, dog, sheep, goat, pig, cow, springbok, stockfish, kingklip, mackerel and dogfish. The generic names for these animals appear in table (4.III.2). The gastrin content of sera from these species was measured using antiserum G 1, and in most cases 2604-7 as well, and a table of the results was prepared. Figure (3.III.2.) shows the position of the species investigated on the evolutionary tree.

The discovery of exceptionally high"gastrin"levels in frog serum collected from the species Xenopus laevis prompted further investigation in this animal. "Gastrin"extracts were prepared by pooling tissues collected from the skin, stomach, intestine, brain and muscle of three animals and boiling in the same manner as for the mammals studied. The frogs were sacrificed by pithing down the spinal cord in order to keep the brain intact. The tissues were lyophilised and weighed and their total protein content was estimated, so the"gastrin" content of the tissues could be expressed per mg dry weight and per mg total protein, as described previously. No trasylol was used in the preparation of frog extracts.

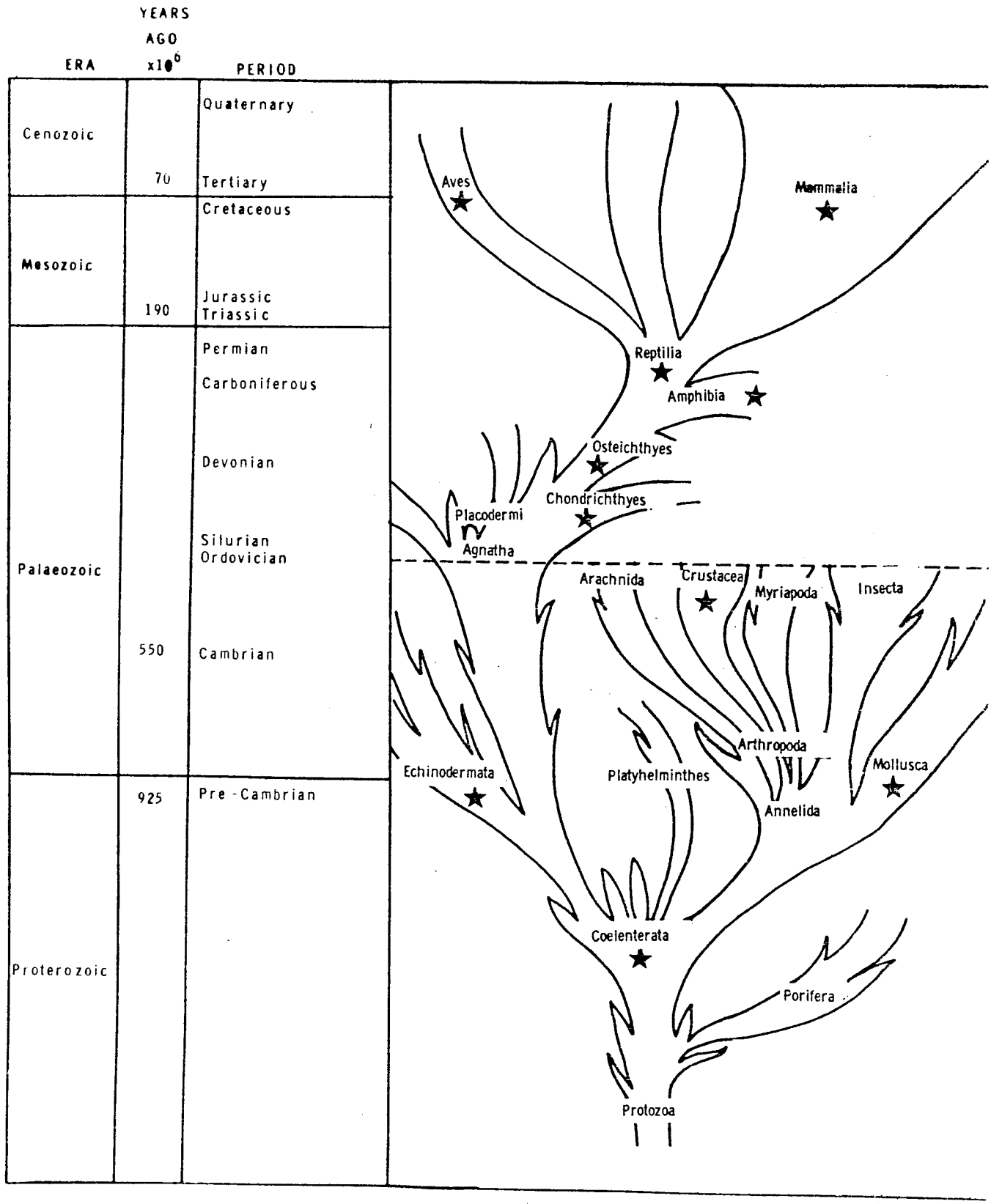
The nature of the gastrin-like material found in frog serum and skin extracts was characterised further by chromatography on a Sephadex G-25/ G-50 fine column, 1,5m x 1,5cm, according to the procedure described in section 3 II. The elution profiles thus obtained using antisera G 1 and 2604-7 were compared with those of the gastrin standards. The effect of serial dilutions of frog serum and frog skin extract on the binding of ^{125}I-labelled gastrin to antisera G 1 and 2604-7 was examined to determine whether the curves of displacement were similar to that found with standard synthetic human gastrin G-17 I.

To examine the distribution of gastrin along the gut of another vertebrate species, extracts of the gut of three snakes were prepared by pooling tissues collected from the skin, stomach, liver, intestine, brain and muscle of these animals and boiling as described. Tissues were lyophilised, weighed and estimated for protein content and the gastrin content was expressed in the same manner as for the mammalian extracts. The species of snake used was the common spotted egg eater, Dasypeltis scabra scabra.

Fig. (3.III.2.) Schematic representation of the evolutionary tree indicating the position of the taxa from which animals were selected for the estimation of gastrin content.

Figure modified from Romer (1947), "Man and the Vertebrates", pages 13 and 17, with permission from the University of Chicago Press.

★ Indicates taxa investigated.


YEARS AGO x10^6
ERA
PERIOD
Cenozoic
Quaternary
70
Tertiary
Mesozoic
Cretaceous
190
Jurassic
Triassic
Palaeozoic
Permian
Carboniferous
Devonian
Silurian
Ordovician
550
Cambrian
Proterozoic
925
Pre-Cambrian
Aves
Mammalia
Reptilia
Amphibia
Osteichthyes
Chondrichthyes
Placodermi
Agnatha
Arachnida
Crustacea
Myriapoda
Insecta
Arthropoda
Mollusca
Annelida
Echinodermata
Platyhelminthes
Coelenterata
Porifera
Protozoa

3 III(iii) Invertebrate Tissue Extracts

Invertebrate animals representative of four phyla were selected to investigate whether gastrin is present in the lower species. The sea anemone, Pseudactinia sp., was chosen as a representative of the Coelenterata. The Mollusca were represented by the mussel (Choromytilus sp.), snail (Helix sp.), limpet (Patella sp.), periwinkle (Oxystele sp.), whelk (Burnupena sp.) and chiton (Chiton sp.). Crustacea were represented by the barnacle (Tetraclita sp.), and the sea urchin (Parechinus sp.) was selected from the phylum Echinodermata. The position of these species on the evolutionary tree is shown in figure (3.III.2.).

Tissue extracts were prepared by boiling the gut, where it was possible to distinguish this from adjoining tissues, or alternatively the entire body contents except for the muscle. The tissues were chopped into small pieces and boiled in a minimal amount of distilled deionized water, without addition of Trasylol, for 20-30 minutes. The extracts were collected and stored frozen for gastrin radioimmunoassay with antiserum G 1 as described for human and mammalian tissue extracts. The gastrin content was expressed per mg dry weight of tissue and per mg total protein after correction for dilutions, as described previously, and then expressed relative to the gastrin content per mg total protein of human antrum, determined in section 3 III(i), which was given the arbitrary value of 100%.

Comments and Criticisms

The extraction procedure in these studies involved boiling the biopsied tissues in water to extract the gastrin, and measurement of the gastrin content of the residual fluid after boiling and centrifugation of the solid matter. As shown in section 4 I(vi), approximately 77% of immunoreactive gastrin was recovered after boiling in water alone and 43% was recovered after boiling in the presence of muscle extract. Since the recovery of a known amount of gastrin from individual tissue extracts was not studied it must be assumed that the recovery probably lies somewhere between 43% and 77%. Boiling tissues in water is an acceptable method for extraction of gastrin; Berson and Yalow (1971) and Yalow and Berson (1972) described extraction of gastrin from human tissues obtained at post-mortem by boiling in water, followed by centrifugation and fractionation of the supernatant on Sephadex G-50 columns (Yalow and Berson, 1972). The first step in extraction and purification of hog antral gastrin described by Gregory and Tracy (1964) involved boiling the antra in tap water.

Control readings of gastrin measured in extracts of rat, rabbit and pig muscle were negligible as shown in table (4.III.1). The same was true in the case of the frog and snake extracts, although the values shown in table (4.III.3) are corrected for the readings obtained in muscle.

Recovery of gastrin added to rat thigh muscle and treated in various ways was assessed in a study described in sections 3 I(vi) and 4 I(vi).

Each gastrin estimation was made in duplicate. The gastrin content of human gut extracts was measured once, as was the case for the measurements in the invertebrate extracts. The invertebrate extracts were prepared by pooling two or three small animals and boiling them or their gut tissues together. In

the case of the mammalian tissues, extracts were prepared from two batches of animals, measurements of gastrin content were made three times, and the final result is a mean of all these determinations.

Section IV Gastrin Kinetic Studies in the Pig

Introduction

Studies of the hepatic handling of gastrin under various conditions were performed in two groups of pigs *in vivo*. Group I consisted of 8 pigs in which basal transhepatic measurements of endogenous gastrin levels were made. Blood flow measurements were taken in each pig and the total gastrin mass entering and leaving the liver was calculated. This group acted as a baseline for subsequent studies (Vinik, Hickman and Grant, 1978).

Group II comprised 7 pigs which were given a stepwise infusion of synthetic human gastrin G-17 I in three increasing doses, infused for one hour at each dose level. This allowed investigation of the response of the liver to increasing doses of exogenous gastrin, and the half-life of synthetic human heptadecapeptide gastrin in the circulation was determined from the fall off in levels from the plateau reached after three hours of infusion. The metabolic clearance rate and space of distribution of gastrin in the pig were calculated, and the daily production rate of gastrin, as an indication of endogenous gastrin secretion was calculated from the metabolic clearance rate estimations. Blood flow measurements were not made in these pigs, and calculations were based on the measurements made for the baseline pigs in Group I.

The nature of the circulating gastrin during the second and third hour of infusion of synthetic human heptadecapeptide gastrin was studied chromatographically to see if circulation *in vivo* produced changes in the type of gastrin which was infused (Vinik, Hickman et al., 1978).

Group I Pigs : Basal Transhepatic Measurements of Endogenous Gastrin

(i) Preparation of Animals

Eight young pigs of either sex, weighing 18-22 kg, were anaesthetised after a 24-hour starvation period. Anaesthesia was induced with sodium pentothal (total dose 2-3 mg/kg) injected into an ear vein, and was maintained with oxygen and nitrous oxide given via an endotracheal tube. Under clean but not sterile conditions the animals were prepared for blood flow measurement by the insertion of catheters at the sites shown in figure (3.IV.1.). Catheters were inserted into the right carotid artery for pressure measurement and arterial sampling, and into the right internal jugular vein for the administration of fluids and Bromsulphthalein (BSP) or Indocyanine Green (ICG). A catheter was introduced into the right external jugular vein and was manipulated into an hepatic vein, usually the left, by intra-abdominal palpation. Hepatic venous blood samples were drawn from this catheter. Two catheters were inserted into the portal vein for proximal and distal portal sampling. The proximal portal sampling site was in the hilum of the liver 2-3 cm beyond the junction of the last tributary, and the distal catheter was positioned 4-6 cm into the superior mesenteric vein, against the flow to ensure mixing of para-amino hippuric acid (PAH) with blood of intestinal and splenic origin, as this catheter was also used for the infusion of PAH.

(ii) Blood Flow Measurements

Total hepatic blood flow was measured by the clearance of BSP initially, and then ICG when no further supplies of BSP could be obtained. Portal flow was measured using PAH, according to the method described by Hickman, Saunders and Terblanche (1974). Blood samples were analysed for BSP

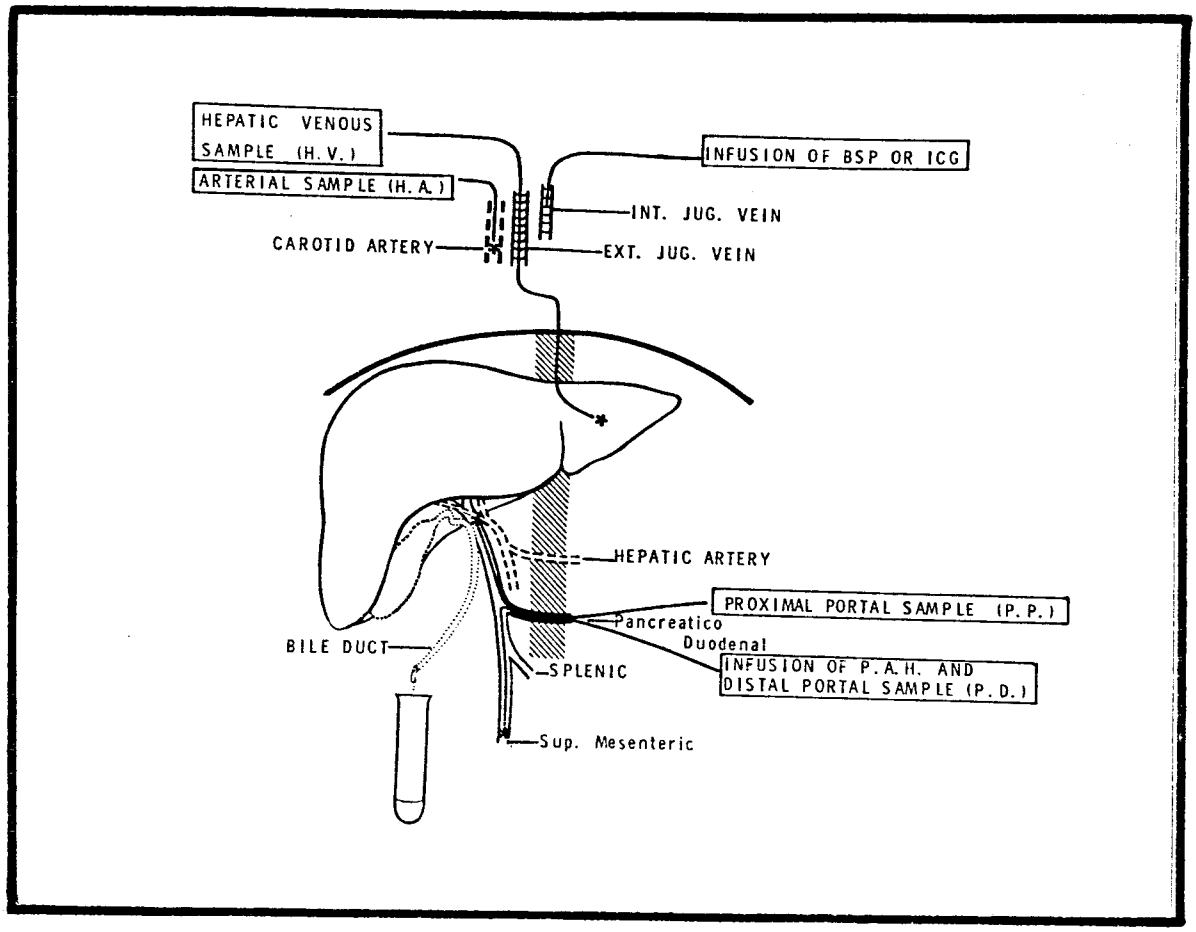


Fig. (3.IV.1.) Sites of catheters for transhepatic sampling and infusion of dye for blood flow measurements in the pig.

(Bradley, Inglefinger, Bradley and Curry, 1945), ICG (Winkler and Tygstrup, 1960), and PAH (Bratton and Marshall, 1939; Harvey and Brothers, 1962). There was no interference by any dye with the measurements of the other. Further details of the blood flow measurement technique can be found in the appendix.

(iii) Sampling Procedure

On completion of the operative preparation the priming dose of dye was given and a constant infusion of dye in saline was commenced. The priming dose of BSP was 300 mg and infusion was continued at the rate of 5 mg/min. The priming dose of ICG was 2,5 mg, followed by infusion at 0,156 mg/min.

Three arterial samples were taken at 10-minute intervals to ensure stabilization of dye levels. Thereafter, infusion continued for a further 30 minutes and simultaneous transhepatic samples were taken from the carotid arterial (representative of hepatic arterial blood, HA), proximal portal (PP), distal portal (PD), and hepatic venous (HV) catheters at 10-minute intervals. At the end of the experiment the animals were sacrificed and the liver was weighed after draining for a standard period of 15 minutes (Hickman, Saunders and Terblanche, 1970).

(iv) Gastrin Measurements

The gastrin content of the serum samples was measured by radioimmunoassay as described in section I of this chapter, using antiserum G 1, which detects non-sulphated forms of gastrin but reacts poorly with sulphated heptadecapeptide gastrin. To ensure that pig serum did not interfere in the assay a curve of displacement of antibody-bound ^{125}I-gastrin was prepared using serial dilutions of a sample containing high endogenous levels of gastrin. This curve was parallel with that of the synthetic human heptadecapeptide gastrin standard, confirming that serum gastrin measurements could be made

in the pig without any problems. With each set of samples from a particular pig, control tubes in which antiserum had been omitted were included in the assay to monitor non-specific binding of labelled gastrin by factors in the serum, which would give artificially low results. Where this value exceeded 5%, appropriate corrections were made. It was found to be unnecessary to substitute "gastrin-free" pig serum for the horse serum used in the separation procedure, since both gave similar results.

(v) Calculations

The rate of liver blood flow was calculated either as flow per whole liver or as flow per gram of liver tissue. The hepatic venous flow was assumed to equal the sum of the hepatic portal and hepatic arterial flow, or total flow. The gastrin concentration reaching the liver (AHg) was calculated as per equation (1):

$$AHg\ (pg/ml) = \frac{(Pf \times PPg) + (HAf \times HAg)}{Tf} \qquad (1)$$

where Pf = absolute portal flow (ml/min)

PPg = proximal portal gastrin concentration (pg/ml)

HAf = hepatic arterial flow (ml/min)

HAg = hepatic arterial gastrin concentration (pg/ml)

Tf = total liver blood flow (ml/min).

Total gastrin reaching the liver (TAHg), or the afferent hepatic gastrin mass, was calculated thus:

$$TAHg\ (pg/min) = AHg \times Tf \qquad (2)$$

Total gastrin leaving the liver (TEHg), or the efferent hepatic gastrin mass, was calculated by equation (3):

$$TEHG\ (pg/min) = HVg \times Tf \qquad (3)$$

where HVg = hepatic venous gastrin concentration (pg/ml).

The net hepatic balance of gastrin was determined as the difference between the afferent and efferent hepatic gastrin mass.

Portal gastrin secretion (PGs), or portal gastrin mass, referring to the contribution of gastrin by the stomach, duodenum and proximal small intestine, was calculated by equation (4):

$$PGs\ (pg/min) = (PPg - PDg) \times Pf \qquad (4)$$

where PDg = distal portal gastrin concentration (pg/ml), and PPg and Pf are as defined above.

Each measurement was the mean of three estimations made at 10-minute intervals. Significant differences in mean basal values were tested for by the paired Student's "t"-test.

Group II Pigs : Stepwise Infusion of Exogenous Gastrin at Three Dose Levels

(i) Preparation of Animals

Ten pigs weighing between 29 and 32 kg were fasted for 24 hours and anaesthetised with sodium pentothal and nitrous oxide in the same manner as the pigs in group I. Catheters were inserted under clean but not sterile conditions at the same sites as before, viz. hepatic venous, hepatic arterial, and proximal and distal portal sampling sites. Blood flow measurements were not made in this group, and for calculations involving flow measurements the values determined for the pigs in group I were used.

(ii) Experimental Procedure

Continuous infusions of synthetic human gastrin G-17 I (I.C.I.) were commenced after a basal period of 1 hour, during which time blood samples were taken at 10-minute intervals. The mean of the 6 measurements made in this period was taken as the basal gastrin level in each pig. Synthetic human heptadecapeptide gastrin was infused at three increasing dose levels for one hour each, via the internal jugular vein, using a Harvard Syringe Pump. A dose of 0,25 µg/kg/hr was administered for the first hour, 0,5 µg/kg/hr for the second hour and 1 µg/kg/hr for the third hour. Assuming that each pig weighed approximately 30 kg, the total doses made up in 14 ml saline each, allowing 2 ml for dead space volume, and infused at a rate of 0,2 ml/minute, were 7,5 µg, 15 µg and 30 µg respectively. Blood samples were drawn via the hepatic venous, hepatic arterial and proximal portal catheters at 15-minute intervals during the infusions, and at 5, 10, 20 and 30 minutes after the infusion of the highest gastrin dose. Gastrin measurements made during this decay period were used to calculate the half-life of synthetic human G-17 I gastrin in the porcine circulation after a plateau level

had been reached. The protocol for this experiment is outlined in figure (3.IV.2.).

All the beakers used for making up dilutions of gastrin in this study were siliconised with Dow Corning Antifoam-A spray (Dow Corning Corporation, Midland, Michigan, U.S.A.) to minimize loss of hormone by adsorption to glass. The infusion solutions contained 0,25% by volume of a gelatin solution (Haemaccel, Hoechst), which was added to prevent loss of gastrin through adsorption to syringes and tubes used for infusion.

(iii) Gastrin Measurements

Gastrin content of the samples was measured by radioimmunoassay with G 1 antiserum, which detects chiefly non-sulphated forms of gastrin. Corrections were made for non-specific binding in the assay where necessary.

(iv) Calculations

Using the mean blood flow measurements obtained for the pigs in group 1, the afferent and efferent hepatic mass of gastrin at each dose level was calculated. The blood flow values used were: hepatic venous or total flow 580,3 ml/min, hepatic arterial flow 147,3 ml/min, and portal flow 433,0 ml/min. Equations used in the calculations were the same as those used for the pigs in group 1, equations (1), (2) and (3). Gastrin concentration values used were the mean of the three most stable readings out of four taken during each hour of infusion. The net hepatic balance of gastrin at each dose level was determined as the difference between the mass of gastrin entering and leaving the liver, and the afferent and efferent gastrin masses at each dose level were compared to see if hepatic uptake of gastrin occurred at any dose level of infused gastrin.

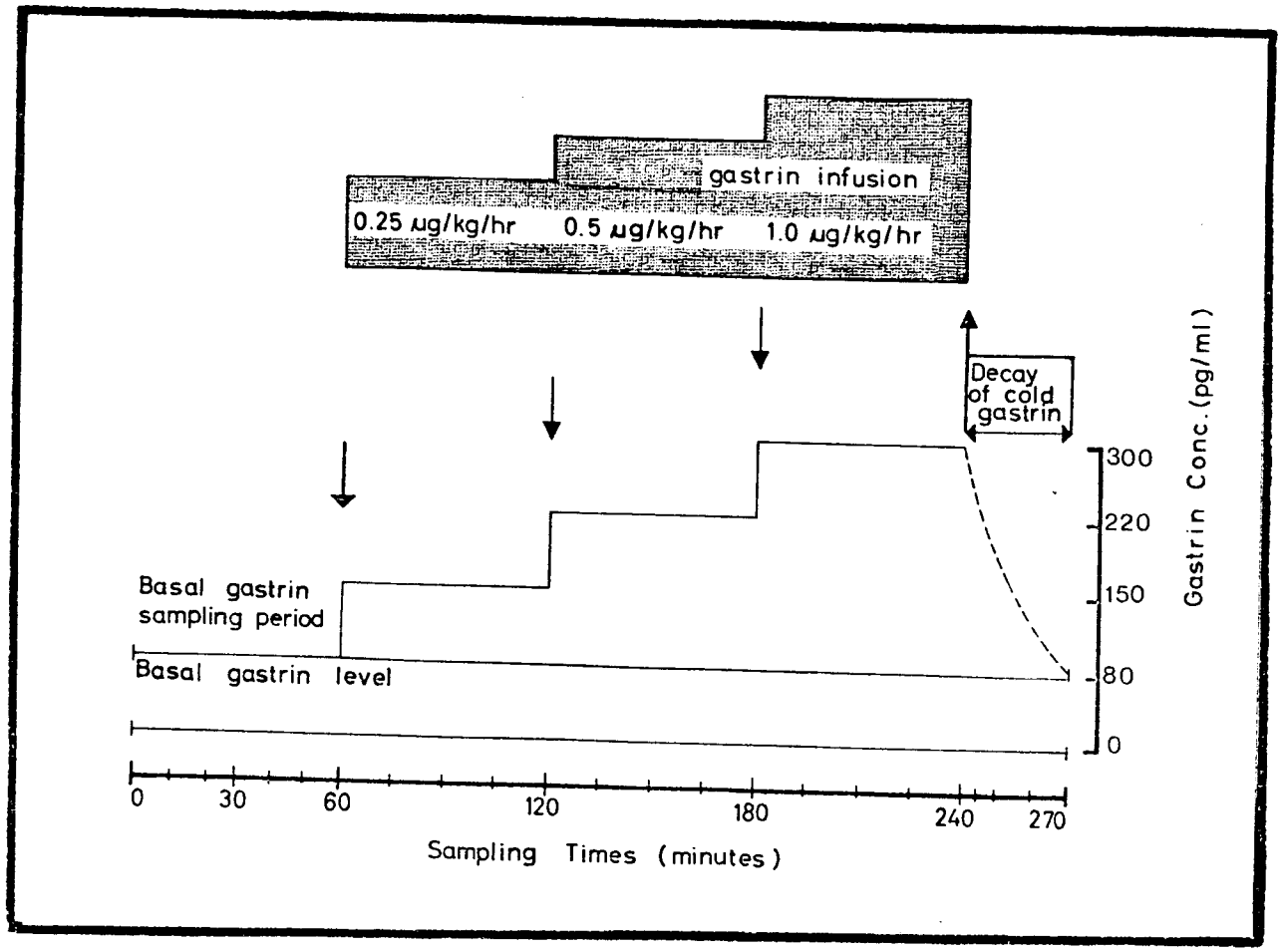


Fig. (3.IV.2.) Outline of protocol for stepwise infusion of synthetic human gastrin G-17 I into group II pigs.
(See section 3 IV in text.)

Plateau serum gastrin levels were calculated as the mean of the closest three out of four serum gastrin measurements made during the last hour of infusion at the highest dose level, in arterial, portal and venous circulations. For calculation of the half-life ($T\frac{1}{2}$) of the infused gastrin in the circulation, basal or endogenous gastrin levels were subtracted from all measurements during and after infusion, and post-infusion gastrin values were calculated as a percentage of the plateau value after converting the values from picograms/ml to picomoles/litre. The natural log of the percent plateau gastrin concentration versus time was computed to yield the elimination constant, K_e, from which the $T\frac{1}{2}$ was calculated in the arterial, portal and venous circulations according to the formula:

$$T\tfrac{1}{2} = \frac{0{,}693}{K_e} \qquad (5)$$

The space (or volume) of distribution of the heptadecapeptide gastrin as a fraction of body weight in arterial, portal and venous circulations was calculated for each pig from the equation derived by Goldstein, using the plateau principle (Goldstein, Aronow and Kalman, 1974):

$$\log V = \log D - \log G - \log K_e \qquad (6)$$

where V = volume of distribution as a fraction of body weight (litres/kg)

D = dose of gastrin infused (p mol/kg-min)

G = actual plateau blood level of gastrin above zero in arterial, portal or venous circulation (p mol/litre)

K_e = elimination constant.

Serum gastrin levels measured as pg/ml were converted to p mol/litre by dividing by 2,2 assuming an approximate molecular weight for gastrin of 2 200. The dose of gastrin infused used for these calculations was 1 µg/kg/hr,

or 7,575 p mol/kg-min.

The space of distribution of gastrin in each pig was determined using the individual weights of each pig, and the mean $\pm$ S.E.M. were calculated.

The metabolic clearance rate (M.C.R.) of infused synthetic human heptadecapeptide gastrin in the arterial, portal and venous circulations was calculated using equation (7):

$$M.C.R. = \frac{D}{G} \qquad (7)$$

where M.C.R. = metabolic clearance rate (litres/kg-min)

D = dose of gastrin infused (p mol/kg-min)

G = plateau gastrin concentration above basal (p mol/litre).

The clearance rate was calculated for each dose of gastrin infused, and expressed as the average of the values obtained for all three doses, and as the average of values obtained for the two highest dose levels. The values for gastrin concentrations used here were the levels above basal, in contrast to the absolute gastrin levels used in the calculation of the space of distribution.

The daily production of gastrin was determined using the metabolic clearance rate. The M.C.R. was converted to litres/day per whole animal, using the individual weights of each pig. The clearance rate thus obtained for the arterial circulation was multiplied by the mean basal serum gastrin level (pg/ml) to obtain the total mass of gastrin which is cleared by the body, and thus presumably produced by the body per day, allowing for discrepancies due to recirculation. The daily production of gastrin, or the daily blood production rate (B.P.R.) was calculated for each pig according to the formula:

B.P.R. (ng/day) = M.C.R. (l/day) x Basal gastrin conc. (ng/l)

and the mean and standard error were determined.

(v) Chromatography

Serum samples from portal and venous sites, taken via the portal and hepatic venous catheters in pig 500, and samples from the portal, venous and arterial sites in pig 521 were selected for chromatography, as very high serum gastrin levels were found in these pigs during the second and third hours of gastrin infusion. Sample volumes of 1-2 ml were chromatographed on a Sephadex G-25/G-50 column of dimensions 1,5m x 1,5cm, as described in section II of this chapter. Internal standards of the molecular weight markers blue dextran and vitamin B_{12} were run with each elution and the column was calibrated with standard preparations of natural human gastrin G-17 I and G-34 I. The gastrin content of the eluted fractions was measured by radioimmunoassay using antiserum G 1, and the elution profile for each sample was plotted as for previous chromatographic procedures.

Comparison of the elution profiles obtained here with those obtained using the standard preparations of natural human gastrin G-17 I and G-34 I gave an indication of whether the infused synthetic human heptadecapeptide gastrin was changed to larger or smaller forms after circulation in the pig for 2-3 hours. Pre- and post-hepatic samples were studied to see whether the gastrin type was altered by passage through the liver.

(vi) Statistical Analysis

Significant differences between the afferent and efferent hepatic gastrin mass in the baseline pigs and following gastrin infusions were tested for by the paired "t"-test and the Wilcoxon Signed Rank test for non-parametric data.

Comments and Criticisms

An attempt was made to measure blood flow in the pigs belonging to group I using a cuff surrounding a blood vessel, connected to an electromagnetic flow meter. Due to technical problems (Hickman, Crosier, Smith, Immelman and Terblanche, 1975) this method had to be abandoned, and blood flow measurements were made by infusion of dye, as described.

The study was commenced with 8 pigs in group I, and satisfactory blood flow measurements were obtained with all 8 pigs. The mean flow rates and fractional flow rates obtained for these pigs were used in calculations of afferent and efferent hepatic gastrin mass for these pigs and group II pigs. However, gastrin measurements at one or two sites in pig number 15/4 gave such bizarre results that the values completely distorted the mean values calculated for hepatic balance of gastrin in these 8 pigs, and this pig was therefore excluded from the study.

Since the weights of the pigs in groups I and II differed to such an extent, extrapolation of blood flow measurements made in group I pigs, which weighed 18-22 kg, for calculations of hepatic gastrin balance in group II pigs, which weighed approximately 30 kg each, is a source of error which must be borne in mind when considering these results. However, since blood flow measurements were made relative to the weight of the pigs, the discrepancy should be small.

The concentrations of gastrin used to make up the doses for infusion into group II pigs were measured by dilution from a stock solution of 1 mg/ml. It is unfortunate that the concentration was not determined spectrophotometrically, using the molar extinction coefficient for gastrin of 12 261

(Walsh, Debas et al., 1974), as this would have given a more accurate representation, but this was overlooked at the time.

The infusion study was commenced on ten pigs. Due to bleeding problems three pigs had to be discarded before the study was completed. The 3-hour infusion was completed on 7 pigs, and the gastrin measurements made in these animals were used to calculate the response of the liver to increasing doses of gastrin. One pig, number 447, developed bleeding problems at the end of the third hour of infusion, so the gastrin measurements for this pig made during the period of fall-off from the plateau level dropped to below the mean basal arterial level within 5 minutes after stopping the infusion. For this reason gastrin values in this pig during the decay period were discarded. All calculations involving measurements in the decay period were made for only 6 pigs. These calculations included K_e and $T\frac{1}{2}$ values, and determination of the space of distribution. Hence some results in this part of the study are for a total of 7 and some are for a total of 6 pigs.

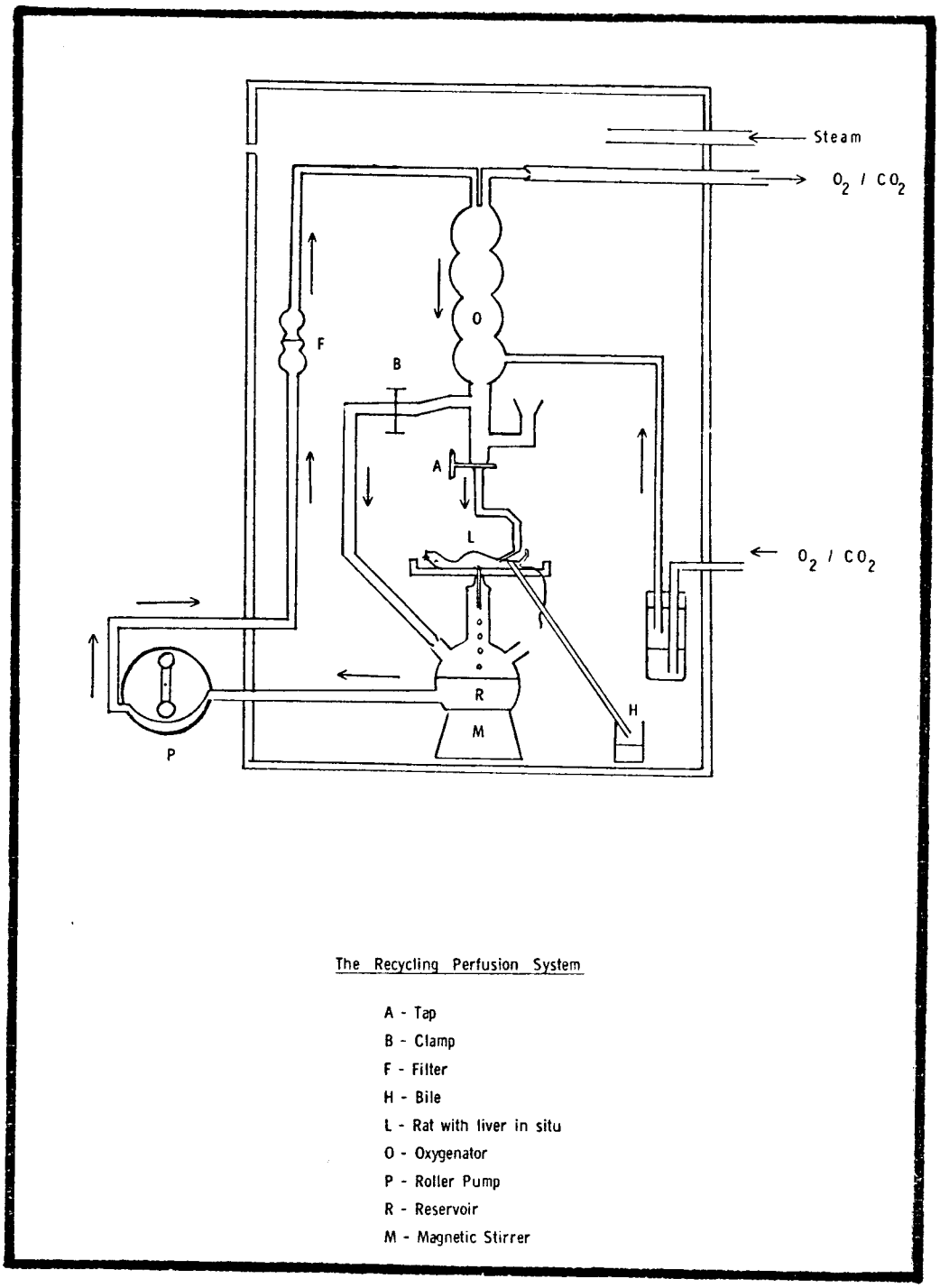
Section V Studies with Rat Liver

3 V(i) Metabolism of Synthetic Human Heptadecapeptide Gastrin by the Isolated Perfused Rat Liver.

Synthetic human heptadecapeptide gastrin G-17 I was perfused cyclically through the isolated rat liver *in situ* in a plasma-free medium at physiologic and supraphysiologic levels, to investigate the role of the rat liver in the metabolism of SHG-17 I. Liver donors were Wistar rats of either sex, aged six weeks, weighing 85-100 grams, and allowed water but no food for 16-18 hours before perfusion. The rats were reared on a 20% casein diet fully supplemented with minerals and vitamins from the age of three weeks (Stead and Brock, 1972).

Perfusions were performed according to the recycling method of Hems, Ross, Berry and Krebs (1966), which involved isolating the liver from the rest of the animal, whilst leaving it *in situ*. The liver was perfused through the portal vein with a semi-synthetic medium under a hydrostatic pressure of about 20 cm of water, maintained by a reservoir of adjustable height. The medium leaving the liver through the vena cava dropped into a reservoir or collecting vessel, from where it was pumped to the top of a multibulb oxygenator, and then returned via the liver to the reservoir. The apparatus was housed in a perspex cabinet and was heated by a thermostatically controlled fan heater. The perfusion system is diagrammatized in figure (3.V.(i).1). The perfusion procedure is described in detail in the appendix.

Perfusions with gastrin were carried out by addition of synthetic human gastrin G-17 I (I.C.I.) to the reservoir after a 30-minute equilibration period. A baseline sample of 0,8 ml was collected 1 minute before the addition of gastrin to ascertain whether there was any endogenous gastrin



The Recycling Perfusion System

A - Tap
B - Clamp
F - Filter
H - Bile
L - Rat with liver in situ
O - Oxygenator
P - Roller Pump
R - Reservoir
M - Magnetic Stirrer

Fig. (3.V.(i).1.) The recycling perfusion system used for perfusion of the isolated rat liver in situ.

in the system which had been leeched out of the liver during this period. Gastrin was added in one of two doses on separate occasions to separate liver preparations, so that three perfusions with a supraphysiological dose of gastrin and three perfusions with physiological gastrin levels were performed:

(a) One hundred and sixty ng synthetic human gastrin G-17 I in 0,8 ml Krebs-Ringer Bicarbonate (KRB) buffer containing 0,25% crystalline human serum albumen (HSA) (Behringwerke) was added to give a perfusate gastrin concentration at equilibration, $2\frac{1}{2}$ minutes after addition, of approximately 1 nM, which is supraphysiological. Samples were withdrawn from the circuit tubing leaving the reservoir after addition of gastrin, centrifuged immediately and the supernatants were stored deep frozen at -20°C until assayed for gastrin using antiserum G 1, as described in section 3 I(iii). Sample volumes of 2,5 ml were withdrawn at $2\frac{1}{2}$, 30 and 60 minutes for chromatography, to investigate whether gastrin was altered in molecular size by passage through the liver. Sample volumes of 0,6 ml were collected at 10, 20 and 45 minutes, so that the total volume removed from the system amounted to 9,3 ml or 15% of the total perfusate volume. No corrections were made for these volume losses. Samples were chromatographed on a Sephadex G-25/G-50 fine column of dimensions 1,5 cm x 1,5 m as described in section 3 II, and the gastrin content of the eluted fractions was measured by radioimmunoassay. The chromatographic profile was plotted as gastrin concentration against the percent elution volume between blue dextran and vitamin B_{12} as these markers were run as internal standards in the fractionation. An estimate of the recovery of gastrin added to the Sephadex column was made.

(b) To follow the fate of more physiological gastrin levels approximately

8 ng gastrin in 0,8 ml KRB buffer containing 0,25% HSA was added to the system to give gastrin concentrations after equilibration of approximately 0,05 nM. Samples were withdrawn from the circuit tubing leaving the reservoir at the same times and in the same volumes as for the high dose perfusion, viz. 0,8 ml at -1 minute, 0,6 ml at 10, 20 and 45 minutes and 2,5 ml at $2\frac{1}{2}$, 30 and 60 minutes after addition of gastrin, so that the volume loss due to sampling was identical in both systems. The samples were centrifuged immediately and the supernatants stored at -20°C until assayed for gastrin using antiserum G I, which detects non-sulphated forms of gastrin but reacts poorly with sulphated heptadecapeptide gastrin.

Gastrin levels measured at each sampling time during the perfusions were compared with the gastrin level at $2\frac{1}{2}$ minutes after addition of gastrin by analysis of variance.

One control perfusion, in which the liver was omitted from the system, was performed at each dose level of gastrin. Perfusate samples from the high dose perfusion were assayed in dilutions and the values corrected appropriately. All standards and unknown samples were diluted in KRB buffer.

In a separate series of experiments the clearance of physiological concentrations of rat insulin (Lot no. R170, Novo Laboratories, Denmark) by the liver in the same system was investigated. The insulin was perfused at an equilibration concentration of approximately 1 nM, and samples of 0,5 ml were collected from the circuit tubing leaving the reservoir at 5, 10, 15, 20, 30, 40, 50 and 60 minutes after addition of insulin. This was repeated in 12 separate liver perfusion preparations. Four control perfusions in the absence of liver were performed. The samples were

centrifuged immediately after collection and the supernatant stored at -20°C and assayed for insulin within three weeks. Insulin was measured by a double-antibody micro-radioimmunoassay, using the same rat insulin as was added to the perfusion system as standard, and ^{125}I-porcine insulin as tracer (Weinkove, Weinkove and Pimstone, 1974).

3 V(ii) In Vitro Binding of Gastrin to Rat Liver Ligandin

Ligandin is a basic protein isolated from supernates of human and rat liver, kidney and intestinal homogenates, which is known to bind organic anions, cortisol metabolites and carcinogens (Levi, Gatmaitan et al., 1969; Litwack, Ketterer et al., 1971; Fleischner, Mishkin et al., 1971). The binding of gastrin to rat liver ligandin was examined to assess the importance of this liver protein in relation to gastrin transport and/or inactivation.

A 25% homogenate of rat liver in 0,25M-sucrose/0,01M-phosphate buffer, pH 7,4, was prepared as described by Kirsch, Vinik, Frith, Gordon, Grant and Saunders (1975). Five ml of the 100 000 xg supernate was mixed with 100 microlitres ^{125}I-albumen and 100 microlitres sodium bromosulphthalein (BSP). This mixture was chromatographed on a Sephadex G-100 column of dimensions 100 cm x 2,5 cm. Elution was carried out using the same sucrose/phosphate buffer in a pump-driven upward flow system at a flow rate of 15 ml/hour. The protein concentration of the eluted fractions was estimated at 280 nanometres (nm), using a Unicam spectrophotometer model SP 1700, and the presence of BSP was monitored by reading the optical density at 580 nm. Radioactivity of the eluted fractions was estimated to locate the ^{125}I-labelled albumen. The ligandin peak was identified by its binding with BSP and by immunodiffusion against rabbit anti-rat liver ligandin as described by Fleischner, Mishkin et al. (1971).

Synthetic human gastrin G-17 I (I.C.I.) was labelled with ^{125}Iodine (Radiochemical Centre Ltd., Amersham, England) to a specific activity of 1 440 μCi ^{125}Iodine/μg hormone, and purified on a small Sephadex G-10 column of dimensions 0,9 cm x 10 cm. An aliquot containing approximately 2 million c.p.m. was mixed with 5 ml liver supernate, and chromatographed without delay on the Sephadex column described above. The protein

concentration and radioactivity of the eluted fractions were monitored and the elution profile was plotted. ^{125}I-labelled unsubstituted synthetic human gastrin G-17 I was also passed through the column in the absence of liver supernate. On another occasion Na ^{125}Iodide was mixed with 5 ml liver supernate and passed through the column.

The displacement of radioactive gastrin from ligandin was achieved by the addition of a gross excess (100 µg) of non-radioactive synthetic human gastrin G-17 I to a mixture of liver supernate and ^{125}I-gastrin before chromatography. In another experiment unlabelled synthetic human gastrin G-17 I was added to liver supernate in high concentration (15 ng) before chromatography, and the gastrin content of the eluted fractions was estimated by radioimmunoassay using antiserum G 1, which has a high affinity for gastrin ($K=4,0 \times 10^{10}$ litres/mole).

Binding of ^{125}I-labelled insulin and ^{125}I-labelled glucagon to ligandin was investigated by mixing aliquots of each labelled hormone with liver supernate and subjecting them to the same chromatographic procedure on separate occasions.

Each chromatographic procedure involving binding of ^{125}I-gastrin, displacement of labelled gastrin by cold gastrin, and binding of cold gastrin by ligandin was repeated at least twice, and the estimations of recovery of radioactivity in the ligandin peak include both or all of the findings on these occasions.

In the section which follows, the results of the investigations described will be presented.

CHAPTER 4

RESULTS

Section 1 Radioimmunoassay

4 1(i) Production of Antisera

Several antisera were produced which were suitable for immunoassay. Antiserum G 5, obtained by this procedure, was used extensively in the radioimmunoassay procedures described in this thesis at a final dilution of 1 : 35 000. Figure (4.1.1.) shows a series of antibody binding curves obtained by testing the ability of another antiserum (G 9), drawn in 10 consecutive bleeds, to bind radioactive gastrin. The dilutions shown in the figure refer to initial dilution of antiserum; the final dilution was ten times greater in each case. It can be seen that the animal became tolerant to the gastrin injections after approximately 6 booster injections, as shown by the fall off in binding of ^{125}I-gastrin in the later bleeds.

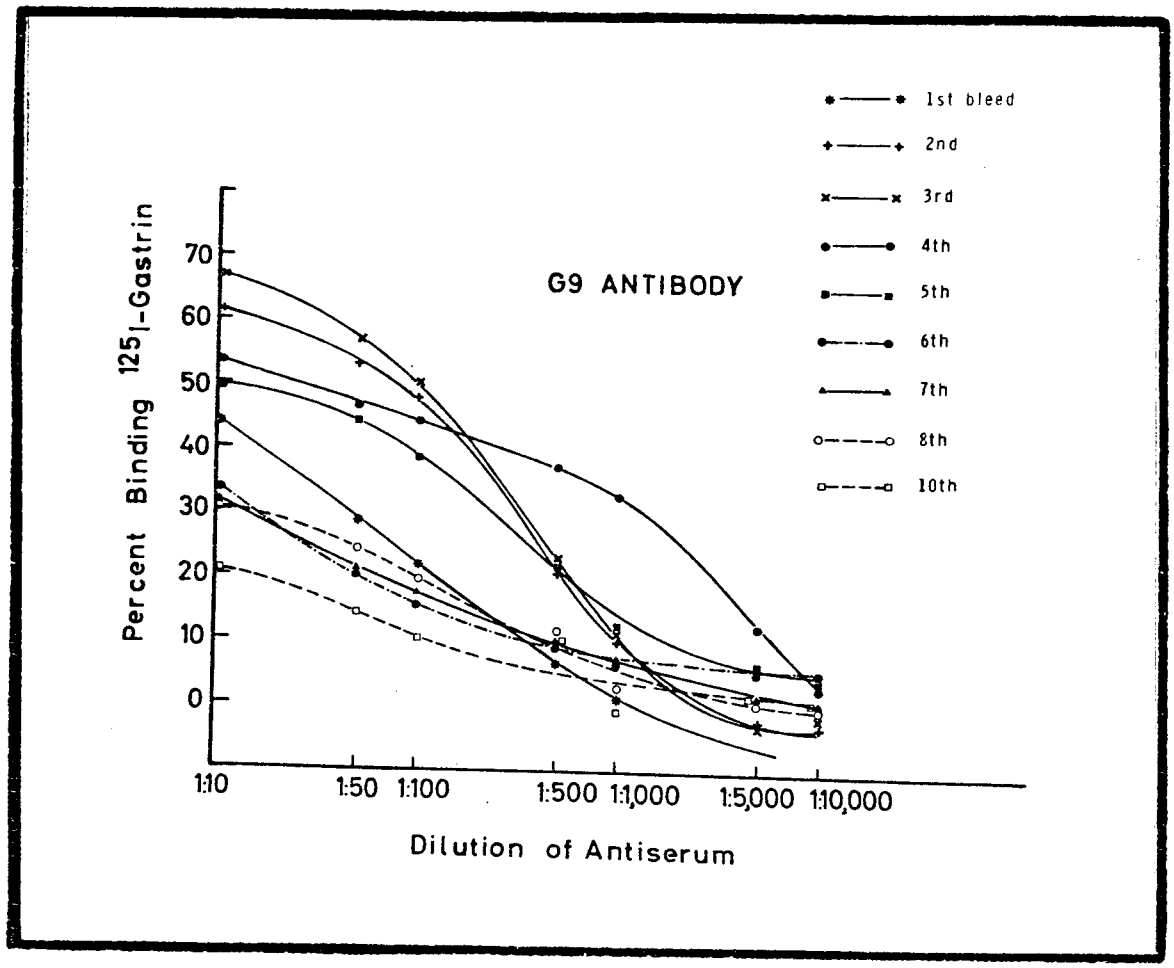


Fig. (4.1.1.) Binding of ^{125}Iodine-labelled synthetic human gastrin by antibodies obtained from a single rabbit (G 9), bled at 6-week intervals.

4 I(ii) The Iodinated Product

The percentage incorporation of ^{125}Iodine into the hormone was usually of the order of 75%, giving a "specific activity" of 1 500 μCi ^{125}Iodine/μg gastrin when the reagents were added in the proportions described in section 3 I(ii). Labelled preparations used in the radioimmunoassay had "specific activities" ranging from 700 to 1 800 μCi ^{125}Iodine/μg hormone. Greater incorporation of radioactive iodine was achieved when a lower "specific activity" was aimed for.

The integrity of the labelled hormone as determined by charcoal adsorption ranged from 88% to 98%. The immunological integrity was found to vary between 74% and 95%.

The iodination procedure described in section 3 I(ii) is not the only method of labelling a hormone with radioactive iodine. More delicate peptides require gentler treatment and in some cases the use of Chloramine-T is too harsh, so that one may have to resort to an iodination method utilising hydrogen peroxide as oxidation agent for example, as described by McFarlane (1956). However, the yield using this method is not nearly as high as that obtained with the Chloramine-T method, which has a yield of 80% (Aubert, 1971). Generally the more delicate the peptide one is trying to label, the lower the "specific activity" one should aim for, so that one should commence by aiming to incorporate approximately 200 μCi ^{125}Iodine/μg hormone. The individual doses of Chloramine-T and sodium metabisulphite are peculiar to the iodination of each hormone and often have to be determined by trial and error.

With labelled hormones the degree of integrity that can be obtained varies from hormone to hormone; for example insulin can be labelled to give a preparation which is 98-99% intact, whereas gastrin does not remain as intact

following labelling. However, it is fortunate that gastrin with an integrity of as low as 85% can be used successfully in the radioimmunoassay. In general, less intact peptides give rise to inappropriately elevated readings in the assay, and improvement of the quality of the labelled preparation may lead to a decrease in "apparently" measured hormone levels, as discussed by Stadil and Rehfeld (1973a).

4 I(iii) Validation of the Assay

The results of the trial to test the adsorption of labelled gastrin to glass and plastic in the presence of albumen and gelatin (Haemaccel) at various concentrations are shown in table (4.1.1). The degree of adsorption of hormone to LKB cuvettes was a mere 2,2% ± 0,11 (S.E.M.) justifying their use in the assay system without additional albumen or gelatin. One percent albumen was the most effective treatment for glass tubes, with only 4,6% ± 0,61 (S.E.M.) of the labelled gastrin remaining adsorbed to their surfaces.

Figure (4.1.2.) shows standard curves obtained for three different incubation times using antiserum G 1. The best curve was obtained with 24 hours' incubation at 4°C, as it was the most sensitive and showed complete displacement of ^{125}I-labelled gastrin from antibody with increasing concentrations of cold hormone.

Figures (4.1.3.), (4.1.4.) and (4.1.5.) show the mean standard curves ± 95% confidence limits obtained using antisera G 1, 2604-7 and G 5. In all cases the standard reference preparation used was synthetic human gastrin G-17 I, and the tracer was ^{125}I-synthetic human gastrin G-17 I.

Details of the gastrin radioimmunoassay with three antisera appear in table (4.1.2). Assays using antiserum G 5 displayed the lowest inter-assay coefficient of variation, which was 17,2%. Assays involving antiserum G 1 showed the highest precision, with an intra-assay coefficient of variation of 4,95%. Values for the index of precision ranged from 13,9% for assays utilising antiserum 2604-7 to 28,1% for assays performed with antiserum G 1. The best correlation coefficient obtained on regression analysis of observed versus expected concentrations of added gastrin was found for assays using antiserum G 5, with a value of r of 0,973.

The Scatchard plots derived from the mean standard curve using each anti-

serum are shown in figure (4.1.6.). The plots are almost linear, suggesting homogeneity of the antibody populations. Since antisera, and not purified antibodies were used, the slopes of the graphs were expressed as the "average" affinity constants. The highest affinity antiserum was 2604-7, which showed the steepest slope and had an average affinity constant of $2,0 \times 10^{11}$ litres/mole. The affinity of antiserum G 1 was almost as high, with a K value of $4,0 \times 10^{10}$ litres/mole. Antiserum G 5 had the highest concentration of antibody binding sites ($10,20 \times 10^{-11}$ moles/litre) and the lowest average affinity constant ($8,3 \times 10^{9}$ litres/mole), which implies that it is a high capacity antiserum of low affinity. The concentrations of binding sites for antisera G 1 and 2604-7 were $24,1 \times 10^{-12}$ and $10,0 \times 10^{-12}$ moles/litre.

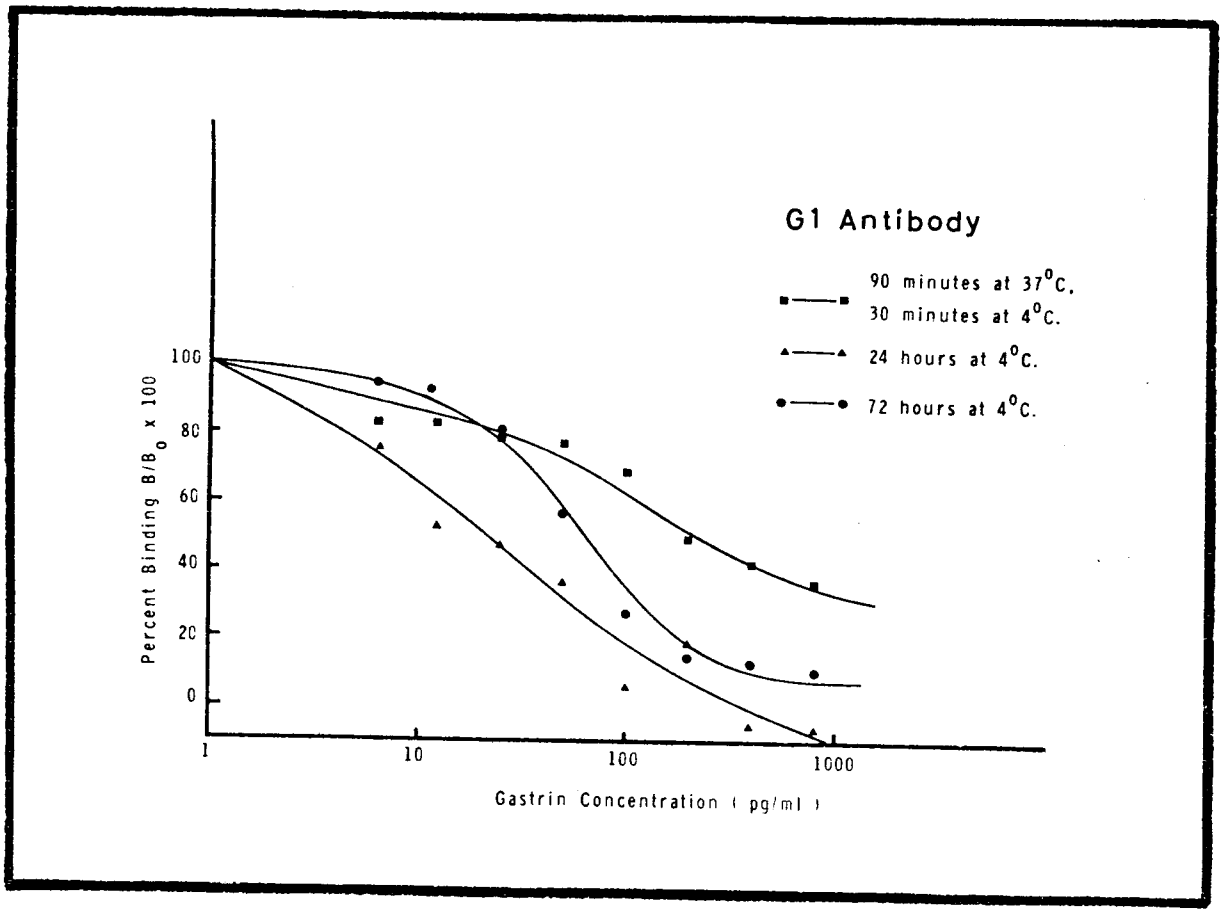


Fig. (4.1.2.) The effect of different incubation times on the slope of the standard curve obtained using antiserum G 1.

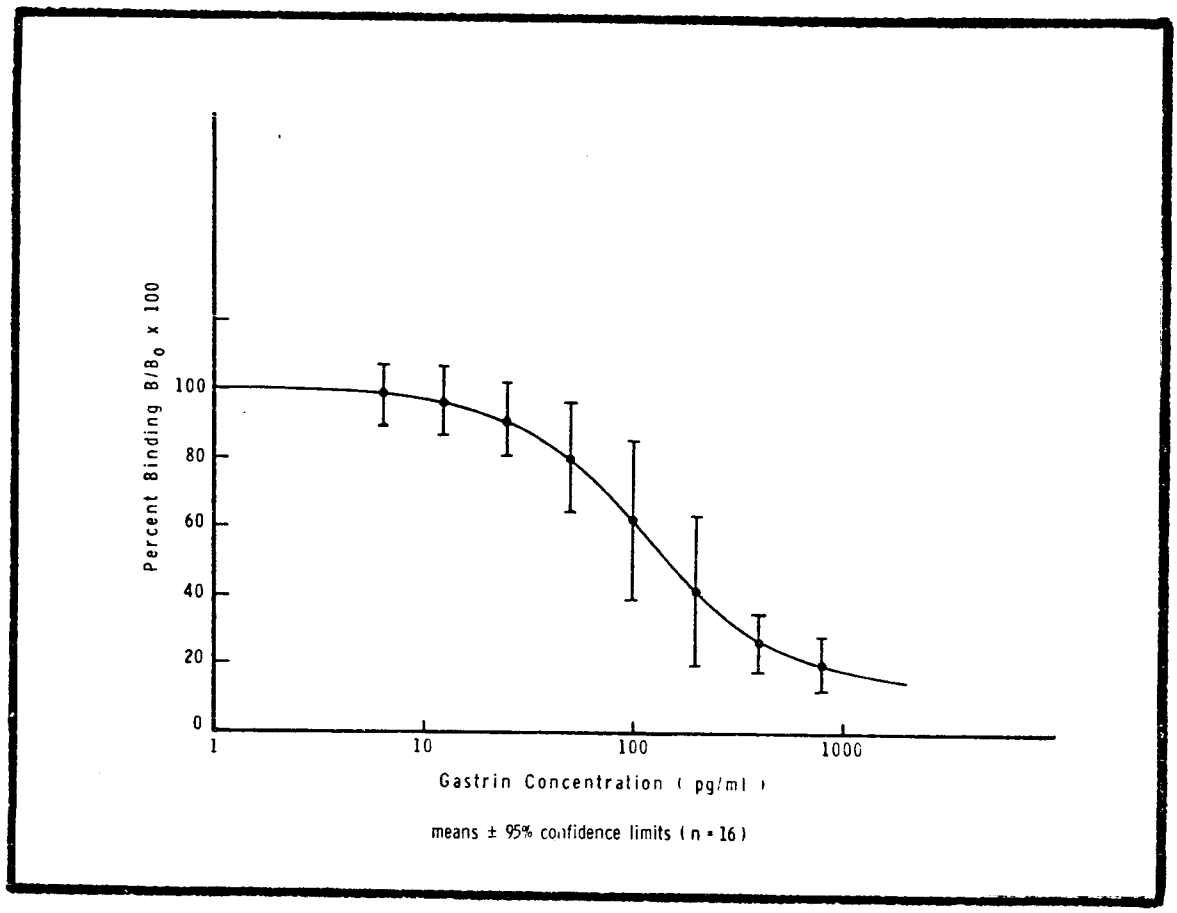


Fig. (4.1.3.) Mean standard curve ± 95% confidence limits for 16 assays using antiserum G 1.

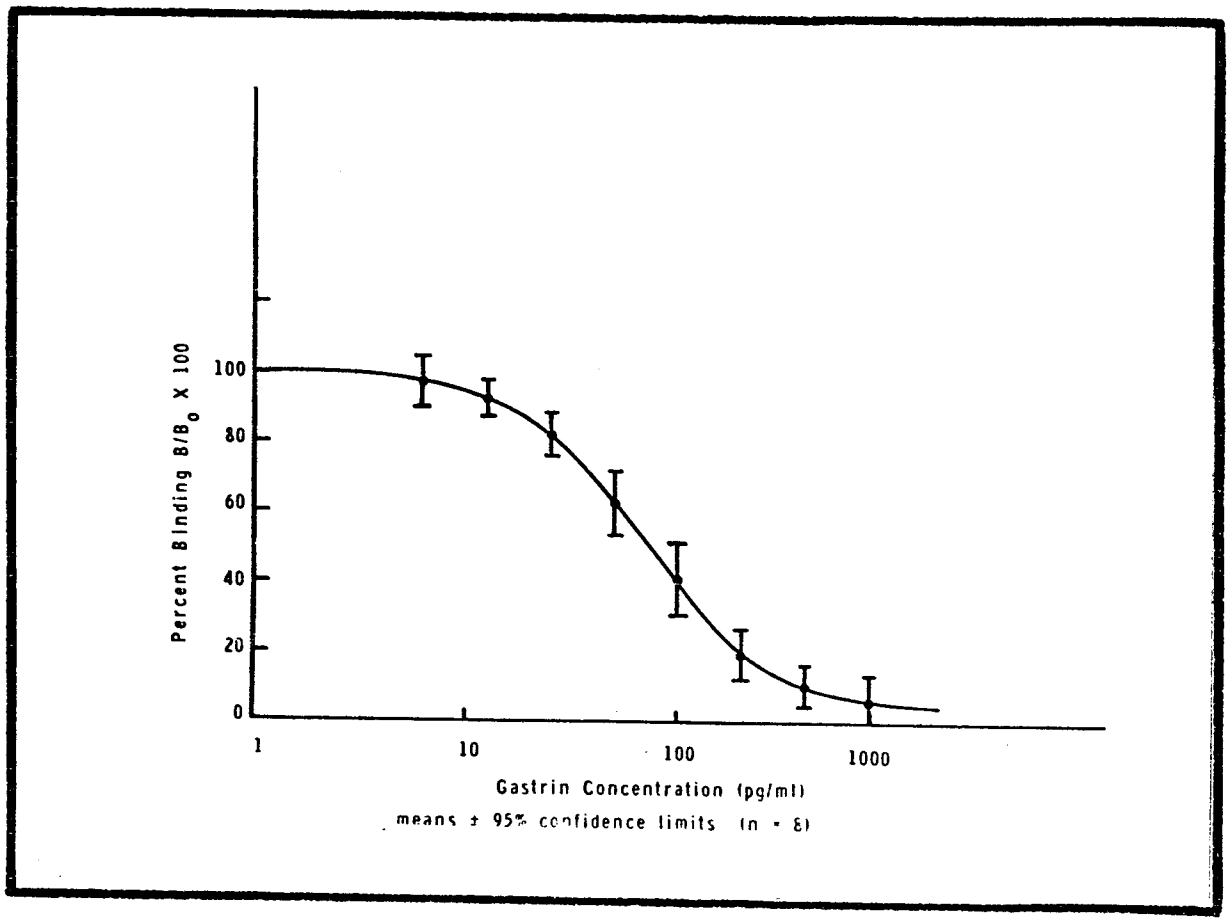


Fig. (4.1.4.) Mean standard curve ± 95% confidence limits for 8 assays using antiserum 2604-7.

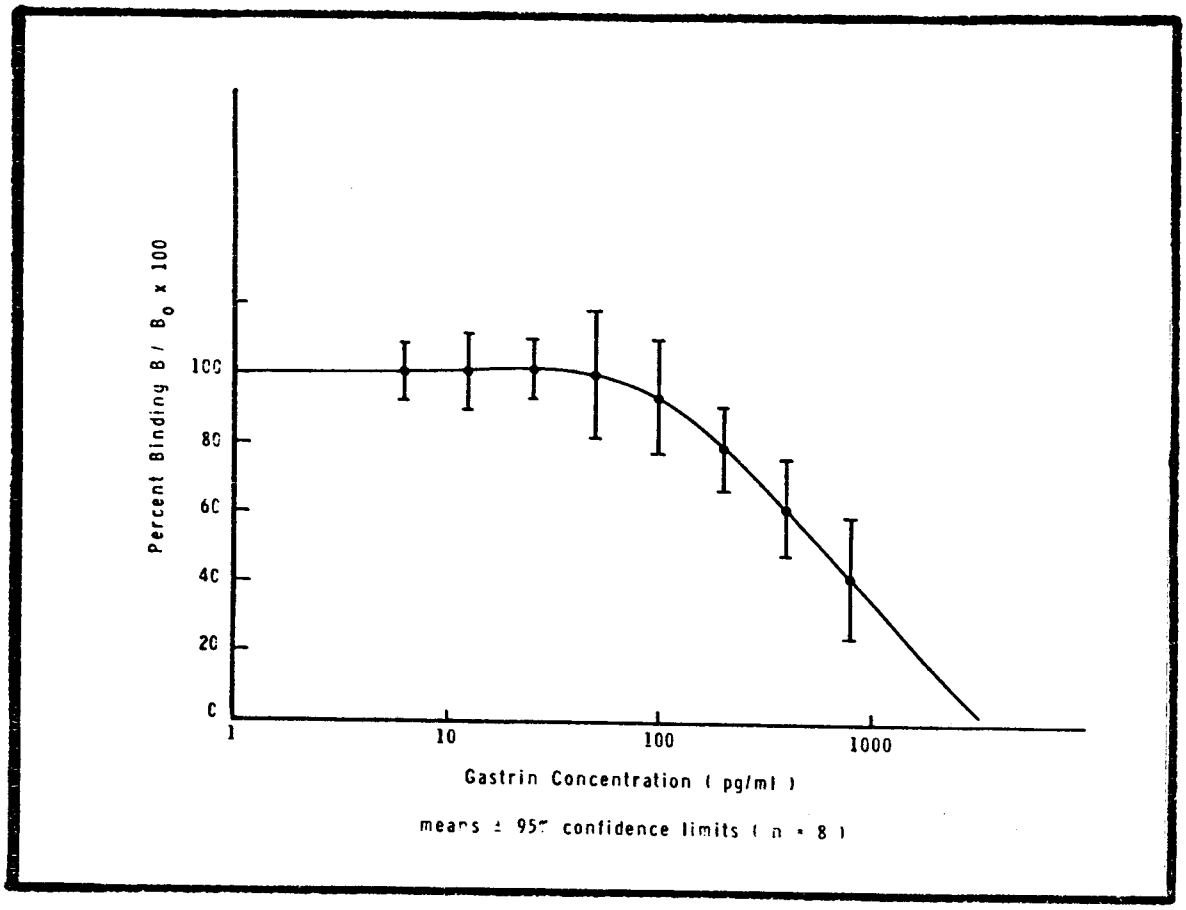


Fig. (4.1.5.) Mean standard curve ± 95% confidence limits for 8 assays using antiserum G 5.

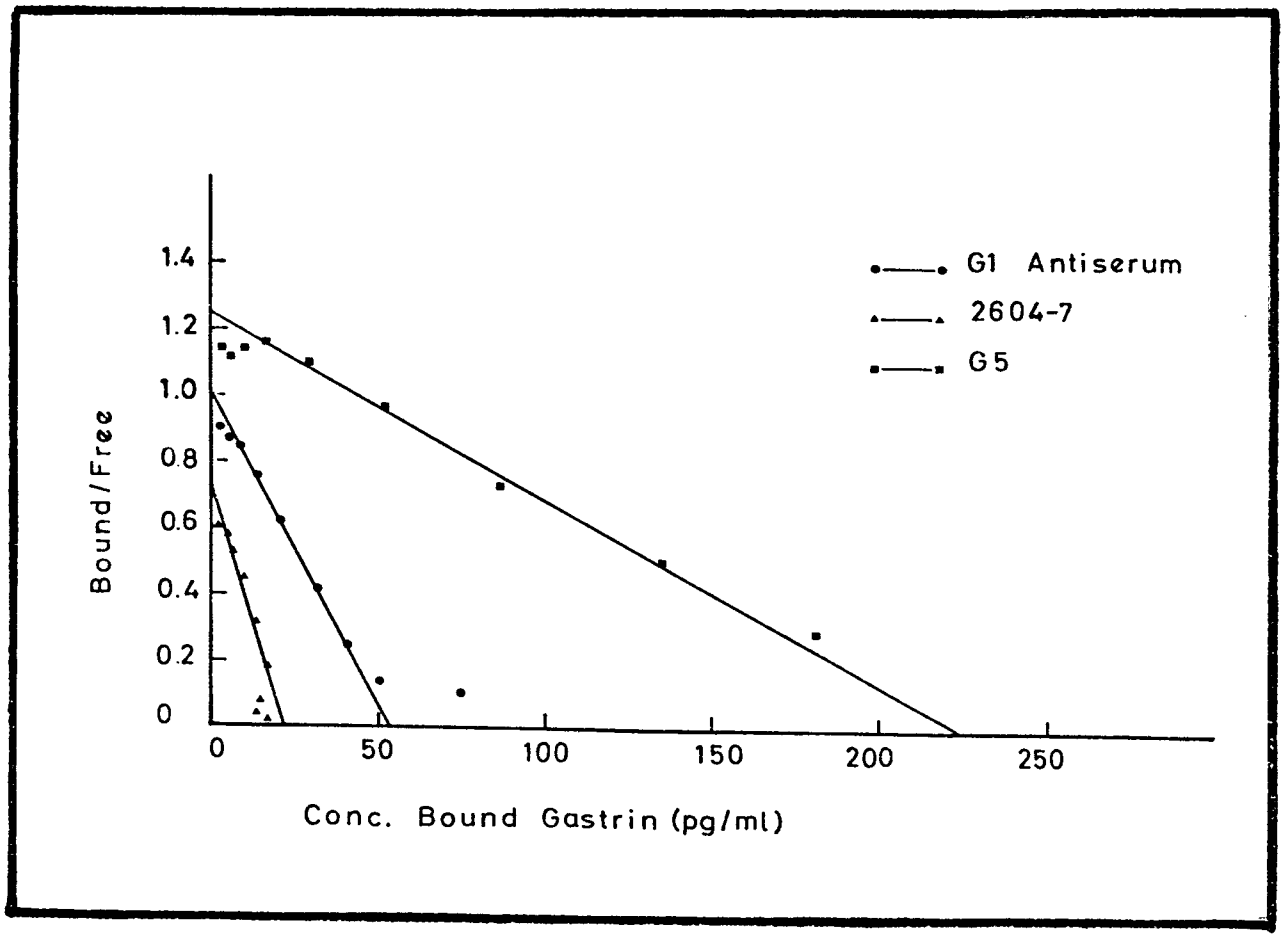


Fig. (4.1.6.) Scatchard (1949) plots of mean standard curves obtained with antisera 2604-7, G 1 and G 5.

TABLE (4.1.1)

Experiment to test the Effect of Addition of Albumen and Gelatin on the Adsorption of ^{125}I-labelled Gastrin to Glass and Plastic Vessels.

(Values are means $\pm$ S.E.M. n=4)

	Percentage of Total Counts remaining in Tube after Decanting	
	Glass Tubes	LKB Plastic Cuvettes
Veronal buffer	18,4% $\pm$ 1,20	2,2% $\pm$ 0,11
Albumen 0,5%	5,8% $\pm$ 0,56	1,5% $\pm$ 0,15
Albumen 1,0%	4,6% $\pm$ 0,61	1,5% $\pm$ 0,13
Gelatin 0,25%	11,5% $\pm$ 1,16	1,7% $\pm$ 0,14
Gelatin 0,5%	9,9% $\pm$ 0,85	1,7% $\pm$ 0,14
Gelatin 1,0%	10,9% $\pm$ 1,17	1,8% $\pm$ 0,12

TABLE (4.1.2)

Details of Gastrin Radioimmunoassay with Three Antisera

	G 1	2604-7	G 5
Lower limit of sensitivity	1,65 pg (p=0,05)	0,82 pg (p=0,05)	13,5 pg (p=0,05)
Inter-assay coefficient of variation	28,9% (n=11)	29,3% (n=6)	17,2% (n=8)
Intra-assay coefficient of variation	4,95% (n=12)	17,7% (n=16)	6,7% (n=12)
Index of precision	28,1%	13,9%	22,1%
Correlation coefficient on regression analysis of observed vs expected concentration of added gastrin	r=0,710 ($p < 0,01$)	r=0,864 ($p < 0,01$)	r=0,973 ($p < 0,01$)
Final dilution of antiserum in assay	1:200 000	1:100 000	1:35 000
Average affinity constant derived from Scatchard Plot	$K=4,0 \times 10^{10}$ l/mol	$K=2,0 \times 10^{11}$ l/mol	$K=8,3 \times 10^{9}$ l/mol
Concentration of binding sites	$24,1 \times 10^{-12}$ mol/l	$10,0 \times 10^{-12}$ mol/l	$10,2 \times 10^{-11}$ mol/l

4 I(iv) Characteristics of Antisera and Immunoreactivity of Gastrin Fragments and Gastrin-Related Peptides

Figure (4.1.7.) shows the dose response curves obtained with dilutions of serum drawn from fasting and arginine-stimulated subjects, as well as those of various gut and gastrin-related peptides compared with synthetic human gastrin G-17 I, using antiserum G 1. In this and the subsequent two figures the concentration of the peptides is expressed as femtomole equivalents, which refers to the amount of gastrin added to the assay in a volume of 0,1 ml. The curve for fasted serum in dilutions was parallel to that of the SHG-17 I standard. The coincidence of the curves of standard synthetic G-17 I and stimulated serum suggests strongly that the heptadecapeptide gastrin species is produced on arginine stimulation. Gastrin (2-17) cross-reacted to the degree of 95% of that of the standard (1-17) gastrin. Porcine crystalline glucagon, highly purified natural porcine secretin, pentagastrin and crude CCK-PZ failed to cross-react, while caerulein and the C-terminal octapeptide of CCK-PZ reacted minimally. The ID_{50} values for these peptides relative to synthetic human gastrin G-17 I appear in table (4.1.3).

The cross-reaction curves obtained by testing the ability of synthetic gastrin fragments to reduce the binding of ^{125}I-labelled gastrin to antisera G 1 and G 5 are shown in figures (4.1.8.) and (4.1.9.) and the ID_{50} values compared to that of SHG-17 I appear in table (4.1.3). Comparison of the relative ID_{50} values of these peptides suggests that antiserum G 1 recognises chiefly the C-terminal portion of heptadecapeptide gastrin in the region of amino acids 8 to 17. Fragments (8-17) and (11-17) were almost equally active, whereas fragments which did not include the C-terminal 4 amino acids, namely (1-13), (3-13), (6-13) and (9-13), did not react in the system at all. This implies that antiserum G 1 requires the presence of the C-terminal tetrapeptide sequence for

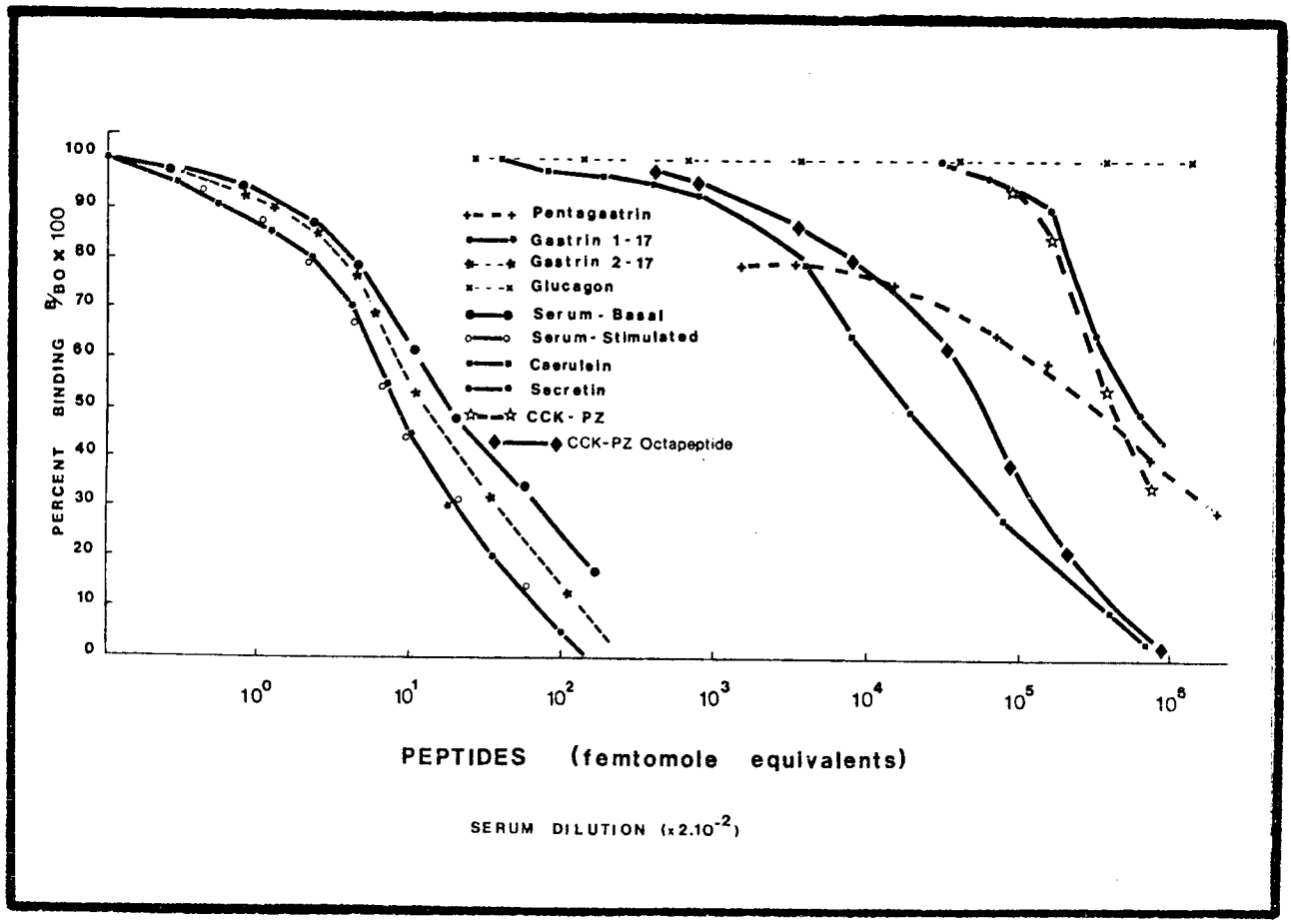


Fig. (4.1.7.) Dose response curves using antiserum G 1 of: serum drawn from fasted and arginine-stimulated subjects; various gut peptides; and gastrin-related peptides, compared with standard synthetic human gastrin G-17 I.

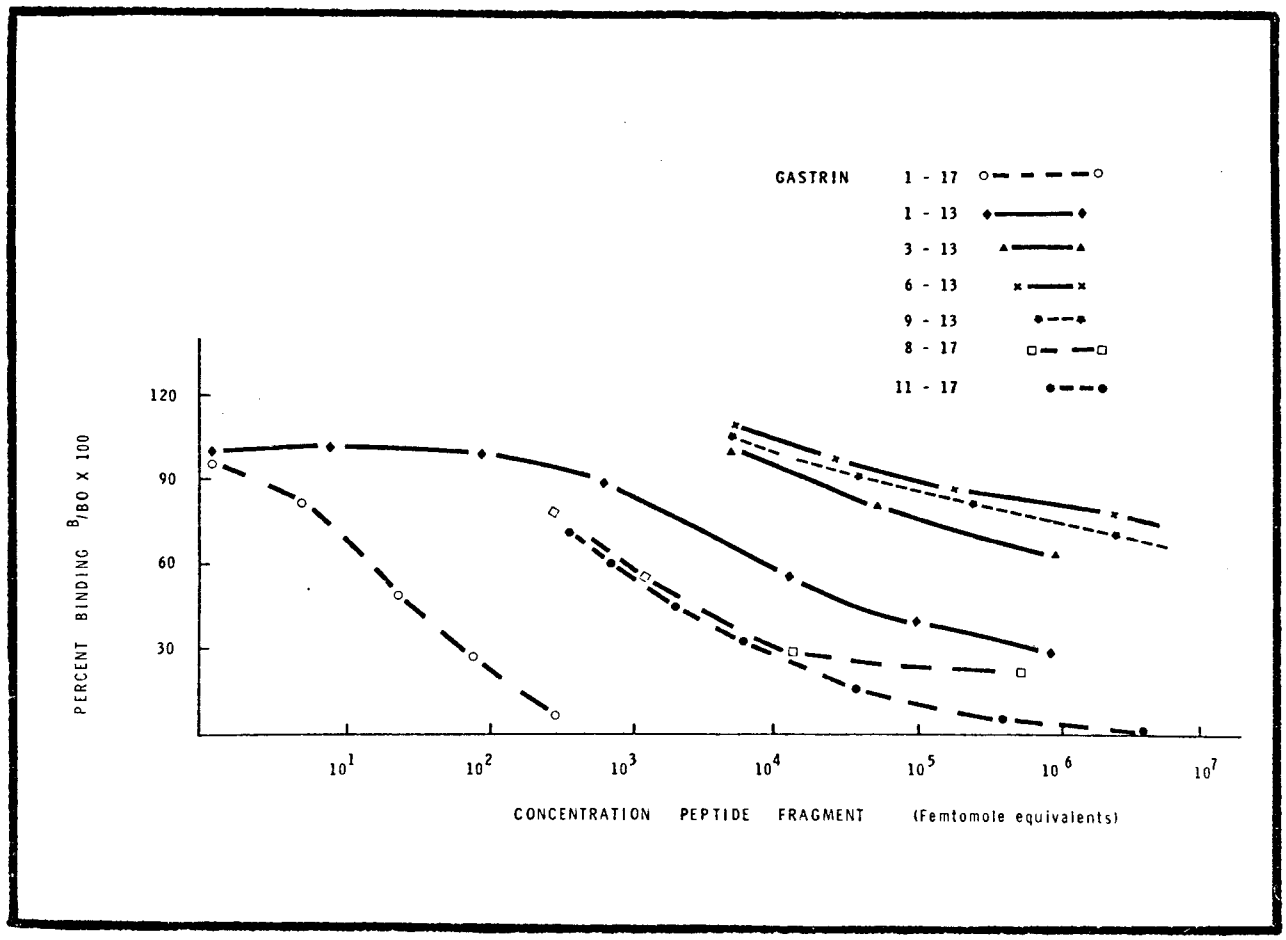


Fig. (4.1.8.) The relative ability of synthetic gastrin fragments to reduce the binding of ^{125}Iodine-labelled synthetic human gastrin G-17 I to antiserum G 1.

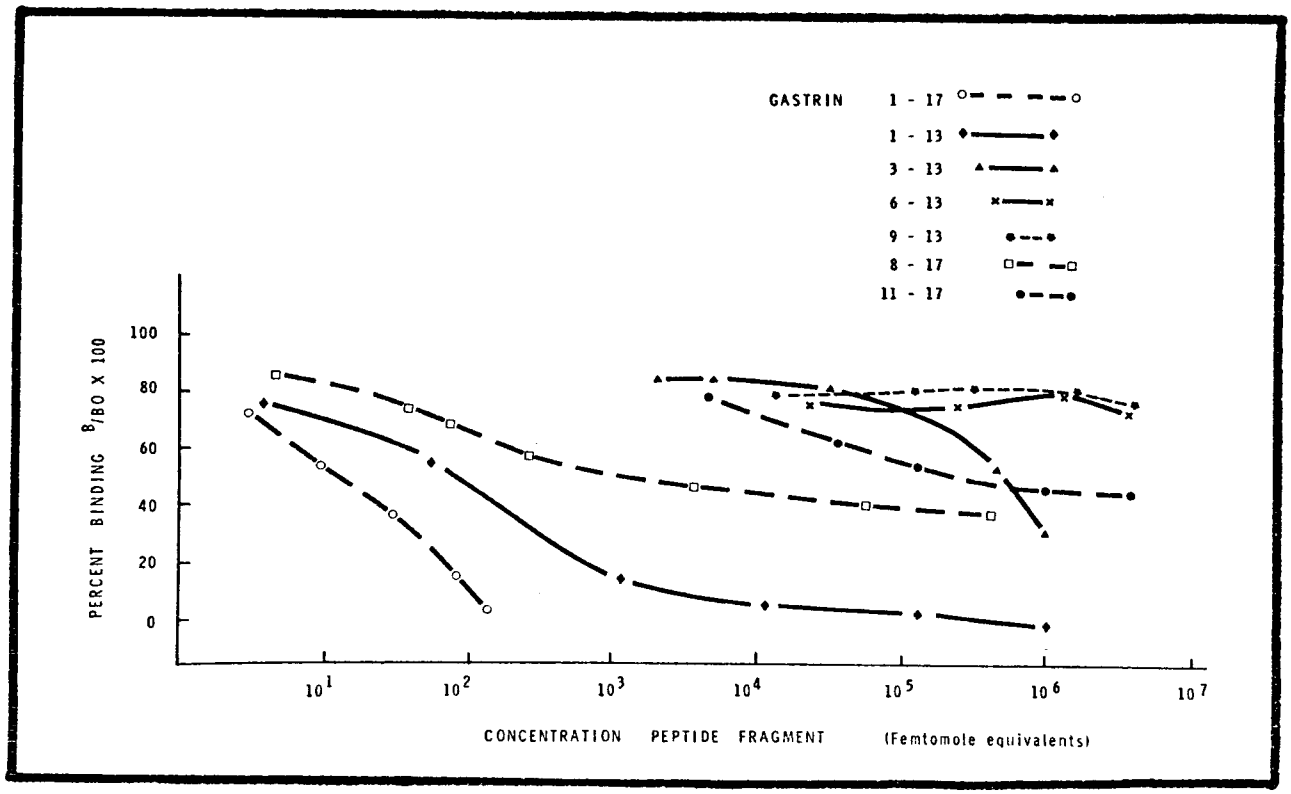


Fig. (4.1.9.) The relative ability of synthetic gastrin fragments to reduce the binding of ^{125}Iodine-labelled synthetic human gastrin G-17 I to antiserum G 5.

TABLE (4.1.3)

Relative Displacement (ID_{50}) of Antibody-Bound ^{125}I-Gastrin by Synthetic Gastrin Fragments and Related Peptides.

Peptide	ID_{50} as a percentage of ID_{50} of SHG-17 I	
	G 1	G 5
Caerulein (Erspamer)	0,033%	-
Pentagastrin (Gly-Trp-Met-Asp-Phe-NH_2)	0,001%	-
Crude CCK-PZ	0,001%	-
Synthetic C-terminal octapeptide of CCK-PZ	0,012%	-
Secretin (highly purified porcine)	0,001%	-
Glucagon, crystalline porcine (Lilly)	0%	-
*Synthetic gastrin peptides		
1-17	100%	100%
1-13	0,1%	16,9%
3-13	0,0033%	0,0023%
6-13	0%	0%
9-13	0%	0%
8-17	1,33%	1,0%
11-17	1,29%	0,003%
2-17	95%	-
5-17 (mini-gastrin)	45,9%	0,86%

* Synthetic gastrin peptides kindly supplied by Dr. John Morley of I.C.I. Pharmaceuticals, except for (1-17) which was obtained from CEA-IRE Sorin.

reaction. The high degree of cross-reaction with mini-gastrin, the (5-17) sequence, and the almost complete cross-reaction with the (2-17) molecule as compared with the intact heptadecapeptide would seem to indicate a preference of antiserum G 1 for the entire molecule.

Antiserum G 5 cross-reacted to a fairly high degree (16,9% of that of the standard) with the sequence (1-13) and hardly at all with any other fragment. The very small degree of recognition for the (8-17) and (5-17) sequences seems to suggest that this antiserum reacts chiefly with the (1-5) sequence of the heptadecapeptide. This correlates with the lack of reaction of G 5 antiserum with G-34 gastrin shown in table (4.1.4), since the N-terminal pyroglutamyl residue of G-17 gastrin is not available for reaction with antibody in the G-34 gastrin sequence.

The relative abilities of natural human gastrin G-34 I, G-17 I and G-17 II and of synthetic human G-17 I to displace ^{125}I-labelled gastrin from antisera G 1, 2604-7 and G 5 are shown in figures (4.1.10.), (4.1.11.) and (4.1.12.) and the corresponding ID_{50} values for these peptides can be found in table (4.1.4). The immunoreactivity of SHG-17 I obtained from I.C.I. was only 46% and 64% of that of SHG-17 I obtained from CEA-IRE Sorin when measured using antisera G 1 and G 5 respectively. It may be that the preparation supplied by CEA-IRE Sorin is purer than that from I.C.I. As was mentioned in section 3 I(iii) the standard reference preparation of gastrin used in all assays for this thesis was obtained from CEA-IRE Sorin.

The most surprising observation to arise from this study was the marked difference in immunoreactivity of NHG-17 I and NHG-17 II with antiserum G 1, to the extent that this antiserum recognised almost exclusively the non-sulphated type I gastrin. In contrast antiserum 2604-7 cross-reacted strongly with both gastrins type I and II, so that with these two antisera sulphated and non-sulphated

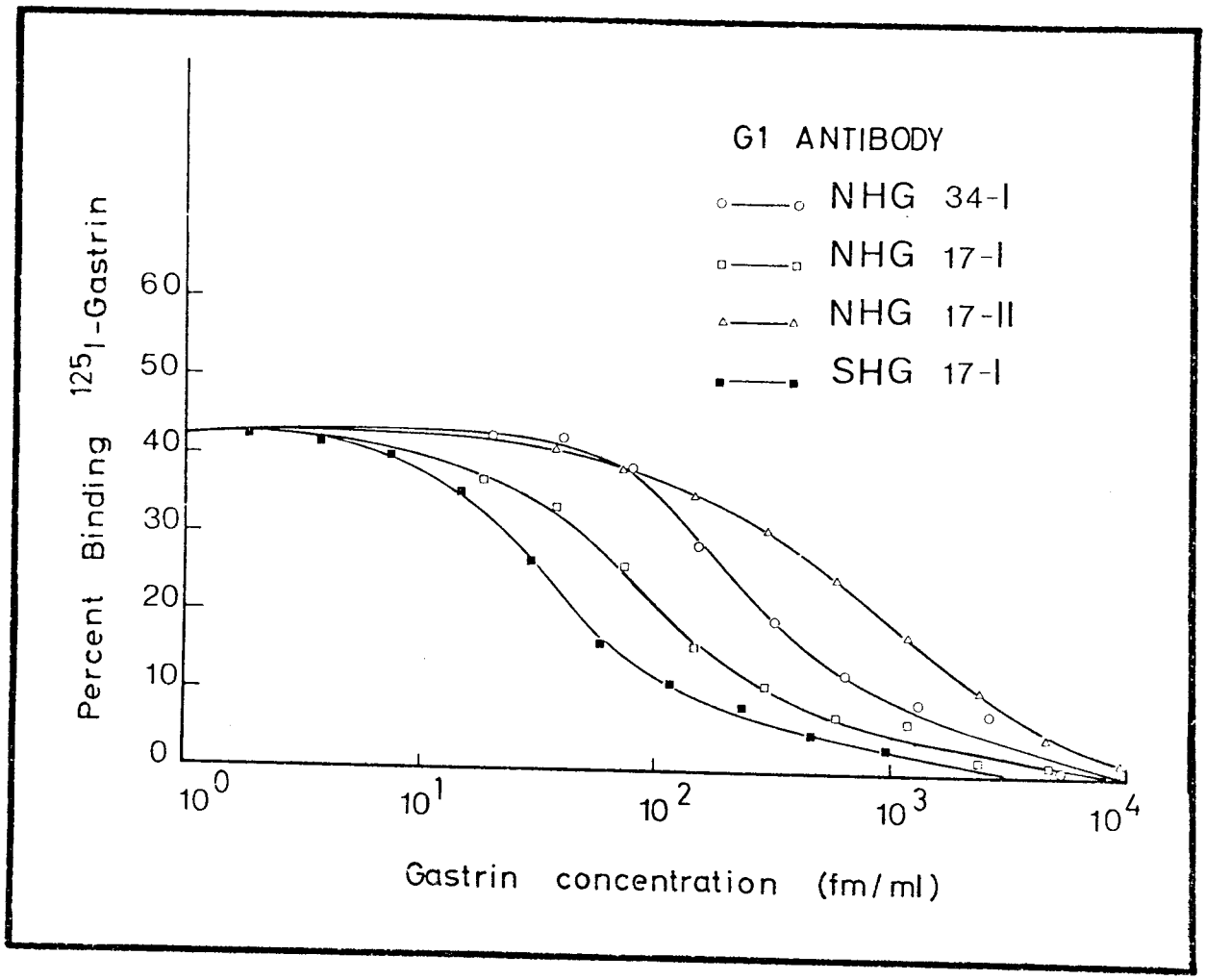


Fig. (4.1.10.) The relative abilities of natural human gastrin G-34 I, G-17 I, G-17 II and synthetic human gastrin G-17 I to displace ^{125}I-labelled synthetic human gastrin G-17 I from antiserum G 1.

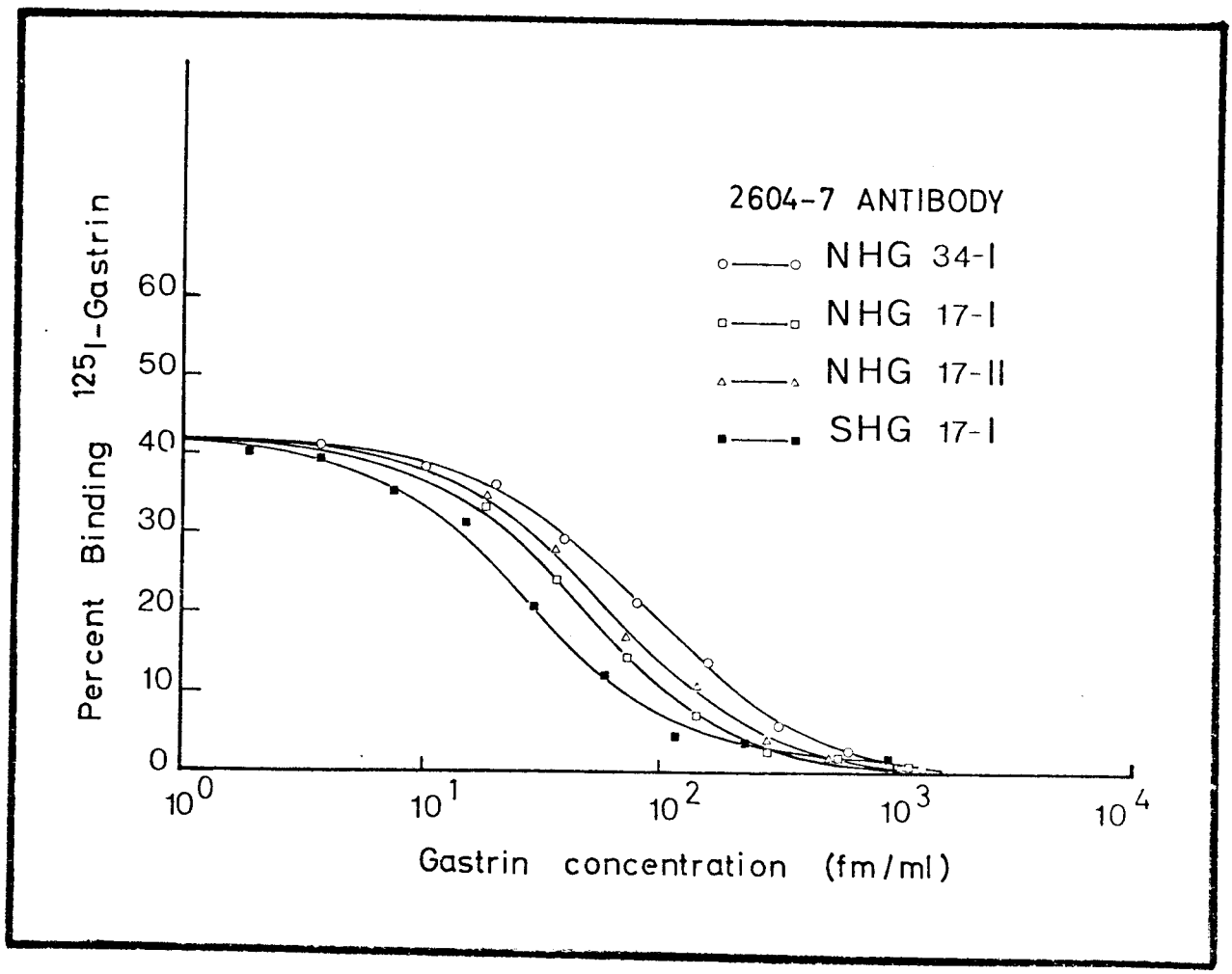


Fig. (4.1.11.) The relative abilities of natural human gastrin G-34 I, G-17 I, G-17 II and synthetic human gastrin G-17 I to displace ^{125}I-labelled synthetic human gastrin G-17 I from antiserum 2604-7.

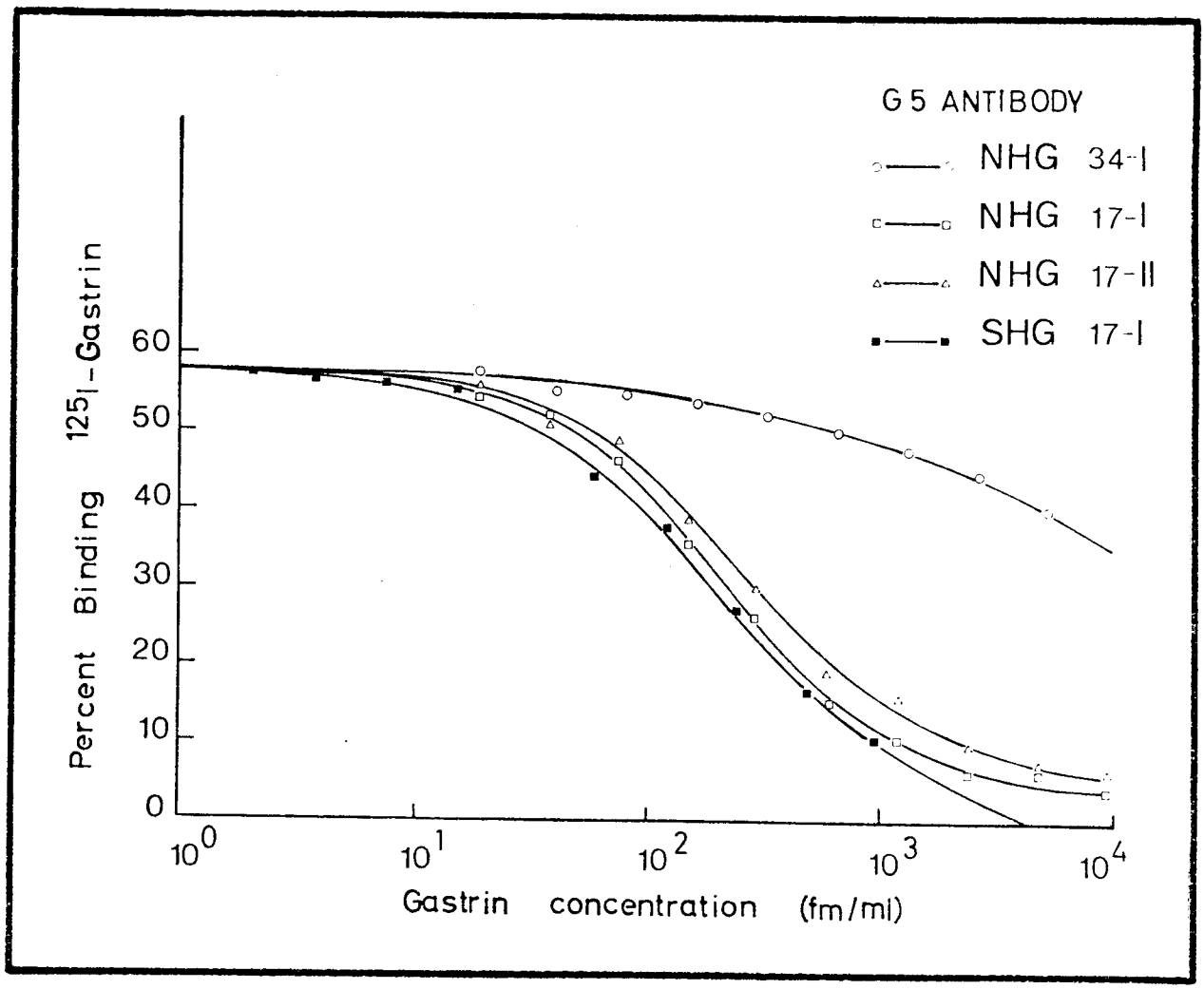


Fig. (4.1.12.) The relative abilities of natural human gastrin G-34 I, G-17 I, G-17 II and synthetic human gastrin G-17 I to displace ^{125}I-labelled synthetic human gastrin G-17 I from antiserum G 5.

TABLE (4.1.4)

Relative Displacement (ID_{50}) of Antibody-Bound ^{125}I-Gastrin by Various Natural and Synthetic Human Gastrins.

Gastrin Type	ID_{50} as a percentage of ID_{50} of SHG-17 I		
	G 1	2604-7	G 5
SHG-17 I (CEA-IRE Sorin)	100%	100%	100%
*NHG-34 I	14,3%	31,3%	0,72%
*NHG-17 I	41,7%	61,9%	78%
*NHG-17 II	5,2%	46,4%	61,9%
SHG-17 I (I.C.I.)	46%	-	64%

*Kindly supplied by Dr. M.Grossman, U.C.L.A., V.A. Hospital, Los Angeles.

types of gastrin could be distinguished. NHG-34 I cross-reacted in both assay systems. Failure of antiserum G 5 to distinguish between sulphated and non-sulphated gastrins is probably due to the fact that the antigenic site does not include the tyrosine residue in position 12 of the heptadecapeptide.

Another surprising finding was the complete lack of recognition of NHG-34 I by antiserum G 5, since earlier chromatographic data had suggested that G 5 measured a gastrin species larger than G-17, which had been thought to be G-34 gastrin (Kalk, Vinik et al., 1973). Possibly the larger gastrin species measured in earlier chromatographic profiles with G 5 antiserum were big gastrins bearing a sequence resembling the (1-13) or (1-5) sequence of the heptadecapeptide. The preparations of natural human gastrin 17 I and II and synthetic human gastrin 17 I all cross-reacted to approximately the same degree with G 5 antiserum, but at higher concentrations of gastrin standard than with antisera G 1 and 2604-7. This movement of the displacement curve to the right with antiserum G 5 is an indication of the lower affinity of this antiserum.

In view of the finding that antiserum G 1 recognised only non-sulphated gastrin, whereas antiserum 2604-7 recognised both sulphated and non-sulphated gastrins, a linear regression analysis of gastrin levels measured in the same samples with these two antisera was carried out. Samples used were human serum samples, extracts prepared by boiling biopsies collected from the gut of mammals, and sera collected from various vertebrate species, as shown in table (4.1.5). Regression analysis of 47 pairs of values gave a correlation coefficient of 0,796 ($p < 0,01$) with an equation for the straight line of $y = 1,048x + 104,8865$. This very good correlation suggests that the two antisera measure similar things. The y-intercept of 104,8865 indicates that antiserum 2604-7 measures approximately 105 pg/ml more than does antiserum G 1 in the same sample. In the light of the cross-reaction studies discussed, it is feasible to

suggest that the extra peptide(s) measured by antiserum 2604-7 are the sulphated forms of gastrin. This material may occur consistently or inconsistently in the different tissue extracts and serum samples.

TABLE (4.1.5)

Gastrin Levels Measured in the Same Samples with Antisera G 1 and 2604-7

Units are pg/ml

Human Serum Samples

G 1	2604-7
220	510
16	117
13	34
37	164
8	35
75	121
49	225
41	159
266	195
44	169
114	335
80	312
6	30
25	47
50	271
20	43
140	245
204	476
194	256
10	27
17	76
31	274
34	55
610	572
191	412

Mammalian Gut Extract Samples

G 1	2604-7
81	172
55	188
59	226
87	188
182	717
205	517
792	826
190	517
149	340
45	155
86	168
27	69

Vertebrate Serum Samples

G 1	2604-7
38	91
95	230
16	39
64	67
67	58
87	188
44	80
19	44
29	46
51	43

$r = 0,7963 \qquad p < 0,01$

Equation for linear regression $y = 1,0480x + 104,8865$

4 I(v) Identity of Gastrin in Biological Fluids

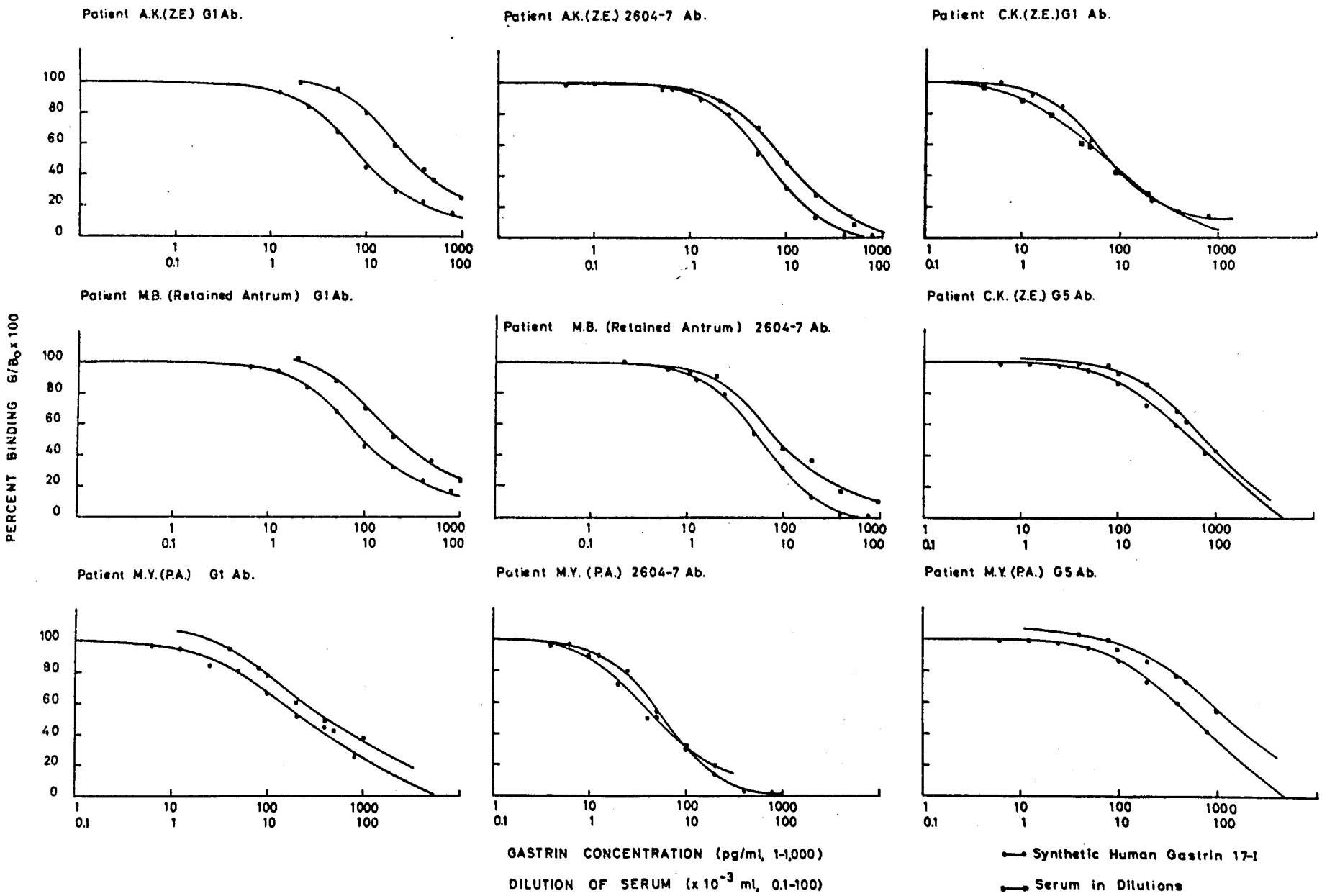
Figure (4.1.13.) shows the curves of displacement of antibody-bound labelled gastrin obtained upon serial dilution of sera drawn from patients with hypergastrinaemic conditions compared with that of synthetic human gastrin G-17 I, measured using three antisera. Serum from Zollinger-Ellison patient A.K. gave displacement curves parallel to that of the standard, using antisera G 1 and 2604-7, and the same result was found when serum from Zollinger-Ellison patient C.K. was investigated using antisera G 1 and G 5, suggesting that these sera contain material identical with, or similar to the gastrin standard. Displacement curves parallel to that of the standard were obtained on serial dilution of serum from patient M.B. with isolated retained antrum using antisera G 1 and 2604-7, providing evidence that heptadecapeptide gastrin is secreted by the portion of antrum remaining in this condition after incomplete gastrectomy. The elevated gastrin levels found in patient M.Y. with pernicious anaemia appear to be due to the presence of heptadecapeptide gastrin in the serum, since parallel displacement curves were obtained using all three antisera.

The curves of displacement of ^{125}I-labelled gastrin from antisera G 1 and G 5 obtained by serial dilution of pig serum exhibited parallelism with that of standard SHG-17 I, as can be seen in figures (4.1.14.) and (4.1.15.). This confirmed that serum gastrin levels could be measured in pig serum without any problems. Since porcine heptadecapeptide gastrin differs from human heptadecapeptide gastrin only by one amino acid residue in position 5 of the heptadecapeptide, as shown in figure (3.1.2.), and it was found that the antigenic site for antiserum G 1 did not include this residue, one would not expect this antiserum to discriminate between porcine and human gastrin. Antiserum G 5 appears not to distinguish between human and porcine gastrin, which suggests that although the antigenic determinant for G 5 antiserum probably lies in the (1-5)

region, substitution of the residue in position 5 does not significantly effect recognition of porcine gastrin by this antiserum.

No measurements of gastrin levels could be obtained in pig bile and gastrin added to bile could not be recovered in the assay. This may be due to interference by the bile salts in the assay.

Fig. (4.1.13.) The curves of displacement of antibody-bound ^{125}I-SHG-17 I obtained by serial dilutions of sera drawn from patients with Zollinger-Ellison syndrome (Z.E.), pernicious anaemia (P.A.) and isolated retained antrum, using antisera G 1, 2604-7 and G 5, compared with that of the synthetic human heptadecapeptide (SHG-17 I) standard.


Patient A.K.(Z.E.) G1 Ab.
Patient A.K.(Z.E.) 2604-7 Ab.
Patient C.K.(Z.E.)G1 Ab.
Patient M.B. (Retained Antrum) G1 Ab.
Patient M.B. (Retained Antrum) 2604-7 Ab.
Patient C.K. (Z.E.) G5 Ab.
Patient M.Y.(P.A.) G1 Ab.
Patient M.Y. (P.A.) 2604-7 Ab.
Patient M.Y.(P.A.) G5 Ab.
PERCENT BINDING B/B0 x 100
100
80
60
40
20
0
1
0.1
10
1
100
10
1000
100
GASTRIN CONCENTRATION (pg/ml, 1-1,000)
DILUTION OF SERUM (x 10-3 ml, 0.1-100)
Synthetic Human Gastrin 17-I
Serum in Dilutions

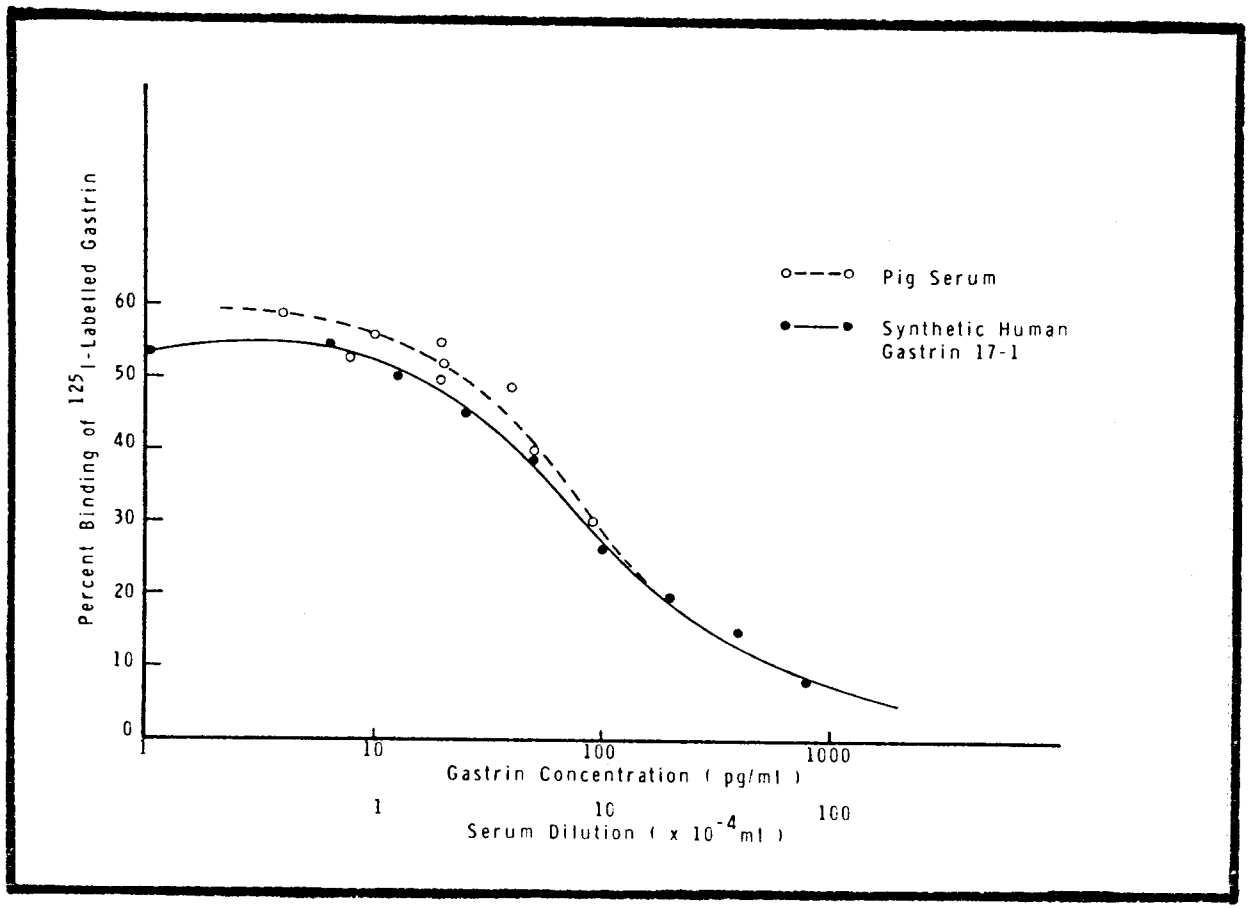


Fig. (4.1.14.) Displacement of G 1 antibody-bound labelled SHG - 17 I by serial dilutions of pig serum, compared with the SHG - 17 I standard.

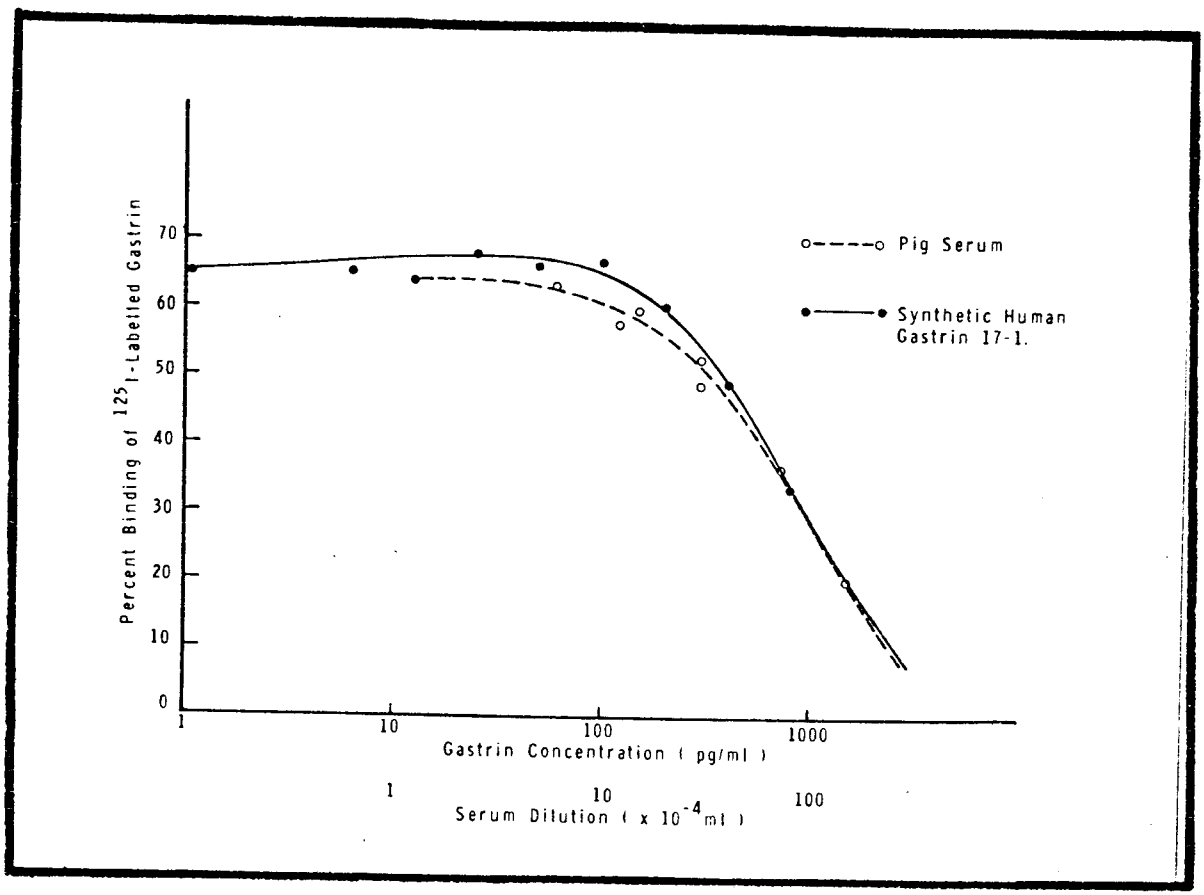


Fig. (4.1.15.) Displacement of G 5 antibody-bound labelled SHG - 17 I by serial dilutions of pig serum, compared with the SHG - 17 I standard.

4 I(vi) Recovery of Gastrin in Tissues

The gastrin levels measured after the treatments detailed in section 3 I(vi) are shown in table (4.1.6). The recovery of gastrin added to glassware for the study was 93,8% and the recovery of gastrin after the other treatments was assessed as a percentage of this concentration, 150 pg/ml.

Boiling led to a loss of 23,3% of immunoreactivity. No gastrin was detected in straight muscle extract before or after boiling, confirming that there were no factors in these extracts which interfered in the assay and produced erroneous values. The slightly higher recovery of gastrin after boiling muscle before addition of gastrin (48%) as opposed to direct addition of gastrin to muscle followed by boiling (43,3%) could indicate the presence of tissue enzymes which degrade gastrin, but such a small difference in recovery after these two treatments may be insignificant. Trasylol had no effect on gastrin measurement when added to muscle extract before boiling. Recovery of gastrin from boiled muscle with added Trasylol was 38%, which differs only slightly from a recovery of 43,3% for the same treatment without Trasylol, suggesting that Trasylol probably had no effect on gastrin in muscle extracts.

TABLE (4.1.6)

Recovery of Gastrin after Various Treatments, Measured with Antiserum G 1

Each extract contained 800 pg gastrin in 1 ml made up to a final volume of 5 ml, to give an expected concentration of 160 pg/ml.

	Gastrin measured
(a) Gastrin in water before boiling	150 pg/ml
(b) Gastrin in water after boiling	115 pg/ml
(c) Muscle in water, before gastrin added	undetectable
(d) Muscle extract after gastrin added and boiled	65 pg/ml
(e) Muscle extract after boiling before gastrin added	undetectable
(f) Muscle extract after boiling, gastrin added, boiled again	72 pg/ml
(g) Muscle + Trasylol + gastrin before boiling	150 pg/ml
(h) Muscle + Trasylol + gastrin after boiling	57 pg/ml

Gastrin loss on addition to glassware	$\frac{150}{160} \times 100 = 93{,}8\%$ recovered
Effect of boiling on gastrin per se	$\frac{115}{150} \times 100 = 76{,}7\%$ recovered
Recovery of gastrin from boiled muscle	$\frac{65}{150} \times 100 = 43{,}3\%$ recovered
Effect of inactivating muscle tissue enzymes by boiling before addition of gastrin	$\frac{72}{150} \times 100 = 48\%$ recovered
Effect of Trasylol on gastrin measurement	$\frac{150}{150} \times 100 = 100\%$ recovered
Effect of Trasylol on recovery of gastrin from boiled muscle	$\frac{57}{150} \times 100 = 38\%$ recovered

4 I(vii) Gastrin Levels in Serum and In Vivo Studies

The mean basal serum gastrin level determined in 27 normal control subjects was 48,5 ± 2,4 pg/ml (± S.E.M.), with a range from 26 to 77 pg/ml. Figure (4.1.16.) shows serum gastrin levels in patients divided according to age, ethnic group and sex. There were no significant differences in the serum gastrin levels in the three different ethnic groups (t-test). Serum gastrin levels in females were significantly higher than in males ($p < 0,025$, t-test). There was no obvious relationship between serum gastrin levels and advancing age, although the highest levels were found in the age group 51-60 years. The difference between the gastrin levels in this age group and in the age group 31-40 years, which were the lowest, was not statistically significant when tested by the t-test.

Serum gastrin levels in various disease conditions are shown in figure (4.1.17.). The grossly elevated levels in pernicious anaemia and Zollinger-Ellison syndrome patients are apparent, with serum gastrin concentrations up to ten- or a hundred-fold greater than in normal subjects in some instances. There was no significant difference between gastrin levels in normals and patients with hiatus hernia (H.H.), gastric ulcer (G.U.) or moderate rheumatoid arthritis (R.A.). Gastrin levels in gastric carcinoma (gastric Ca) and duodenal ulcer (D.U.) were significantly elevated above the normal value ($p < 0,05$, t-test).

Although the mean serum gastrin level in 11 patients with moderate rheumatoid arthritis was higher than that in controls and patients with mild rheumatoid arthritis, as shown in table (4.1.7), the differences were not significant, because one patient in this group had much higher serum gastrin levels than the others, producing a falsely elevated mean value. When this patient was excluded from the group the mean serum gastrin level in 10

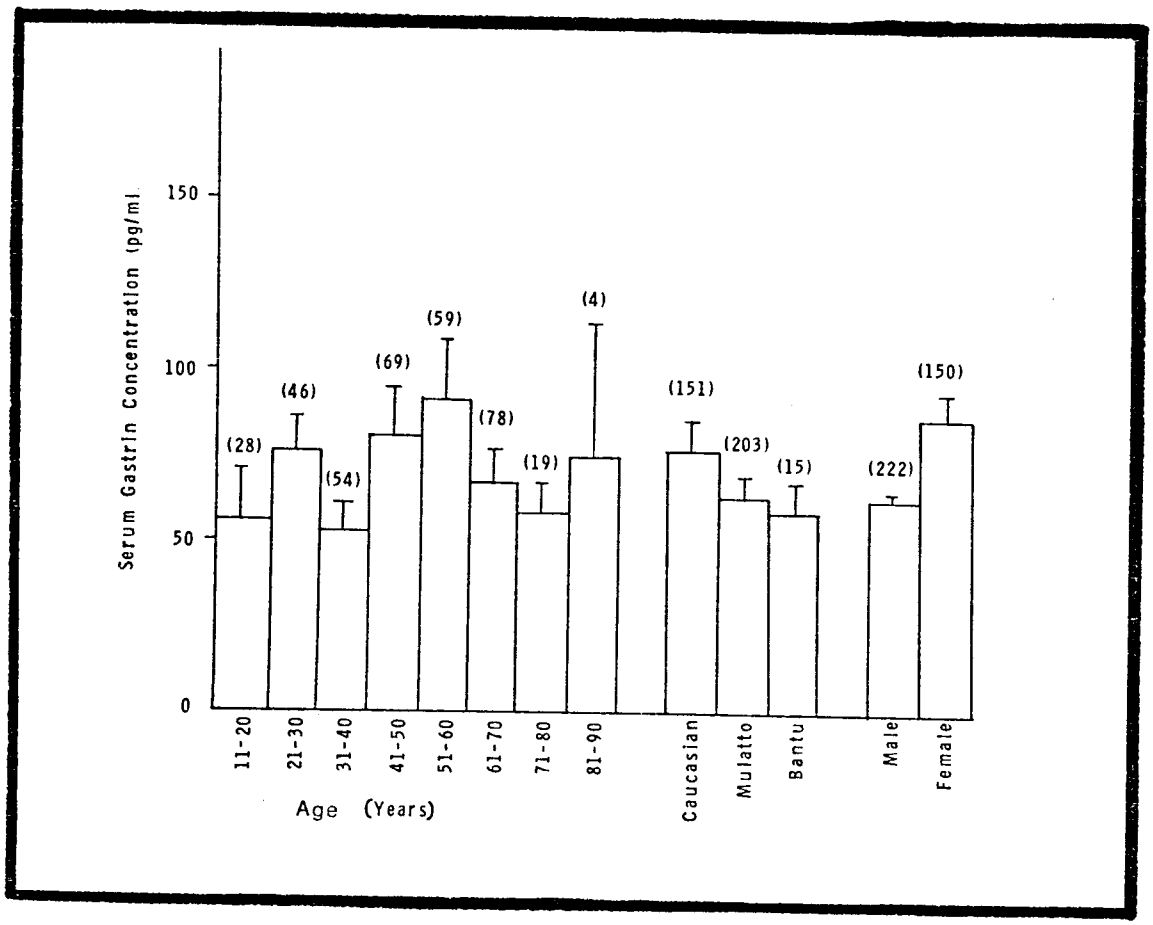


Fig. (4.1.16.) Serum gastrin levels, measured using G 1 antiserum, in a large group of patients classified according to age, race and sex. Figures in parentheses indicate the number of samples assayed in each category.

Vertical bars indicate S.E.M.

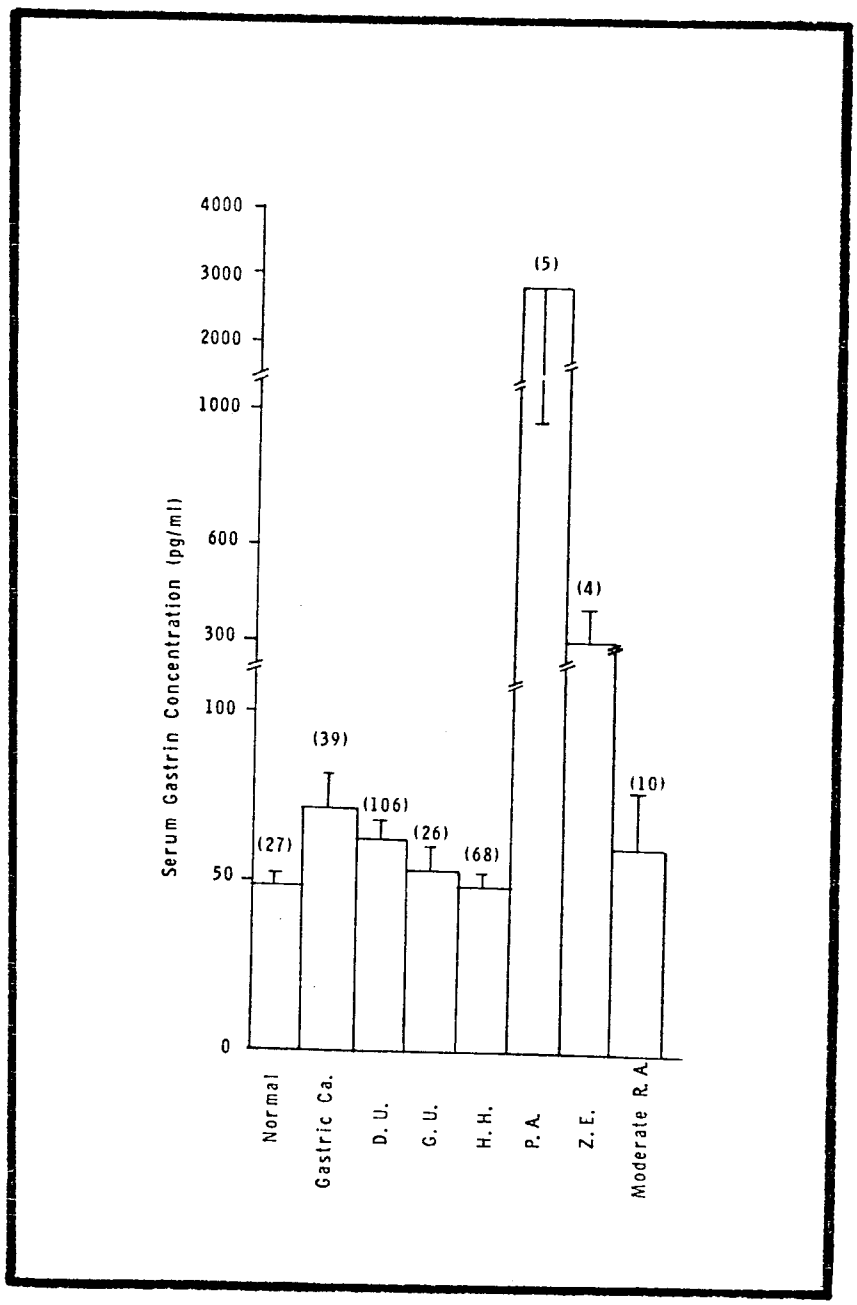


Fig. (4.1.17.) Serum gastrin levels, measured using G 1 antiserum, classified according to various disease conditions. Figures in parentheses indicate the number of samples assayed in each category.

Gastric Ca : Gastric Carcinoma
G.U. : Gastric Ulcer
P.A. : Pernicious Anaemia
Moderate R.A. : Moderate Rheumatoid Arthritis
D.U. : Duodenal Ulcer
H.H. : Hiatus Hernia
Z.E. : Zollinger-Ellison Syndrome

Vertical bars indicate S.E.M.

TABLE (4.1.7)

Serum Gastrin Levels in Rheumatoid Arthritis (pg/ml)

	$\overline{X}$	S.E.M.	n
Moderate Rheumatoid Arthritis	112	55,4	11
Mild Rheumatoid Arthritis	81	19,5	24
Gout	52	11,5	4
Reiter's Syndrome	38	7,4	4
Osteoarthritis	54	11,9	11

patients with rheumatoid arthritis was 59 pg/ml, as indicated in figure (4.1.17.). The mean serum gastrin level in 24 patients with mild rheumatoid arthritis was 81 pg/ml and in the related arthritic conditions values were not elevated. Thus there appears to be no relation between serum gastrin levels and the degree of severity of rheumatoid arthritis.

Antrectomised subjects had undetectable gastrin levels and the mean basal gastrin level in 6 patients who had had a truncal vagotomy and partial antrectomy was 26,4 $\pm$ 3,4 pg/ml ($\pm$ S.E.M.), which was significantly less than that in control subjects ($p < 0,001$), as shown in table (4.1.8) (Vinik, Kalk, Dent, Barbezat, Grant and Bank, 1975).

Intravenous infusion of arginine into control subjects for 30 minutes caused a sharp early rise in serum gastrin levels to almost three times the basal concentration after 15 minutes. Because the peak values in some patients were found later than 15 minutes the mean maximum increment over the basal value was 389,7%. The levels returned to basal between 45 and 60 minutes after the start of the arginine infusion, as shown in figure (4.1.18.) (Vinik, Kalk et al., 1975).

There was no rise in serum gastrin concentration with orally administered arginine. Oral oxo produced a twofold increase in gastrin levels above the basal concentration in normal subjects ($p < 0,05$) with the peak value occurring at 45 minutes and a sustained plateau type of response (figure (4.1.18.)) (Vinik, Kalk et al., 1975).

Intravenous arginine failed to elicit a response in antrectomised patients or in vagotomised and antrectomised subjects. The serum gastrin responses to these stimuli appear in table (4.1.8).

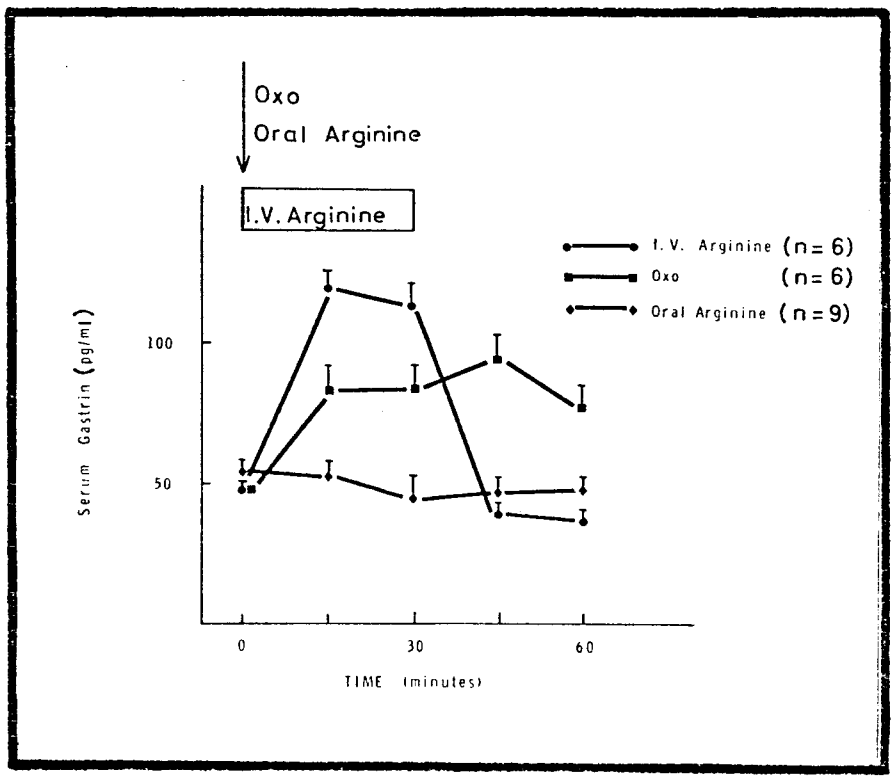


Fig. (4.1.18.) Serum gastrin responses to intravenous arginine infused at a rate of 10 mg/kg/min for 30 minutes, a 30 g dose of oral arginine, and 4 cubes of Oxo in 150 ml warm water given orally. (Oxo is a commercial ox-muscle extract rich in glutamic acid and sulphur amino acids with a smaller proportion of hydrolyzed protein and yeast extract.)

TABLE (4.1.8)

Serum Gastrin Responses to Various Stimuli

Subjects	Test		Basal Gastrin (pg/ml) Mean ± S.E.M.	Maximum % of Basal
Controls	I.V. Arginine	n=6	48,3±2,14	389,7±154,1
	Oral Arginine	n=9	44,3±2,8	108,0±11,4[c,d]
	Oral Oxo	n=6	49,9±2,19	190,1±16,7[d]
Antrectomy	I.V. Arginine	n=3	< 1,6[a]	0[b]
Vagotomy	I.V. Arginine	n=6	26,4±3,4[a]	43,9±15,0[b]

a. Basal concentration in vagotomy and antrectomy subjects is significantly ($p < 0,001$) less than that in all control groups.

b. Significant impairment of gastrin responses to I.V. arginine.

c. Oral arginine causes less ($p < 0,001$) gastrin release compared with I.V. arginine.

d. Oxo causes less of a rise than I.V. arginine ($p < 0,05$) but greater than oral arginine ($p < 0,05$).

Figure (4.1.19.) shows the serum gastrin response of 8 pigs to eating creep meal. The basal serum gastrin level measured in 11 pigs was 57 ± 16 pg/ml (± S.D.) with a range of 31-118 pg/ml (Arnot, Vinik, Grant, Hickman, Terblanche and Louw, 1974). The postprandial serum gastrin measured in 8 pigs was 65 ± 18 pg/ml at 30 minutes, 72 ± 22 pg/ml at 60 minutes and thereafter remained constant up to 120 minutes. Values quoted and shown in the figure are the mean ± S.D. The 30-minute value did not differ significantly from the basal level, but the 60-minute and subsequent values were significantly increased ($p < 0,05$). Basal serum gastrin values in pigs thus appeared to be similar to those in humans, although the rise in gastrin levels after eating was relatively small in pigs compared with that in humans, and remained elevated for a longer period.

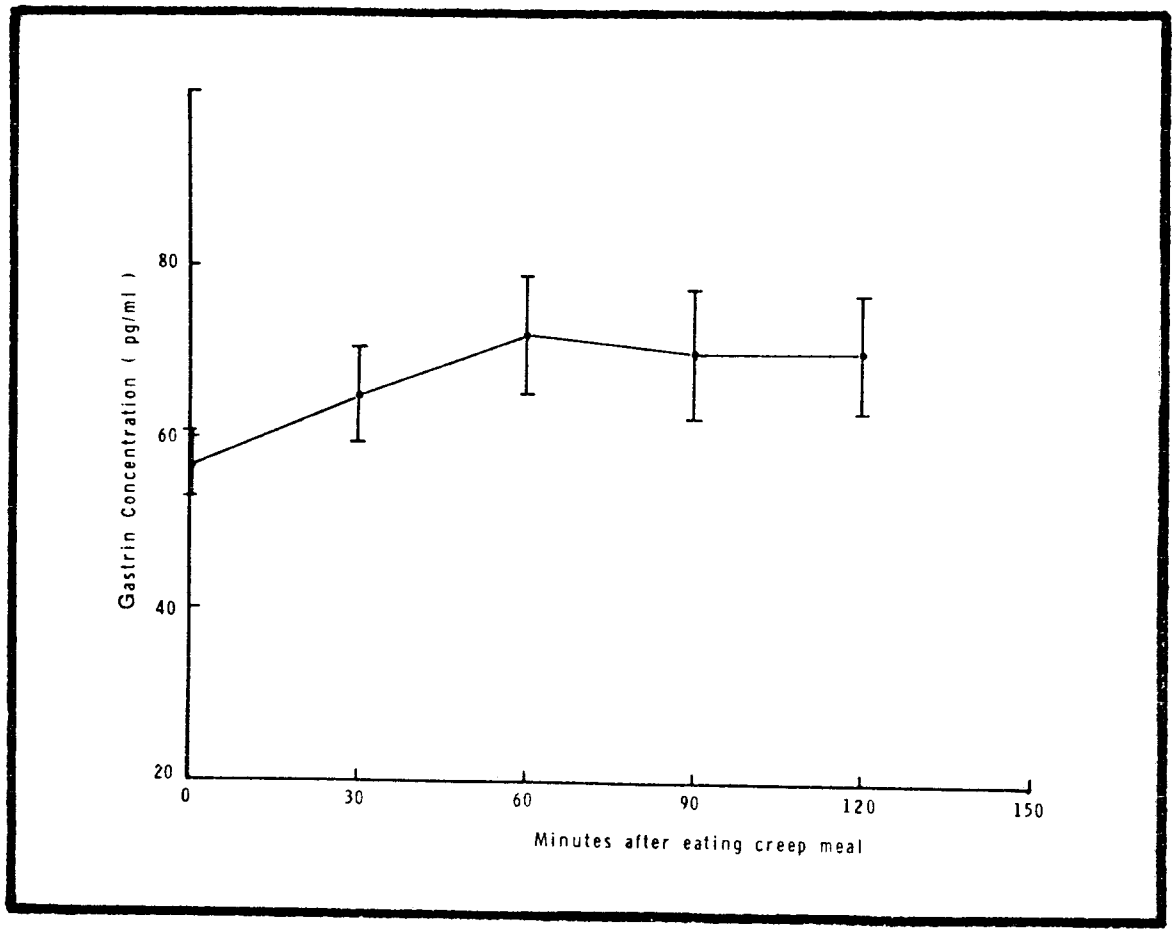


Fig. (4.1.19.) Serum gastrin responses to a creep meal (a mixture of corn meal, bran, pollard, fish meal and ground nuts; 3 060 cal/kg; 18% protein) in 8 pigs. Vertical bars indicate S.D.

Section II Chromatographic Characteristics of Gastrins

4 II(i) Heterogeneity of Gastrin in Serum

The chromatographic elution profile of gastrin in serum drawn from patient K.V., with pernicious anaemia, is shown in figure (4.II.1.). A small amount of a component eluting in the void volume was followed by two apparently paired components eluting between the albumen and ^{125}I-gastrin markers. Another paired component eluted between the ^{125}I-gastrin and Vitamin B_{12} markers, and this was followed by a small amount of low molecular weight material eluting in the region of the salt volume, as indicated by the ^{22}Na marker.

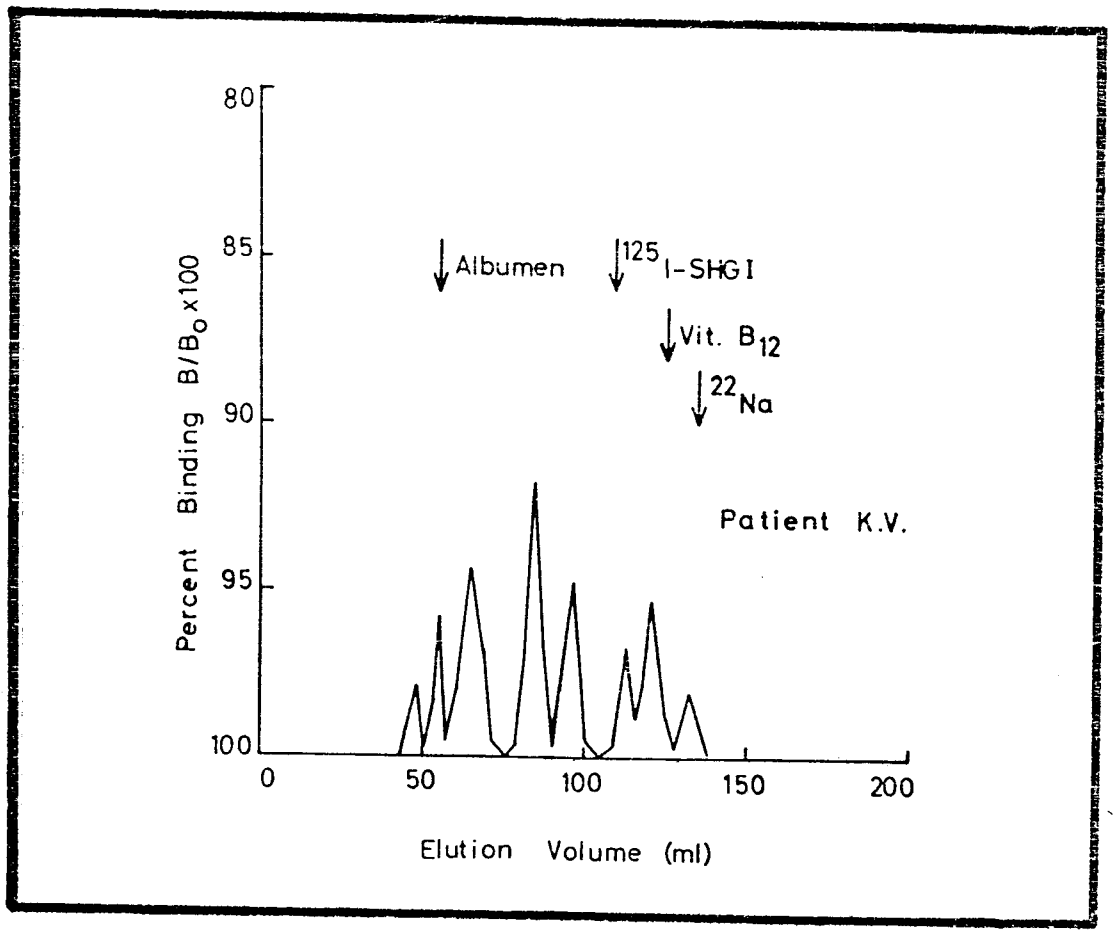


Fig. (4.II.1.) Chromatographic elution profile of immunoreactive gastrin measured with G 5 antiserum in serum drawn from pernicious anaemia patient K.V.

4 II(ii) Antibody Recognition of Different Gastrin Standards

Fractionation of the natural gastrin standards was carried out using the molecular weight markers blue dextran and Vitamin B_{12} as internal standards, so that the elution volumes could be plotted as a percentage of the elution volume between these two markers. This allowed comparison of elution patterns obtained with natural gastrin standards with elution patterns obtained on chromatography of various serum samples.

Figures (4.II.2.), (4.II.3.) and (4.II.4.) show the chromatographic profiles obtained with the natural gastrin standards G-34 I, G-17 I and G-17 II, using antisera G 1, 2604-7 and G 5 respectively. The pattern obtained with antiserum G 1 shows that this antiserum detects the natural G-34 I and G-17 I gastrin species, but fails to measure the sulphated heptadecapeptide gastrin, G-17 II. In contrast to this, all three species of natural gastrin tested were measured using antiserum 2604-7, as shown in figure (4.II.3.). Antiserum G 5 failed to detect the natural G-34 I gastrin to any noticeable extent, but reacted equally well with the NHG-17 I and 17 II peptides. These findings correlate with the poor cross-reaction of antiserum G 1 with G-17 II and of antiserum G 5 with G-34 I shown in section 4 I(iv). The ^{125}I-gastrin marker coincided with the natural gastrin type I in each case, as would be expected since the labelled preparation was prepared using synthetic human gastrin G-17 I. The sulphated heptadecapeptide gastrin eluted before the non-sulphated type in each fractionation, suggesting that the presence of the sulphate group on Tyrosine-12 significantly alters the behaviour of the hepta-decapeptide on Sephadex column chromatography. The fact that ^{125}I-labelled gastrin elutes in the same position as the natural peptide, as shown in figures (4.II.2.), (4.II.3.) and (4.II.4.) suggests that the position of the labelled marker ^{125}I-gastrin in earlier fractionations gives a close representation of the elution position of the natural heptadecapeptide.

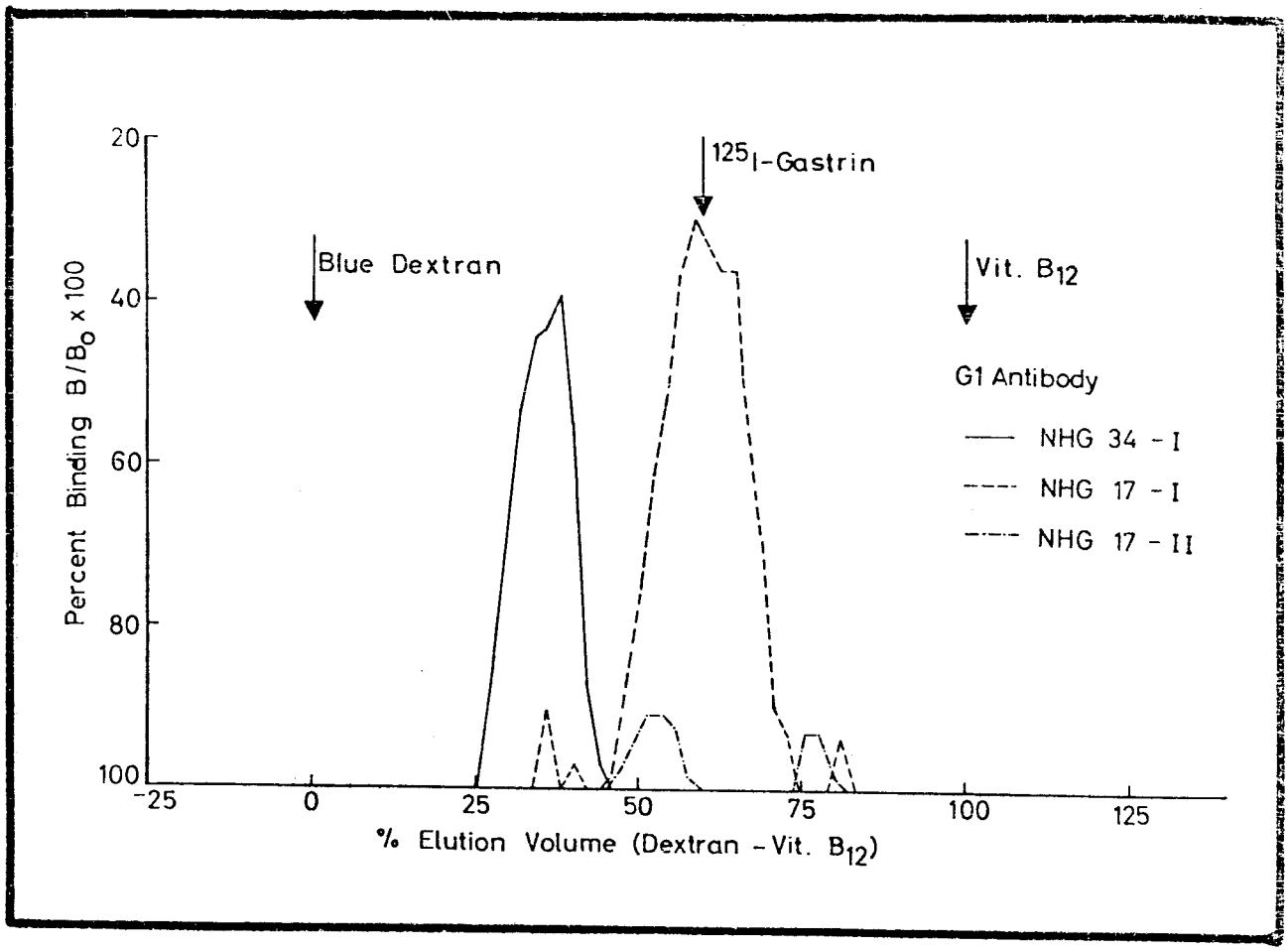


Fig. (4.11.2.) Elution profiles obtained on chromatography of natural human gastrins G-34 I, G-17 I and G-17 II using antiserum G 1.

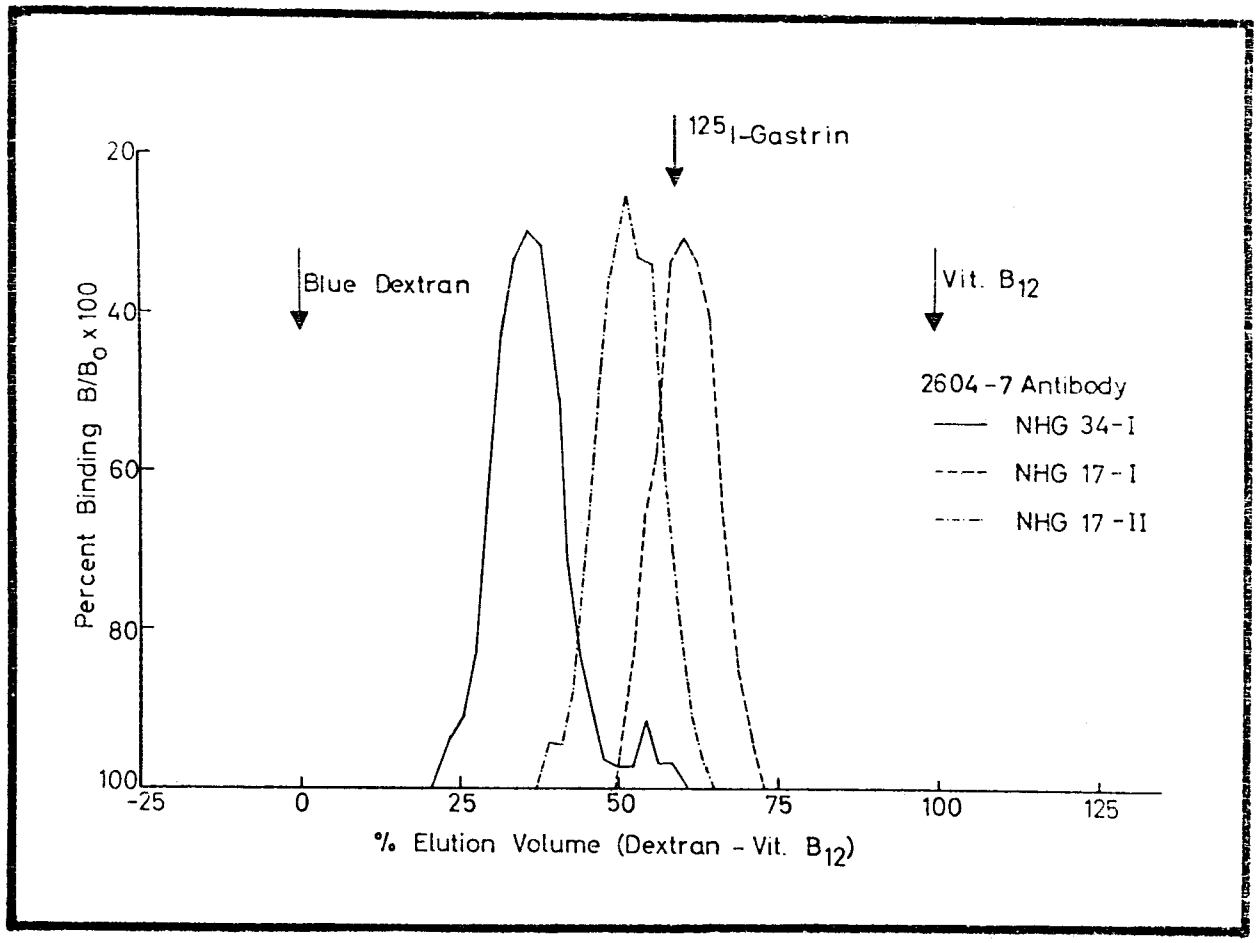


Fig. (4.11.3.) Elution profiles obtained on chromatography of natural human gastrins G-34 I, G-17 I and G-17 II using antiserum 2604-7.

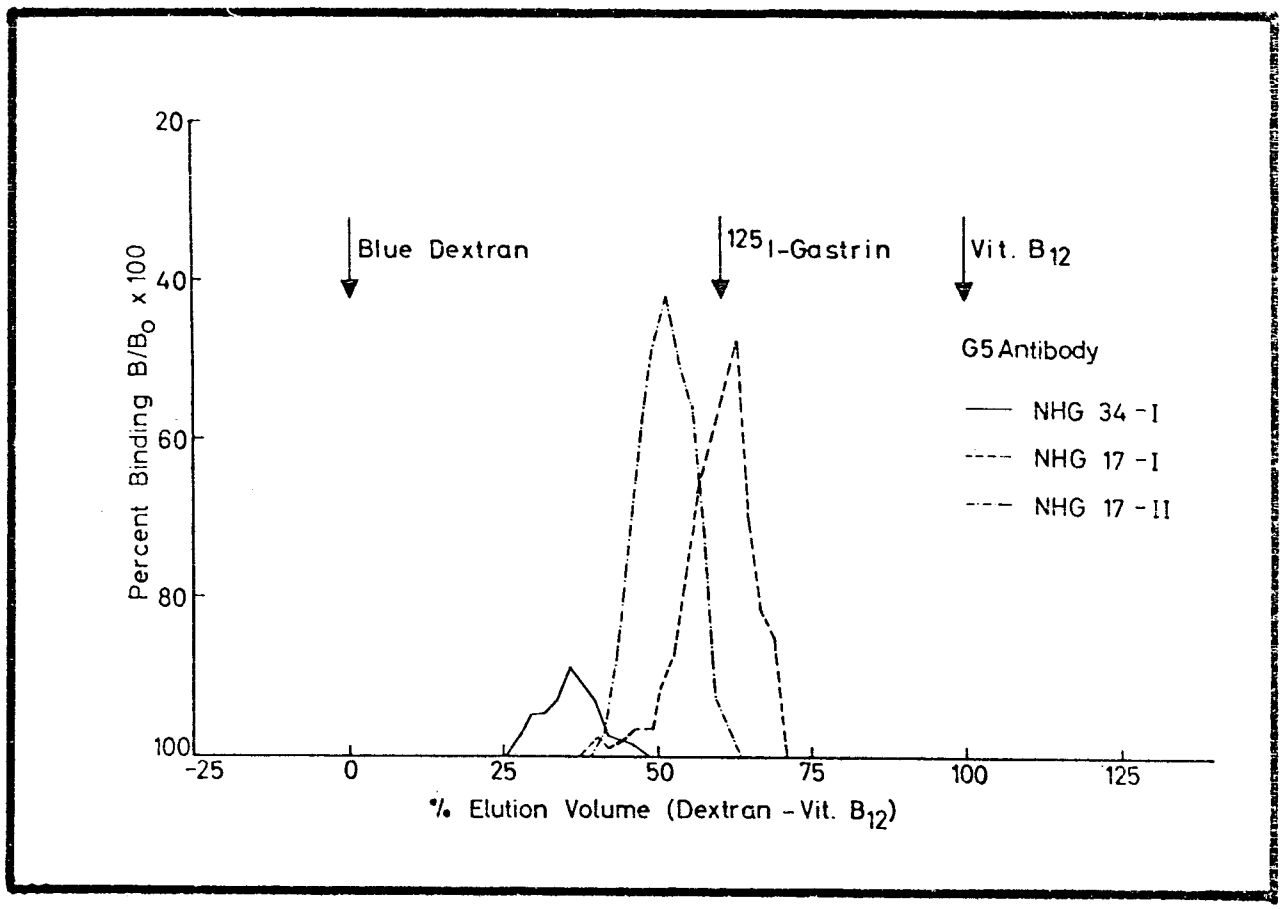


Fig. (4.II.4.) Elution profiles obtained on chromatography of natural human gastrins G-34 I, G-17 I and G-17 II using antiserum G 5.

Section III Tissue Distribution of Gastrin in Humans, Mammals and Invertebrates, and Heterogeneity of Gastrin in Human Tissue Extracts

4 III(i) Human Tissue Extracts

The highest concentration of gastrin was found in the extracts prepared from the human antral biopsies, which had a mean gastrin content of 2 034 ± 50,76 pg/mg total protein (± S.E.M.), or 1 420 ± 35,45 pg/mg dry weight of tissue. Very high gastrin levels were also found in the duodenal cap and in the biopsies from the second part of the duodenum, which had gastrin contents of 1 432 ± 392,56 pg/mg total protein, and 552 ± 78,20 (± S.E.M.) pg/mg total protein respectively. These measurements were made with antiserum G 1, which detects almost exclusively the non-sulphated gastrin species. The gastrin content of all the biopsies taken from the human expressed relative to the total protein and per mg dry weight of tissue is shown in table (4.III.1), and is illustrated diagrammatically in figure (4.III.1.).

The elution profiles following chromatography of the antral and duodenal extracts prepared from biopsies collected from a control subject, measured with antiserum G 5, are shown in figures (4.III.2.) and (4.III.3.). In both figures the peak preceding the albumen marker is thought to represent "big big" gastrin (Yalow and Berson, 1972) and the next peak following the albumen is presumed to be component I (Rehfeld, 1972; Rehfeld and Stadil, 1973a; Rehfeld, Stadil et al., 1974). The three paired peaks following this in both figures represent big gastrin (Yalow and Berson, 1970b; 1971a; Berson and Yalow, 1971), heptadecapeptide gastrin (Gregory, Tracy and Grossman, 1966), and mini-gastrin (Kalk, Vinik et al., 1973; Rehfeld, Stadil et al., 1974) respectively, reading in the direction of increasing elution volume (Vinik,

TABLE (4.III.1)

Gastrin Levels Measured in Gut Extracts from Mammalian Species Expressed per mg Dry Weight and per mg Total Protein, Measured with Antisera G 1 and 2604-7.

All Values are expressed as (pg/mg).

		Rat		Rabbit		Guinea Pig		Dog		Pig	
		Dry Wt.	Total Protein	Dry Wt.	Total Protein	Dry Wt.	Total Protein	Dry Wt.	Total Protein	Dry Wt.	Total Protein
Fundus	G 1	568,3	778	0,44	0,95	0,24	0,67	0,41	2,24	1,76	7,88
	2604-7	853	1168	1,75	3,72	0,51	1,41	0,66	3,63	6,95	31,1
Antrum	G 1	912,4	-	785	2 270	960	6 147	6 130	22 700	15 500	61 480
	2604-7	2 085	-	1 950	5 640	1 680	10 770	13 670	50 640	25 900	102 800
Duodenum	G 1	857,5	623,6	3,33	11,10	1,6	8,0	4,6	22,4	13,5	25,7
	2604-7	1 505	1 085	1,83	6,11	3,74	18,69	4,34	20,97	36,26	69,32
Jejunum	G 1	1,29	11,0	0,31	1,36	1,6	13,5	1,43	6,8	2,5	12,18
	2604-7	2,99	25,5	1,22	5,28	2,12	17,86	3,09	14,7	6,31	30,7
Ileum	G 1	0,11	0,7	0,81	3,68	0,15	0,57	0,65	1,78	9,3	34,7
	2604-7	0,84	5,12	2,38	10,79	0,44	1,58	1,38	3,78	9,72	36,17
Colon	G 1	0,25	0,56	13,7	38,5	0,38	0,95	0,41	1,47	1,99	9,12
	2604-7	0,67	1,48	35,35	99,66	1,66	4,15	1,42	5,04	5,41	24,81
Pancreas	G 1	0,39	0,94	0,39	-	0,092	-	0,47	1,6	0,72	2,4
	2604-7	1,76	4,19	1,54	-	0,72	-	1,77	6,09	1,66	5,50
Muscle	G 1	0,14	0,43	0,03	0,15					0,39	1,8
	2604-7	0,64	1,97	0,18	0,80					1,28	3,75

	Human (Means ± S.E.M.)	
	Dry Wt.	Total Protein
Cardia	32,2 ±8,71	44,7 ±12,12
Gastric Body	18,4 ±5,88	27,1 ±8,63
Antrum	1 420 ±35,45	2 034 ±50,76
Duodenal Cap	970,4 ±265,96	1 432 ±392,56
Duodenum 2nd Part	386,9 ±54,86	551,6 ±78,20

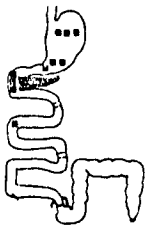
Human tissues measured with G 1 antiserum only. (n=4)

The value for adsorption of tracer by charcoal in the absence of antibody was > 95% with all tissue extracts, indicating little non-specific adsorption of tracer to tissue extract and little inactivation of the tracer during incubation.

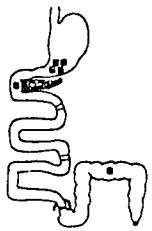
Fig. (4.III.1.) Diagrammatic representation of the gastrin content of tissues collected at sites along the alimentary canal in mammalian species, expressed as gastrin content relative to total protein content of the tissues; measurements made with antiserum G 1 appear on the left and those made with antiserum 2604-7 appear on the right hand side of the figure.

G1

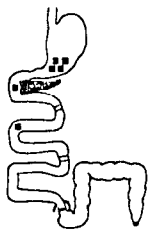
Rat



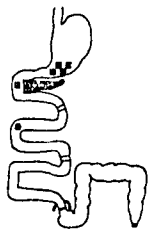
Rabbit



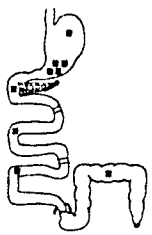
Guinea Pig



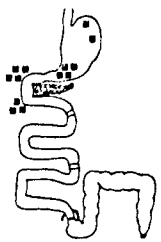
Dog



Pig

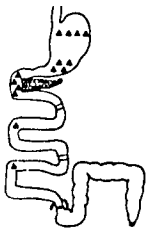


Human

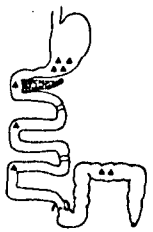


2604-7

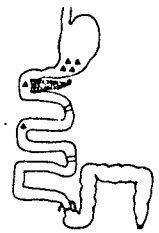
Rat



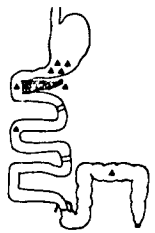
Rabbit



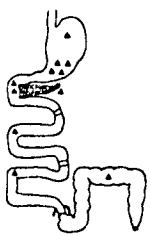
Guinea Pig



Dog



Pig



KEY

5 - 50 pg/mg

50 - 500 pg/mg

500 - 1000 pg/mg

>1ng/mg

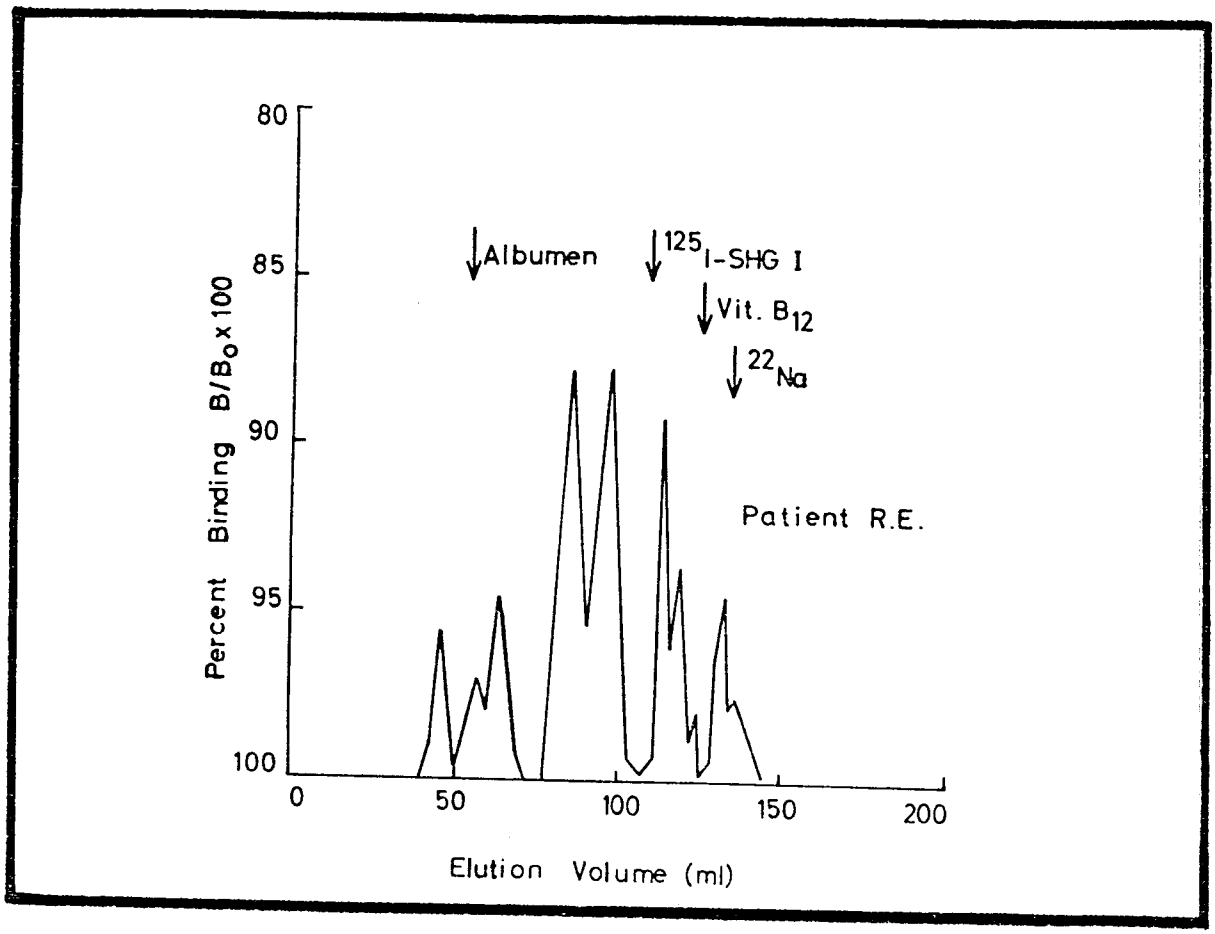


Fig. (4.III.2.) Elution profile of immunoreactive gastrin measured with antiserum G 5, obtained on chromatography of a boiled extract of human antrum. For details of chromatographic procedure used in this and subsequent figure see section 3.II. in text.

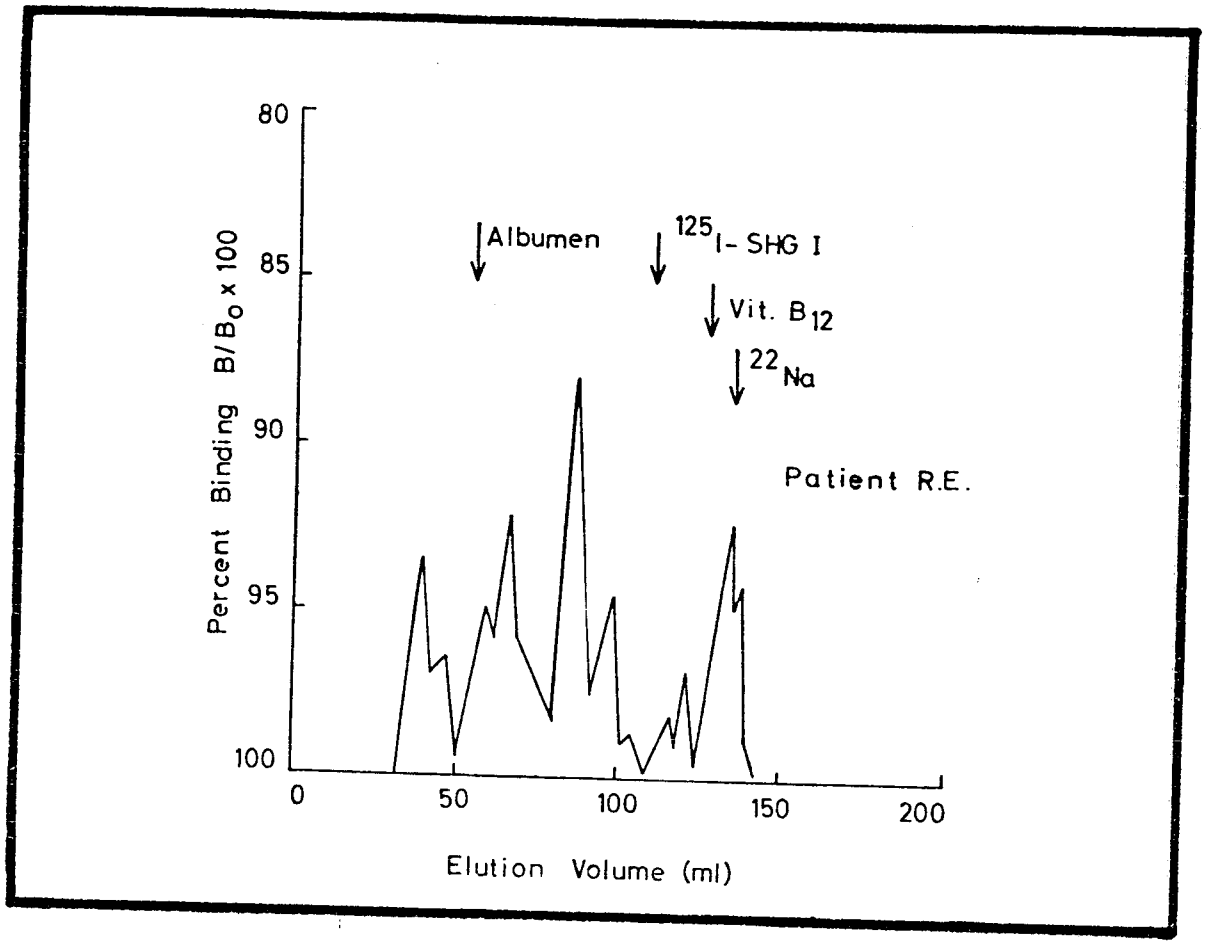


Fig. (4.III.3.) Elution profile of immunoreactive gastrin measured with antiserum G 5, obtained on chromatography of a boiled extract of human duodenum.

Grant et al., 1975). Each peak appears to consist of two components which are thought to be the sulphated (type II) and the non-sulphated (type I) gastrin species respectively. The amount of heptadecapeptide gastrin, indicated as eluting between the ^{125}I-SHG-I and Vitamin B_{12} molecular weight markers, was much greater in the antral than in the duodenal extract, as can be seen from the smaller peak in this region in figure (4.III.3.). This difference in antral and duodenal gastrin is borne out by the curves of displacement of ^{125}I-labelled gastrin from antiserum G 1 by dilutions of the antral and duodenal extracts, shown in figure (4.III.4.). The antral extract gave a displacement curve similar to that of the synthetic human gastrin standard G-17 I, whereas the duodenal extract did not, indicating that there was less heptadecapeptide-like material in this extract, as confirmed in the elution profile. Both extracts in dilutions produced curves of displacement of ^{125}I-labelled gastrin from antiserum G 5 parallel to that of the standard heptadecapeptide gastrin, as shown in figure (4.III.5.), due to the fact that G 5 antiserum measures a greater range of molecular gastrin species than does antiserum G 1.

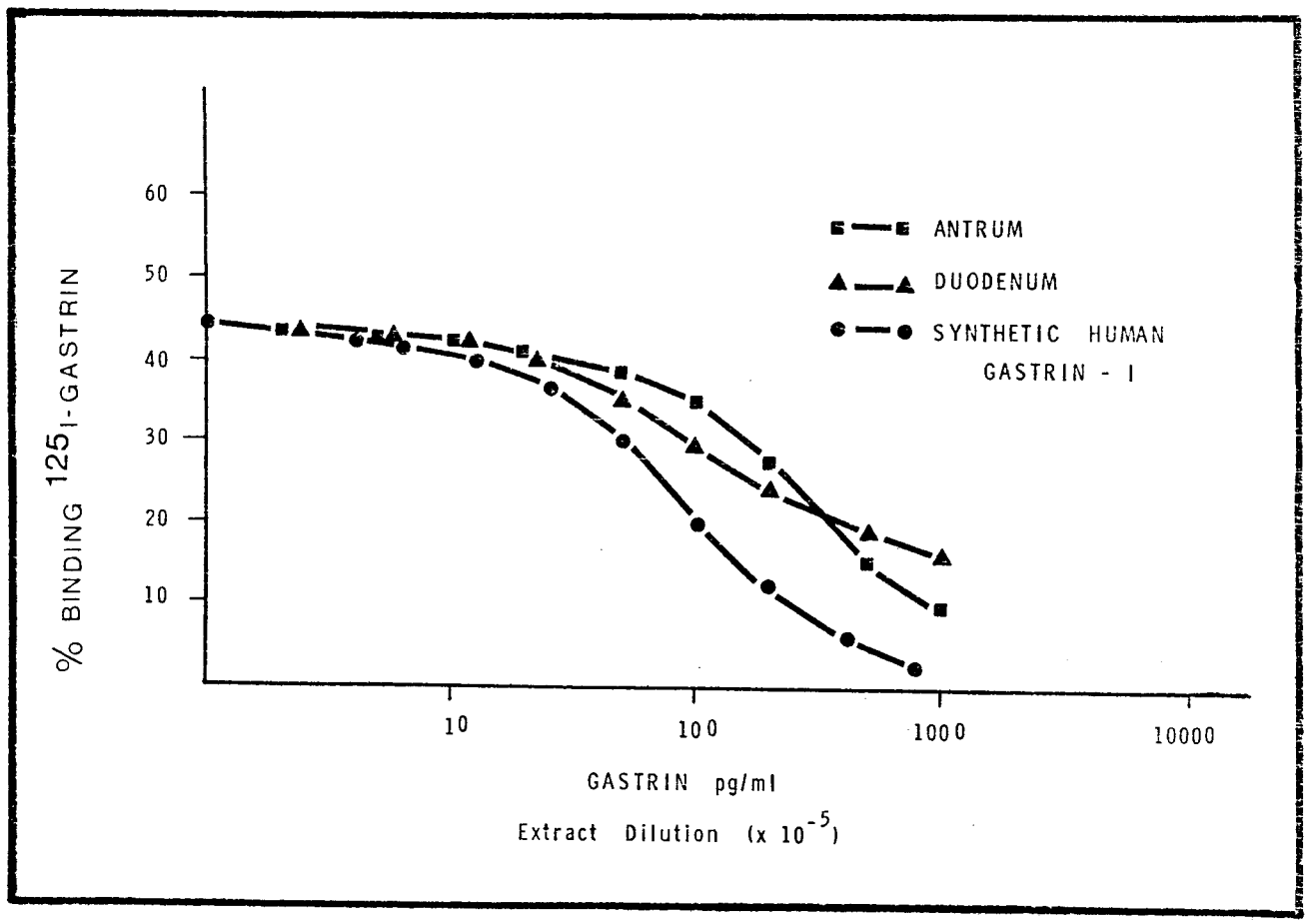


Fig. (4.III.4.) Displacement of ^{125}I-labelled synthetic human gastrin G-17 I from antiserum G 1 by synthetic human gastrin and extracts of human pyloric antrum and duodenum.

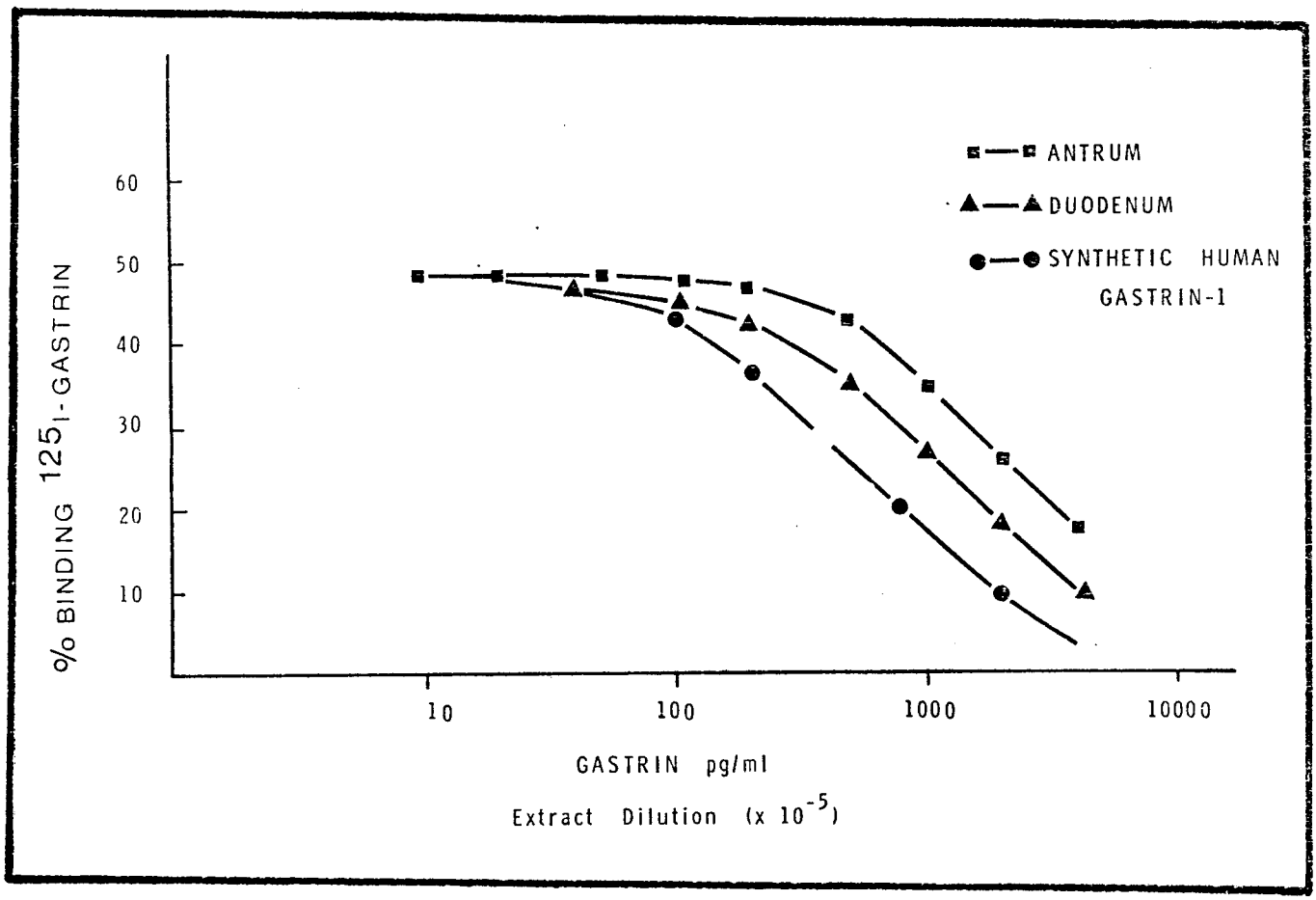


Fig. (4.III.5.) Displacement of ^{125}I-labelled synthetic human gastrin G-17 I from antiserum G 5 by synthetic human gastrin and extracts of human pyloric antrum and duodenum.

4 III(ii) Gastrin Measurements in Serum and Tissue Extracts from Vertebrates

The gastrin content of tissues collected from the gut of the rat, rabbit, guinea pig, dog and pig at the sites indicated in figure (3.III.1.) is tabulated in table (4.III.1). Measurements of the gastrin content of extracts from these sites were made with both antisera G 1 and 2604-7, and in almost every instance the levels measured with antiserum 2604-7 were greater than those measured with antiserum G 1. This suggests the presence of both sulphated and non-sulphated gastrin in these extracts, because antiserum G 1 detects only non-sulphated gastrin, while antiserum 2604-7 measures both types of gastrin. In almost every instance the gastrin content of the tissue expressed per mg total protein was greater than the gastrin content per mg dry weight of tissue, as would be expected. The consistency of this finding and of the higher gastrin levels measured with antiserum 2604-7 as opposed to measurements with antiserum G 1 throughout the study suggests that the same material was being measured in the different extracts. The gastrin content of these tissues expressed per mg total protein, estimated by the Lowry method, is shown in figure (4.III.1.), and quantitated diagrammatically according to the key shown in this figure. No readings of total protein were obtained for rat antral tissue, so this result was expressed as gastrin content per mg dry weight of tissue.

The highest levels of gastrin were found in the antral extracts prepared from all the species studied with minimal amounts in the duodenum except in the case of the rat, where the duodenal gastrin content was 72-94% of that in the antrum. In the rabbit, guinea pig, dog and pig, duodenal gastrin comprised less than 0,5% of that in the antrum when measured with either antiserum. High concentrations of immunoreactive gastrin were found in the rat fundus, where a ratio of fundic to antral gastrin content of 40-62% was

found with both antisera. A considerable amount of gastrin was detected in the rabbit colon, 1,7-1,8% of that in the antrum, which is approximately a hundred times greater than the ratio of colonic to antral gastrin found in the other species investigated.

Extremely high levels of gastrin were found in porcine antral mucosa, and higher levels of intestinal gastrin were found in the pig than in most of the other species examined. The canine antral mucosa was also found to contain large amounts of immunoreactive gastrin. No significant levels of gastrin were found in pancreatic extracts of any species investigated, nor in the muscle extracts prepared from the rat, rabbit and pig.

Table (4.III.2) shows the gastrin concentrations measured in the serum of various mammalian and vertebrate species. In most cases the gastrin levels measured with antiserum 2604-7 were higher than those measured with antiserum G 1, suggesting the presence of both gastrins type I and II in these sera. In three bony fish and one cartilaginous fish investigated (all marine in origin) no gastrin was detected in the sera using antiserum G 1, whereas measurable levels in all four sera were found using antiserum 2604-7. This strongly suggests that only the sulphated form of gastrin occurs in these species, and may be an important clue in the evolution of the gastrin molecule, which may have arisen for the first time in the fishes in the sulphated form. The serum samples from these fish were haemolysed when the gastrin estimations were performed, due to the manner in which they were collected. The fish blood was drawn from fish caught on a trawler and was kept on ice until the following day when it could be centrifuged in the laboratory. Haemolysis is known to interfere in some radioimmunoassay systems (Bloom, 1974) causing artificially elevated levels. However, since the gastrin levels in this instance were undetectable using

Serum samples kindly donated by:

Dr. B.Shapiro, Isotope Unit, Dept. of Medicine,
University of Cape Town

Dr. R.Millar, Dept. of Chemical Pathology,
University of Cape Town

Mr. G.Craye, Dept. of Zoology,
University of Cape Town

Mr. J. van Velden, Irvin and Johnson Trawling
Division, Cape Town

TABLE (4.III.2)

Gastrin Levels in the Serum of Various Vertebrate Species

Common Name	Genus species	G 1 Measurement (pg/ml)	2604-7 Measurement (pg/ml)
Mammalia			
Baboon	Papio ursinus	67	58
Vervet monkey	Cercopithecus aethiops	64	67
Pig	Sus scrofa	29	46
Cow	Bos taurus	51	43
Sheep	Ovis aries	44	80
Goat	Capra hircus	19	44
Springbok	Antidorcas marsupialis	32	-
Rabbit	Oryctolugus cuniculus	95	230
Dassie	Procavia capensis	143	-
Rat	Rattus rattus	38	91
Guinea Pig	Cavia porcellus	11	-
Dog	Canis familiaris	87	188
Aves			
Chicken	Gallus gallus	16	39
Reptilia			
Snake	Dasypeltis scabra scabra	25	61
Amphibia			
Clawed toad	Xenopus laevis	570-950*	3 500-10 750*
Osteichthyes			
Stockfish	Merluccius capensis	0	20
Kingklip	Genypterus capensis	0	55
Mackerel	Scomber japonicus	0	51
Chondrichthyes			
Dogfish	Squalus sp.	0	81

*Range given because serum did not give a displacement curve parallel to that of the gastrin standard in the assay.

antiserum G 1, it is felt that the measurements made using antiserum 2604-7 reflect genuine gastrin levels and are not a consequence of haemolysis.

Exceptionally high levels of"gastrin"or a gastrin-like material were found in the serum of the frog, Xenopus laevis, ranging from 570-950 pg/ml detected using antiserum G 1, and 3 500-10 750 pg/ml measured using 2604-7 antiserum. Because the frog serum did not give a displacement curve parallel to that of the gastrin standard in the assay, actual"gastrin" levels could not be determined, hence the range of levels measured after correction for dilution of serum in the assay is given.

"Gastrin"measurements made in extracts from pooled tissues collected from 3 frogs (Xenopus laevis) are shown in table (4.III.3). Values were expressed per mg total protein and per mg dry weight of tissue, corrected for the readings obtained in muscle, measured using both antisera G 1 and 2604-7. The highest levels of"gastrin"or a gastrin-like material were found in the skin, where 3,982 and 2,653 pg/mg total protein were detected using antisera G 1 and 2604-7 respectively. The very high apparent levels of"gastrin"per mg total protein in the brain are thought to be a result of the very low protein content of brain tissue, giving rise to an erroneously high"gastrin"to total protein ratio. The"gastrin"levels relative to dry weight and total protein content of tissue measured in extracts of skin and stomach were greater when measured with antiserum G 1 than when measured with antiserum 2604-7. This is in direct contrast to gastrin levels measured in the mammalian gut extracts, where 2604-7 levels were higher than G 1 levels in almost every case (table (4.III.1)). The deviation from this trend by frog skin and stomach extracts may point to the fact that a peptide similar to but not identical with gastrin was detected in these extracts.

TABLE (4.III.3)

Gastrin Levels in Extracts Prepared from the Frog and Snake, Expressed per mg Dry Weight and per mg Total Protein, Measured with Antisera G 1 and 2604-7. All values are expressed as (pg/mg).

		Frog		Snake	
		Dry Wt.	Total Protein	Dry Wt.	Total Protein
Skin	G 1	2,198	3,982	0	0
	2604-7	1,464	2,653	0	0
Stomach	G 1	0,348	0,608	0	0
	2604-7	0,139	0,243	0,044	0,124
Intestine	G 1	0,075	0,097	0	0
	2604-7	0,792	1,028	0,456	1,461
Liver	G 1			0	0
	2604-7			0	0
Brain	G 1	0,397	3,310	0	0
	2604-7	0,929	7,741	0,026	0,158
Muscle	G 1	0	0	0	0
	2604-7	0	0	0	0

Gastrin measurements made in extracts of snake gut prepared by pooling tissues collected from three snakes of the genus Dasypeltis scabra scabra, the common spotted egg eater, appear in table (4.III.3). Values were corrected for readings found in muscle. The only site where a significant amount of gastrin was detected in the snake, apart from the serum, was in an extract of intestine, and this was only detected with 2604-7 antiserum, suggesting the presence of a small amount of sulphated type gastrin.

The elution profiles of frog serum and frog skin extract measured with antisera G 1 and 2604-7 are shown in figures (4.III.6.) and (4.III.7.). Both profiles show a large broad peak which eluted in a molecular weight region smaller than that of the natural human gastrin G-17 I marker, while the serum contained an additional large molecular weight component eluting in the void volume. The elution profiles obtained with the two antisera gave a very similar pattern in each case, and antiserum 2604-7 detected more material than did antiserum G 1, suggesting the presence of sulphated and non-sulphated material. A component was detected in the serum with antiserum 2604-7 which was not measured with antiserum G 1, at approximately 20% elution volume. This could be component I, or a component I-like peptide, as this was detected with Rehfeld's antiserum. He has described this component (Rehfeld, 1972; Rehfeld and Stadil, 1973a; Rehfeld, Stadil and Vikelsøe, 1974) which elutes later than "big big" gastrin but before big gastrin. The large broad peaks representing most of the eluted material in both serum and skin extract could be caerulein (Anastasi, Erspamer et al., 1967) or a related peptide of molecular weight smaller than heptadecapeptide gastrin. This suggestion is upheld by the demonstration of caerulein in skin extracts of Xenopus laevis and another species of frog by Anastasi, Bertaccini, Cei, De Caro, Erspamer, Impicciatore and Roseghini (1970).

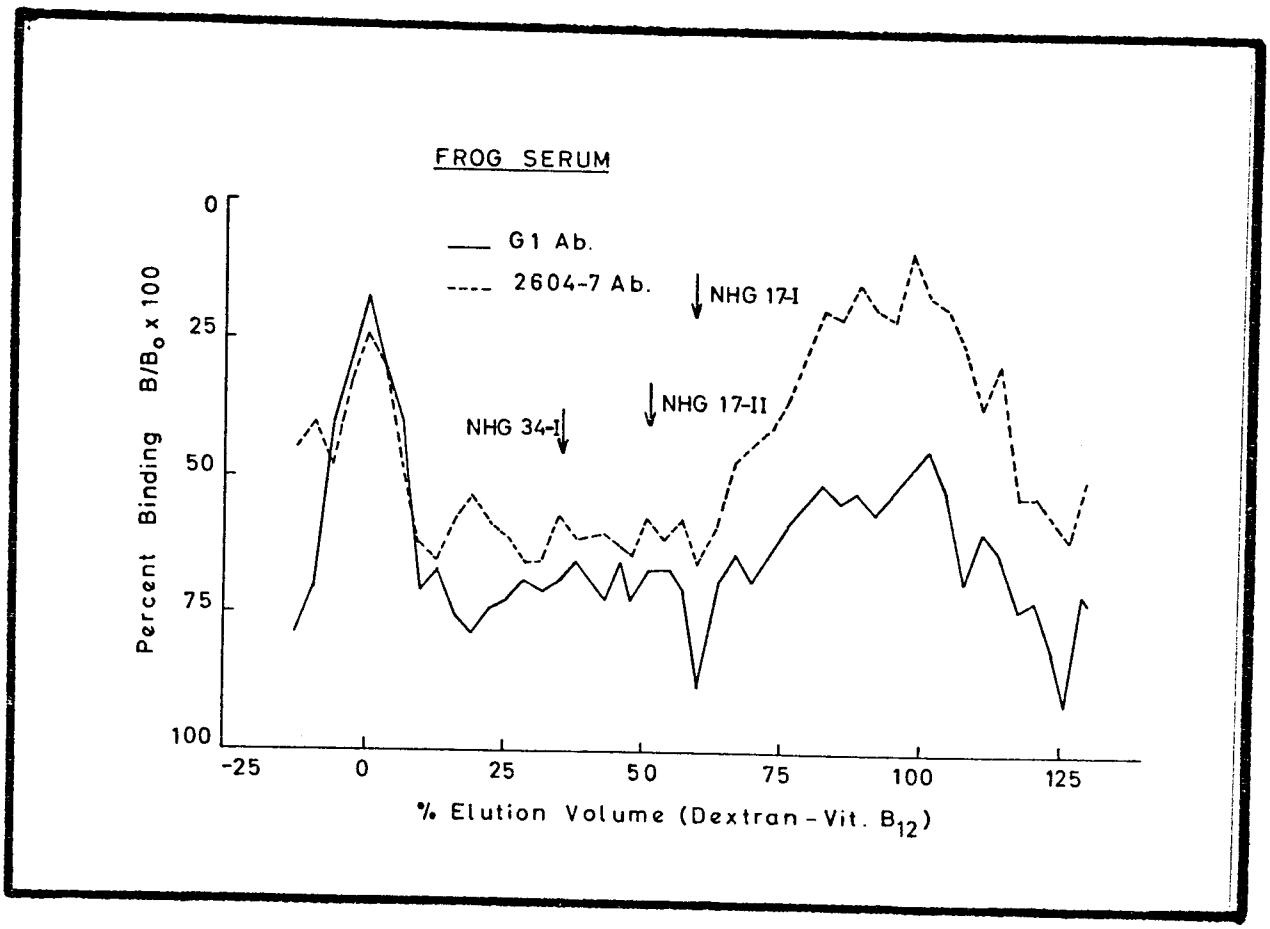


Fig. (4.III.6.) Elution profile of immunoreactive material obtained on chromatography of frog serum, measured with antisera G 1 and 2604-7. Details of chromatographic procedure used for this and subsequent figure appear in section 3.II. in the text.

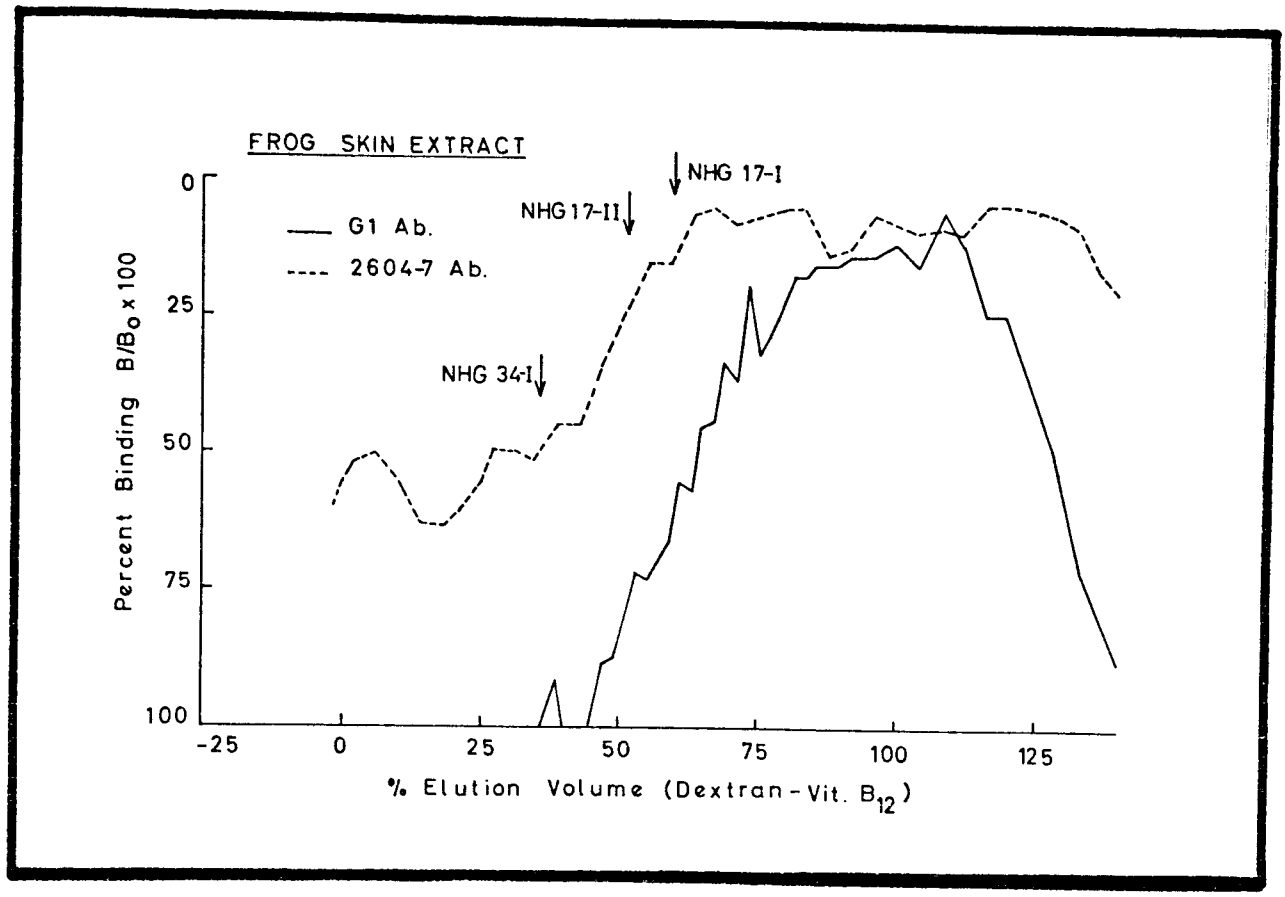
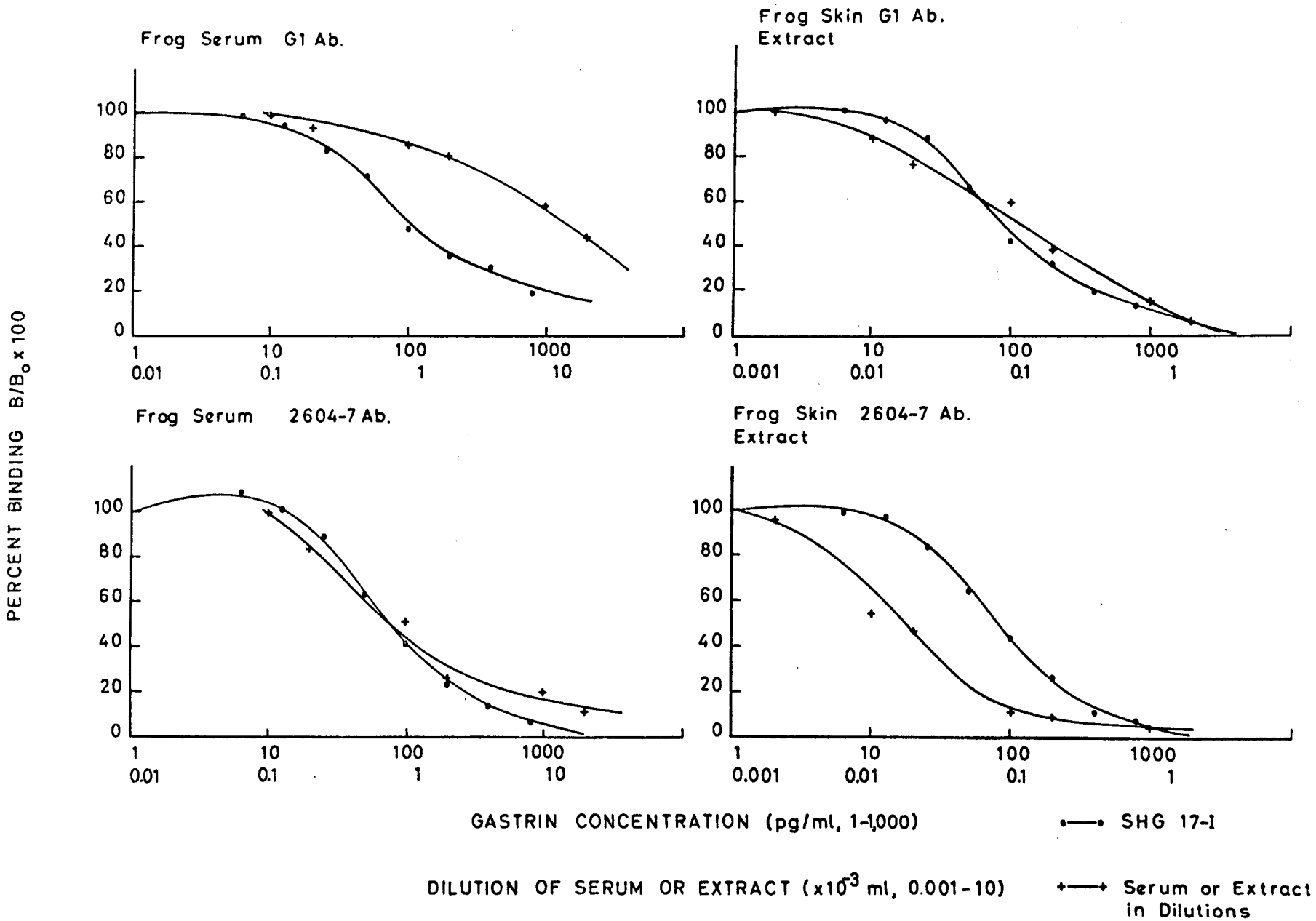


Fig. (4.III.7.) Elution profile of immunoreactive material obtained on chromatography of a boiled extract prepared from frog skin, measured with antisera G 1 and 2604-7.

The displacement curves obtained on serial dilution of frog serum and frog skin extract using both antisera, appear in figure (4.III.8.). Frog serum caused almost parallel displacement of radioactive gastrin from both antisera 2604-7 and G 1, but although the curve of displacement in the first instance was almost parallel to that of the standard, in the latter it deviated considerably. Coupling these findings with the elution profile suggests that frog serum contains large amounts of sulphated material bearing a heptadecapeptide gastrin-like sequence, occurring in a large and a small molecular weight form. Frog skin extract appeared to cause parallel displacement of ^{125}I-labelled gastrin from both antisera, and at approximately ten-fold greater dilutions than the serum. This does not necessarily mean that there is more "gastrin" in the frog skin compared with the serum, because the "gastrin" content of the serum and the skin was not quantitated. Antiserum 2604-7 reacted more strongly with the skin extract material, shifting the curve to the left, but the curve had the same shape as that of the standard. This greater sensitivity may explain why 2604-7 detected more high molecular weight material than did G 1 in the chromatographic profile. Frog skin extract appeared to contain only one component of gastrin-like material eluting in a region with molecular weight comparable to that of caerulein, occurring in both sulphated and non-sulphated forms.

Fig. (4.III.8.) The curves of displacement of antibody-bound ^{125}I-SHG-17 I obtained by serial dilutions of frog serum and skin extract using antisera G 1 and 2604-7, compared with that of the synthetic human heptadeca-peptide (SHG-17 I) standard.


Frog Serum G1 Ab.
Frog Skin G1 Ab. Extract
Frog Serum 2604-7 Ab.
Frog Skin 2604-7 Ab. Extract
PERCENT BINDING B/B0 x 100
100
80
60
40
20
0
1
10
100
1000
0.01
0.1
1
10
0.001
0.01
0.1
1
GASTRIN CONCENTRATION (pg/ml, 1-1,000)
SHG 17-I
DILUTION OF SERUM OR EXTRACT (x10-3 ml, 0.001-10)
Serum or Extract in Dilutions

4 III(iii) Gastrin Measurements in Lower Species

Gastrin levels detected in extracts from the animals representative of the phyla Coelenterata, Mollusca, Crustacea and Echinodermata were tabulated as gastrin content per mg dry weight of tissue and per mg total protein, and appear in table (4.III.4). Gastrin levels per mg total protein were expressed as a percentage of the gastrin content per mg total protein of human antrum, which was 2 034 pg/mg protein. The levels obtained ranged from 0,007% in the sea urchin up to 0,03% in the limpet gut, and the control measurements in muscle ranged from 0,001% in limpet muscle to 0,01% in whelk muscle. Since the background gastrin measurement in whelk muscle was greater than some of the values measured in the animals or gut tissues themselves, the gastrin content of these tissues is probably negligible. There was no obvious trend of higher gastrin content in tissues of invertebrates higher up the evolutionary scale.

TABLE (4.III.4)

Gastrin Levels in Invertebrate Gut Extracts Measured with G 1 Antiserum

Phylum	Animal		Gastrin Conc. in Extract (pg/ml)	Gastrin Content per mg dry wt. (pg/mg)	Gastrin Content per mg total protein (pg/mg)	Gastrin Content per mg protein relative to human antrum
Coelenterata	Sea Anemone	Pseudactinia sp.	11	0,050	0,097	0,0048%
Mollusca	Mussel	Choromytilus sp.	16	0,102	0,167	0,0082%
	Snail	Helix sp.	31	0,088	0,348	0,0171%
	Limpet	Patella sp.	50	0,370	0,687	0,0338%
	Periwinkle	Oxystele sp.	40	0,260	0,559	0,0275%
	Whelk	Burnupena sp.	42	0,381	0,579	0,0284%
	Chiton	Chiton sp.	36	0,137	0,263	0,0129%
Crustacea	Barnacle	Tetraclita sp.	15	0,042	0,580	0,0285%
Echinodermata	Sea Urchin	Parechinus sp.	13	0,075	0,144	0,0071%
Control Measurements	Limpet muscle		2	0,016	0,030	0,0015%
	Periwinkle muscle		5	0,083	0,096	0,0047%
	Whelk muscle		12	0,163	0,226	0,0111%

Each gastrin level is the average of 2 estimations.

Gastrin content of human antrum = 2 034 pg/mg protein.

Section IV Gastrin Kinetics in the Pig

Blood flow measurements obtained in the 8 pigs in group I are shown in table (4.IV.1). The mean ± S.E.M. values for blood flow were: portal 432 ± 35,6 ml/min, hepatic arterial 147,3 ± 34,4 ml/min and total flow 580,3 ± 24,0 ml/min. Since each of these values is the mean of 8 separate estimations, the sum of the mean portal and arterial blood flow measurements is not exactly the same as the mean total flow rate estimation. Values for fractional flow per gram liver weight also appear in table (4.IV.1), from which it is apparent that total blood flow was not dependent upon liver mass. Table (4.IV.2) shows individual gastrin measurements made at various sampling sites in 7 pigs belonging to group I. Values for the sites A5, PD, PP and HV are a mean of three estimations made at 10-minute intervals. Arterial serum gastrin levels were remarkably constant throughout the study, with mean values for the different sampling times ranging from 71,0 to 78,6 pg/ml. Gastrin levels at the proximal portal site were significantly higher than those at the distal portal site ($p<0,001$); hepatic venous gastrin levels were similar to proximal portal levels, whereas arterial gastrin concentrations were significantly lower ($p<0,001$).

The afferent and efferent hepatic mass and the net hepatic balance of endogenous gastrin, determined as the difference between these parameters for each pig, appear in table (4.IV.3a). Five pigs showed a positive balance and two pigs had a negative hepatic balance of gastrin, as shown in figure (4.IV.1.), although the difference between the mass of gastrin entering and leaving the liver in the group of 7 pigs was not significant ("t"-test). Table (4.IV.3b) shows the afferent and efferent hepatic gastrin mass corrected for the individual weights of the liver in each pig, which did not change these results.

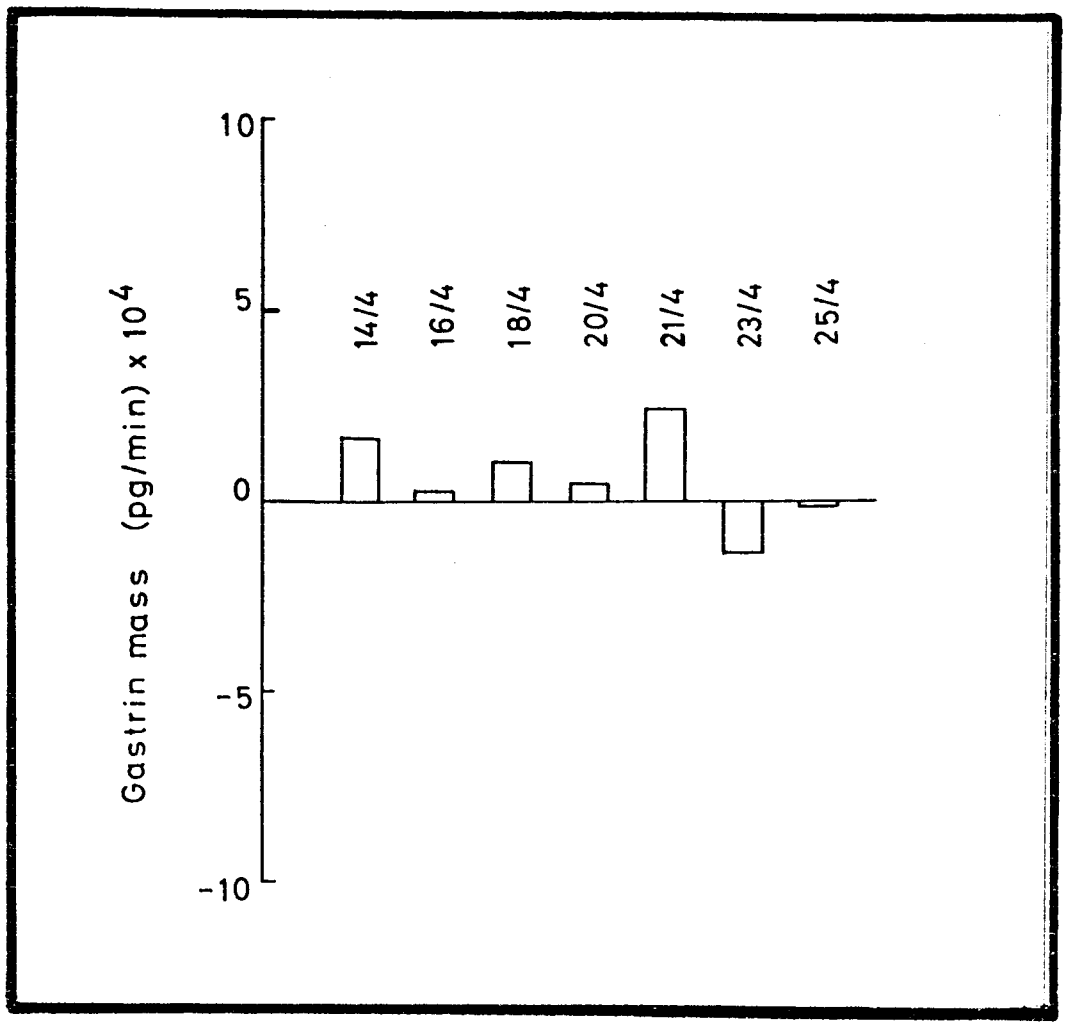


Fig. (4.IV.1.) Measurement of endogenous gastrin mass (concentration (pg/ml) x blood flow (ml/min)) entering and leaving the liver. A positive balance indicates net hepatic uptake of gastrin. Numbers refer to pig numbers which belong to group I. (See section 3.IV. in text.)

TABLE (4.IV.1)

Hepatic Blood Flow and Liver Weight in Group I Pigs (n = 8)

	Absolute Flow (ml/min)			Liver Weight (g)	Fractional Flow per Gram Liver Weight (ml/g/min)		
Animal	Total	Portal	Arterial		Total	Portal	Arterial
14/4	627	360	267	689	0,91	0,52	0,39
15/4	613	555	58	766	0,80	0,73	0,07
16/4	548	469	79	630	0,87	0,74	0,13
18/4	653	332	321	768	0,85	0,43	0,42
20/4	602	489	113	627	0,96	0,78	0,18
21/4	601	523	78	626	0,96	0,70	0,26
23/4	434	268	166	477	0,91	0,56	0,35
25/4	564	460	96	641	0,88	0,70	0,18
$\bar{X}$	580,3	432	147,3	653	0,893	0,645	0,248
S.E.M.	24,0	35,6	34,4	32,8	0,019	0,044	0,045

Each blood flow value is the mean of 3 estimations.

TABLE (4.IV.2)

Gastrin Concentrations in Group I Pigs (pg/ml) (n = 7)

Animal	A1+	A2+	A3+	A4+	A5*	PD*	PP*	HV*
14/4	94	82	85	67	100	73	121	138
16/4	65	65	54	49	64	45	71	75
18/4	81	83	92	74	80	71	85	98
20/4	65	62	58	68	80	32	76	84
21/4	85	86	84	77	87	65	84	125
23/4	55	63	64	65	67	41	109	62
25/4	72	65	70	97	72	62	80	76
$\bar{X}$	73,9	72,3	72,4	71,0	78,6	55,6	89,4	94,0
S.E.M.	5,1	4,1	5,6	5,5	4,7	6,1	7,0	10,6

+A1 = Arterial sample 10-15 minutes post-induction)
+A2-4 = Arterial samples ± 45 min. thereafter at 10-minute intervals) Individual values

*A5 = Arterial)
*PD = Portal Distal)
*PP = Portal Proximal) Each is a mean of 3 estimations at 10-minute intervals
*HV = Hepatic Venous)

Significant differences A vs PP, PP vs PD, HV vs A all $p < 0,001$.

TABLE (4.IV.3a)

Gastrin in Hepatic Afferent and Efferent Circulation in Group I Pigs (n = 7)

Animal	Mass In (pg/min.)	Afferent Conc. (pg/ml)	Mass Out (pg/min.)	Efferent Conc. (pg/ml)	Net Hepatic Balance (pg/min)
14/4	70 260	112,1	86 526	138	16 266
16/4	38 355	70,0	41 100	75	2 745
18/4	53 900	82,5	63 994	98	10 094
20/4	46 204	76,8	50 568	84	4 364
21/4	50 718	84,4	75 125	125	24 407
23/4	40 334	92,9	26 908	62	-13 416
25/4	43 712	77,5	42 864	76	- 848
$\overline{X}$	49 069,0	85,2	55 297,9	94,0	6 230,3
S.E.M.	4 091,5	5,2	7 910,9	10,6	4 623

Afferent flow = portal flow + hepatic arterial flow

Efferent flow = hepatic venous flow = portal flow + arterial flow

Afferent concentration = (portal flow x gastrin conc. + hepatic arterial flow x gastrin conc.)/total flow

Efferent concentration = (hepatic venous flow x gastrin conc.)/total flow

TABLE (4.IV.3b)

Gastrin in Hepatic Afferent and Efferent Circulation Expressed per Gram Liver Weight in Group I Pigs (n = 7)

Animal	Aff. Mass/g (pg/g)	Aff. Conc./g (pg/ml-g)	Eff. Mass/g (pg/g)	Eff. Conc./g (pg/ml-g)
14/4	102,0	0,163	125,5	0,200
16/4	60,9	0,111	65,2	0,119
18/4	70,1	0,107	83,3	0,127
20/4	73,7	0,122	80,7	0,133
21/4	81,0	0,135	120,0	0,199
23/4	84,6	0,195	56,4	0,129
25/4	68,2	0,121	69,5	0,123
$\overline{X}$	77,2	0,136	85,8	0,147
S.E.M.	5,1	0,01	10,2	0,01

The portal contribution of gastrin was determined as the difference in gastrin levels measured at the proximal and distal portal sampling sites and converted to portal gastrin mass using the measurement of portal flow. The mean portal contribution of gastrin in 7 pigs was 33,9 ± 7,6 pg/ml or 13 154 ± 2 289 pg/min (± S.E.M.), as shown in table (4.IV.4). The mean daily secretion rate of gastrin from the portal site was 18,9 µg, with a range from 6,69 to 30,98 µg/day.

Table (4.IV.5) shows the actual gastrin levels and the levels above basal measured in each of the group II pigs during the three-hour stepwise infusion of synthetic human heptadecapeptide gastrin I. The mean basal arterial levels of serum gastrin in these 7 pigs ranged from 19 to 81 pg/ml. The total mass of gastrin entering and leaving the liver, and the net hepatic balance of gastrin during each hour of infusion for each pig is shown in table (4.IV.6). These results are shown diagrammatically in figure (4.IV.2.). Using the paired "t"-test no significant difference was found between the afferent and efferent gastrin mass at any dose level, a fact which is borne out by the linear relationship between afferent and efferent hepatic gastrin mass at each dose level, with the slope of the line approximating 1, as shown in figure (4.IV.3.). Significant differences were found between the afferent gastrin mass at the first and second, and second and third dose levels of gastrin, and the same for the efferent gastrin masses at each dose level, using the paired "t"-test. The p values for these tests are shown in table (4.IV.7) and in the legend to figure (4.IV.2.).

Table (4.IV.8) shows the gastrin levels measured in the arterial, portal and venous circulations in each of 6 pigs during the 30-minute decay period after stopping the gastrin infusion. The elimination constants and half-life values calculated for each pig in the arterial, portal and venous circulation

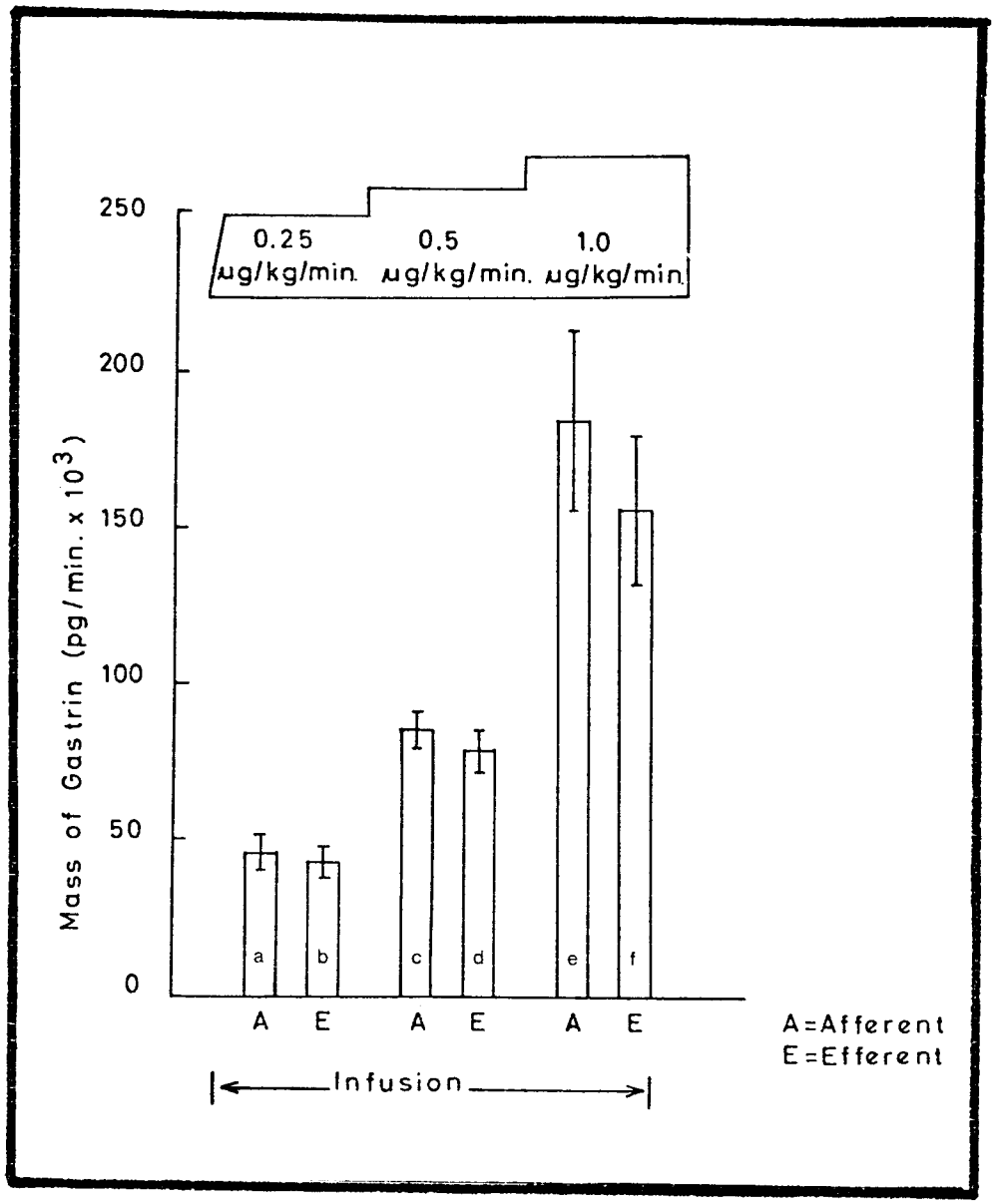


Fig. (4.IV.2.) The mass of endogenous and exogenous gastrin entering (A) and leaving (E) the liver during infusions of synthetic human gastrin G-17 I in the doses indicated.

(a) vs (b), (c) vs (d), (e) vs (f) : not significant.

(a) vs (c)
(c) vs (e)
(b) vs (d)
(d) vs (f)

all $p < 0,01$ (Paired "t" test)

Vertical bars indicate S.E.M.

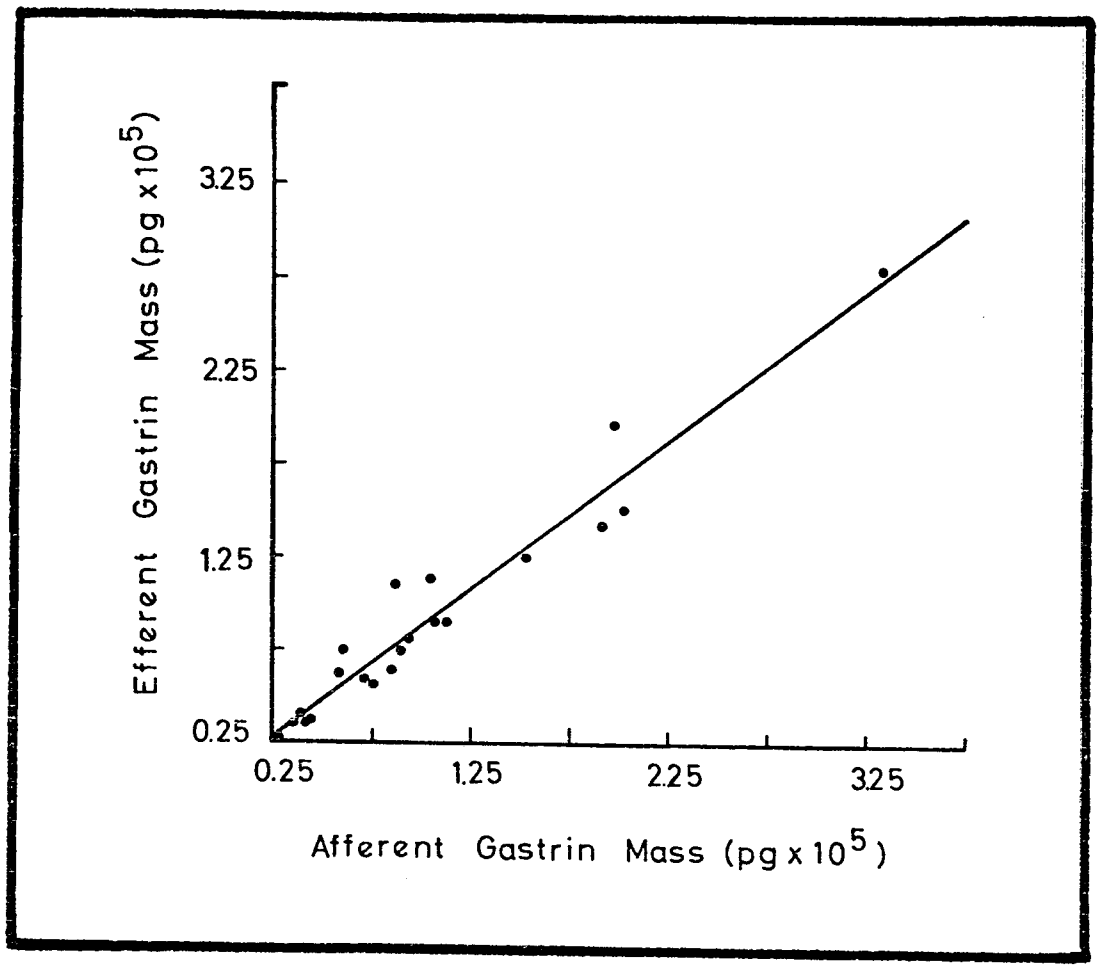


Fig. (4.IV.3.) A graph demonstrating the linear relationship between the mass of gastrin entering and that leaving the liver.

$y = 0,7991x + 8405,21$

$r = 0,9721$

$n = 21$

$p < 0,01$

TABLE (4.IV.4)

Portal Gastrin Contribution in Group I Pigs

Animal	PP - PD (pg/ml)	Portal Gastrin Mass (pg/min)
14/4	48	17 280
16/4	26	12 194
18/4	14	4 648
20/4	44	21 516
21/4	19	9 937
23/4	68	18 224
25/4	18	8 280
$\bar{X}$	33,9	13 154
S.E.M.	7,6	2 289

The difference between PP and PD gastrin concentration is significant, $p < 0,005$.

TABLE (4.IV.5)

Gastrin Concentrations in Group II Pigs Before and During Gastrin Infusion (pg/ml)

Animal		452		447		466		500		501		494		521	
Mean Basal Gastrin		50		81		19		28		35		45		34	
S.E.M.		2,50		4,05		0,90		1,41		1,57		1,91		1,37	
Gastrin Concentration		Actual	Above Basal	Actual	Above Basal	Actual	Above Basal	Actual	Above Basal	Actual	Above Basal	Actual	Above Basal	Actual	Above Basal
0,25 µg/kg/hr	A	81	31	127	46	52	33	110	82	81	46	99	54	74	40
	P	63	13	123	42	45	26	65	37	55	20	100	55	75	41
	V	69	19	102	21	45	26	65	37	63	28	108	63	63	29
0,5 µg/kg/hr	A	177	127	130	49	129	110	215	187	199	164	156	111	310	276
	P	135	85	94	13	130	111	176	148	140	105	148	103	110	76
	V	110	60	128	47	97	78	155	127	129	94	190	145	140	106
1,0 µg/kg/hr	A	260	210	214	133	307	288	850	822	640	605	433	388	610	576
	P	172	122	170	89	250	231	480	452	225	190	310	265	260	226
	V	155	105	195	114	216	197	-	-	245	210	338	293	260	226

Values are the mean of the three closest readings out of four taken during each hour of infusion.

TABLE (4.IV.6)

Total Mass of Gastrin Entering and Leaving Liver During Each Stage of Gastrin Infusion in Group II Pigs (pg/min.)

Animal	452	447	466	500	501	494	521	$\bar{X}$	S.E.M.
				Afferent Hepatic Gastrin Mass (pg/min.)					
0,25 µg/kg/hr	39 210	71 966	27 144	44 348	35 747	57 882	43 375	45 667	5 630
0,5 µg/kg/hr	84 527	59 851	75 291	107 877	89 932	87 062	93 293	85 405	5 662
1,0 µg/kg/hr	112 774	105 132	153 471	333 045	191 697	198 010	202 433	185 223	28 878
				Efferent Hepatic Gastrin Mass (pg/min.)					
0,25 µg/kg/hr	40 040	59 191	26 114	37 720	36 559	62 672	36 559	42 693	5 008
0,5 µg/kg/hr	63 833	74 278	56 289	89 947	74 859	110 257	81 242	78 672	6 702
1,0 µg/kg/hr	89 947	113 159	125 345	278 544	142 174	196 141	150 878	156 598	23 895
				Net Hepatic Balance (Afferent - Efferent) (pg/min.)					
0,25 µg/kg/hr	-830	12 775	1 030	6 628	-812	-4 790	6 816	2 974	2 273
0,5 µg/kg/hr	20 690	-14 427	19 002	17 930	15 073	-23 195	12 051	6 732	6 747
1,0 µg/kg/hr	22 827	-8 027	28 126	54 501	49 523	1 869	51 555	28 624	9 418

TABLE (4.IV.7)

Mean Gastrin Mass Entering and Leaving Liver in Pigs in the Basal State (Group I) and Pigs Given Gastrin Infusion (Group II)*

		Gastrin Mass Entering Liver	Gastrin Mass Leaving Liver
Basal	$\bar{X}$	49 069 (a)	55 298 (b)
	S.E.M.	4 092	7 911
	n	7	7
0,25 µg/kg/min	$\bar{X}$	45 667 (c)	42 693 (d)
	S.E.M.	5 630	5 008
	n	7	7
0,5 µg/kg/min	$\bar{X}$	85 405 (e)	78 672 (f)
	S.E.M.	5 662	6 702
	n	7	7
1,0 µg/kg/min	$\bar{X}$	185 223 (g)	156 598 (h)
	S.E.M.	28 878	23 895
	n	7	7

(a) vs (b), (c) vs (d), (e) vs (f), (g) vs (h) : not significant

(c) vs (e)
(e) vs (g)
(d) vs (f)
(f) vs (h)

all $p<0,01$ (paired "t"-test)

*Units are picograms/minute.

TABLE (4.IV.8)

Gastrin Concentrations During Decay Period from Plateau Level in Group II Pigs

Values are Gastrin Levels above Basal (pg/ml)

	Animal	452			466			500			501			494			521			Mean and S.E.M.		
		A	P	V	A	P	V	A	P	V	A	P	V	A	P	V	A	P	V	A	P	V
	Plateau (pg/ml)	210	122	105	288	231	197	822	452	-	605	190	210	388	265	293	576	226	226			
	Plateau (pmol/l)	95,5	55,5	47,7	130,9	105	89,5	374	206	-	275	86,4	95,5	176	120	133	262	103	103			
	% Plateau level	100%	100%	100%	100%	100%	100%	100%	100%	-	100%	100%	100%	100%	100%	100%	100%	100%	100%			
	$\bar{X}$ Basal Arterial	50			19			28			35			45			34					
5 minutes	pg/ml	46	39	39	176	101	116	207	197	-	42	48	60	110	100	80	40	61	61			
	pmol/l	20,9	17,7	17,7	80,0	45,9	52,7	94,1	89,5	-	19,1	21,8	27,3	50,0	45,5	36,4	18,2	27,7	27,7			
	% Plateau level	21,9	31,9	37,1	61,1	43,7	58,9	25,1	43,4	-	6,9	25,2	28,6	28,4	37,9	27,4	6,9	26,9	26,9	25,1	34,8	35,8
																				8,1	3,3	6,1
10 minutes	pg/ml	26	20	12	81	68	96	124	117	-	18	37	36	45	55	39	37	40	17			
	pmol/l	11,8	9,1	5,5	36,8	30,9	43,6	56,4	53,2	-	8,2	16,8	16,4	20,5	25	17,7	16,8	18,2	27,7			
	% Plateau level	12,4	16,4	11,5	28,1	29,4	48,7	15,1	25,8	-	3,0	19,4	17,2	11,6	20,8	13,3	6,4	17,7	7,5	12,8	21,6	19,6
																				3,5	2,1	7,4
20 minutes	pg/ml	53	85	74	93	59	61	42	62	-	16	26	33	12	23	13	29	31	66			
	pmol/l	24,1	38,6	33,6	42,3	26,8	27,7	19,1	28,2	-	7,3	11,8	15,0	5,5	10,5	5,9	13,2	14,1	30,0			
	% Plateau level	25,2	69,6	70,4	32,3	25,5	30,9	5,1	13,7	-	2,7	13,7	15,7	3,1	8,8	4,4	5,0	13,7	29,1	12,2	24,2	30,1
																				5,3	9,4	11,2
30 minutes	pg/ml	25	33	35	56	51	59	31	27	-	29	7	13	2	0	3	33	47	16			
	pmol/l	11,4	15,0	15,9	25,5	23,2	26,8	14,1	12,3	-	13,2	3,2	5,9	0,9		1,4	15,0	21,4	7,3			
	% Plateau level	11,9	33,3	-2,9	19,5	22,1	29,9	3,8	6,0	-	4,8	3,7	6,2	0,5		1,1	5,7	20,8	7,1	7,7	17,2	8,3
																				2,8	5,5	5,7

over 10 minutes and over 30 minutes appear in table (4.IV.9). Half-life values for heptadecapeptide gastrin calculated during the first 10 minutes of the decay period in the arterial, portal and venous circulations respectively were 3,19; 4,23; and 4,32 minutes. Half-life values calculated over the first 30 minutes of the decay period were 6,51; 7,98; and 7,39 minutes for the arterial, portal and venous circulations respectively. The longer half-life calculated over 30 minutes was due to recirculation of gastrin, as shown by the marked levelling of the decay graph after 10 minutes. This is exemplified by the graph of decay of infused gastrin in the arterial circulation constructed using the mean gastrin concentrations in 6 pigs, shown in figure (4.IV.4.). The longer half-life found in the portal and venous circulations may be due to the endogenous gastrin produced at the portal site.

The mean $\pm$ S.E.M. space of distribution of heptadecapeptide gastrin I in the arterial circulation was 6,401 $\pm$ 2,095 litres, in the portal circulation it was 13,677 $\pm$ 1,976 litres, and in the venous circulation it was 16,614 $\pm$ 4,958 litres. The individual values in 6 pigs appear in table (4.IV.10). The space of distribution of 6,401 litres in the arterial circulation amounts to 21,6% of the total body weight.

The metabolic clearance rate (M.C.R.) of infused heptadecapeptide gastrin was calculated at each dose level, and expressed as the average of all three infusion doses, as well as the average of the two highest infusion doses. The mean $\pm$ S.E.M. for the metabolic clearance rate in the arterial, portal and venous circulations, calculated as the average of 3 doses was 2,014 $\pm$ 0,233; 3,232 $\pm$ 0,440; and 3,170 $\pm$ 0,470 litres/minute respectively. Expressed relative to body weight these values were 67,8 ml/kg/min, 108,8 ml/kg/min and 106,7 ml/kg/min respectively. The corresponding values

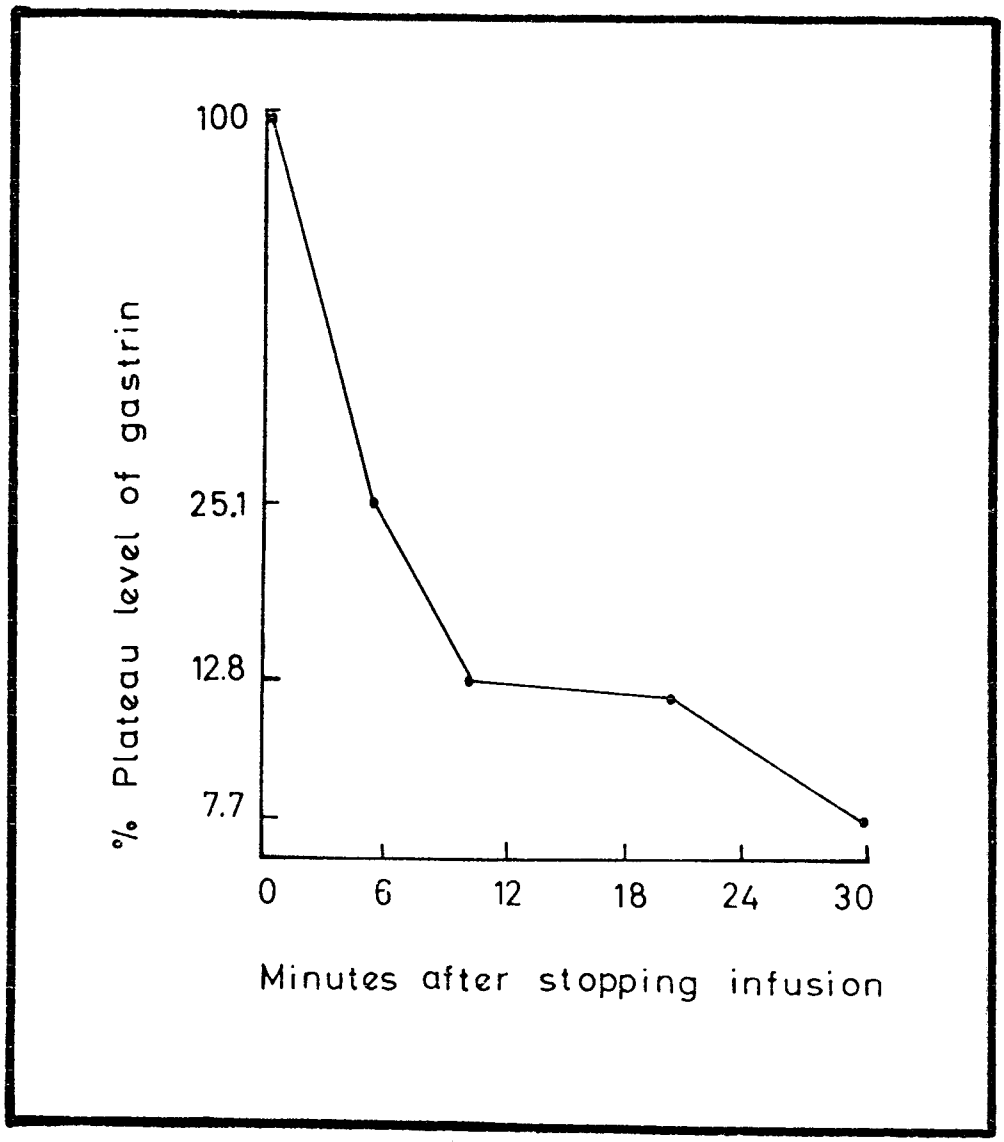


Fig. (4.IV.4.) The disappearance of gastrin from the arterial circulation during the 30-minute period after stopping the gastrin infusion; the graph is drawn from the mean values obtained in 6 pigs.

TABLE (4.IV.9)

Elimination Constants (K_e) and Half Life Values ($T\frac{1}{2}$) of Heptadecapeptide Gastrin in Arterial, Portal and Venous Circulations of 6 Pigs, calculated during the first 10 minutes and during the first 30 minutes of the decay period.

	Over 10 minutes		Over 30 minutes	
Pig	K_e	$T\frac{1}{2}$ (mins.)	K_e	$T\frac{1}{2}$ (mins.)
Arterial Circulation				
452	0,2275	3,05	0,0834	8,31
466	0,1213	5,71	0,0607	11,42
500	0,2063	3,36	0,1282	5,41
501	0,3870	1,79	0,1472	4,71
494	0,2226	3,11	0,1796	3,86
521	0,3262	2,12	0,1298	5,34
$\bar{X}$	0,2485	3,19	0,1215	6,51
S.E.M.	0,0384	0,563	0,0176	1,1574
n	6	6	6	6
Portal Circulation				
452	0,1902	3,64	0,0629	11,01
466	0,1309	5,29	0,0621	11,17
500	0,1417	4,89	0,0993	6,98
501	0,1861	3,72	0,1132	6,12
494	0,1643	4,22	0,1943	3,57
521	0,1908	3,63	0,0770	9,00
$\bar{X}$	0,1674	4,23	0,1015	7,98
S.E.M.	0,0107	0,2903	0,0203	1,215
n	6	6	6	6
Venous Circulation				
452	0,2127	3,26	0,1693	4,09
466	0,0787	8,81	0,0486	14,26
500	-	-	-	-
501	0,1907	3,63	0,1008	6,88
494	0,2130	3,25	0,1572	4,41
521	0,2597	2,67	0,0951	7,29
$\bar{X}$	0,1910	4,32	0,1142	7,39
S.E.M.	0,0302	1,132	0,0221	1,834
n	5	5	5	5

TABLE (4.IV.10)

Space of Distribution of Heptadecapeptide Gastrin in Arterial, Portal and Venous Circulations of 6 Pigs, calculated during the first 10 minutes of Decay Period. Units are litres.

		Space of Distribution, V (litres)		
Pig	Weight (kg)	Arterial	Portal	Venous
452	30	10,464	21,543	22,383
466	31	14,722	17,078	33,344
500	32	3,146	8,326	-
501	28	1,994	13,199	11,651
494	29	5,597	11,098	7,743
521	28	2,484	10,819	7,949
$\overline{X}$	29,7	6,401	13,677	16,614
S.E.M.	0,7	2,095	1,976	4,958
n	6	6	6	5

calculated as the average of the two highest doses were 1,685 ± 0,280; 2,663 ± 0,364; and 2,777 ± 0,401 litres/minute respectively. The individual values of M.C.R. for 7 pigs are shown in table (4.IV.11).

Table (4.IV.12) shows the daily production of gastrin in the arterial circulation for each pig as calculated from the metabolic clearance rate. The mean daily production of gastrin in 7 pigs was found to be between 113,9 ± 40,3 and 125,9 ± 32,0 (± S.E.M.) micrograms/day. Since only approximately 20 µg gastrin was produced at the portal site per day in the case of the pigs in group I it would appear that gastrin is produced at an additional site or sites in the body to account for a daily blood production rate of gastrin of approximately 120 µg.

The chromatographic elution profiles of arterial, portal and venous samples collected at various times during infusion of gastrin into pigs 500 and 521 are shown in figures (4.IV.5.), (4.IV.6.) and (4.IV.7.). In the portal and venous samples from both pigs 500 and 521 it appeared that smaller gastrin species as well as the heptadecapeptide form were present, whereas the elution profile of the arterial sample collected from pig 521 150 minutes after commencement of the gastrin infusion suggested the presence of an additional higher molecular weight gastrin species, eluting in the region of the G-34 marker.

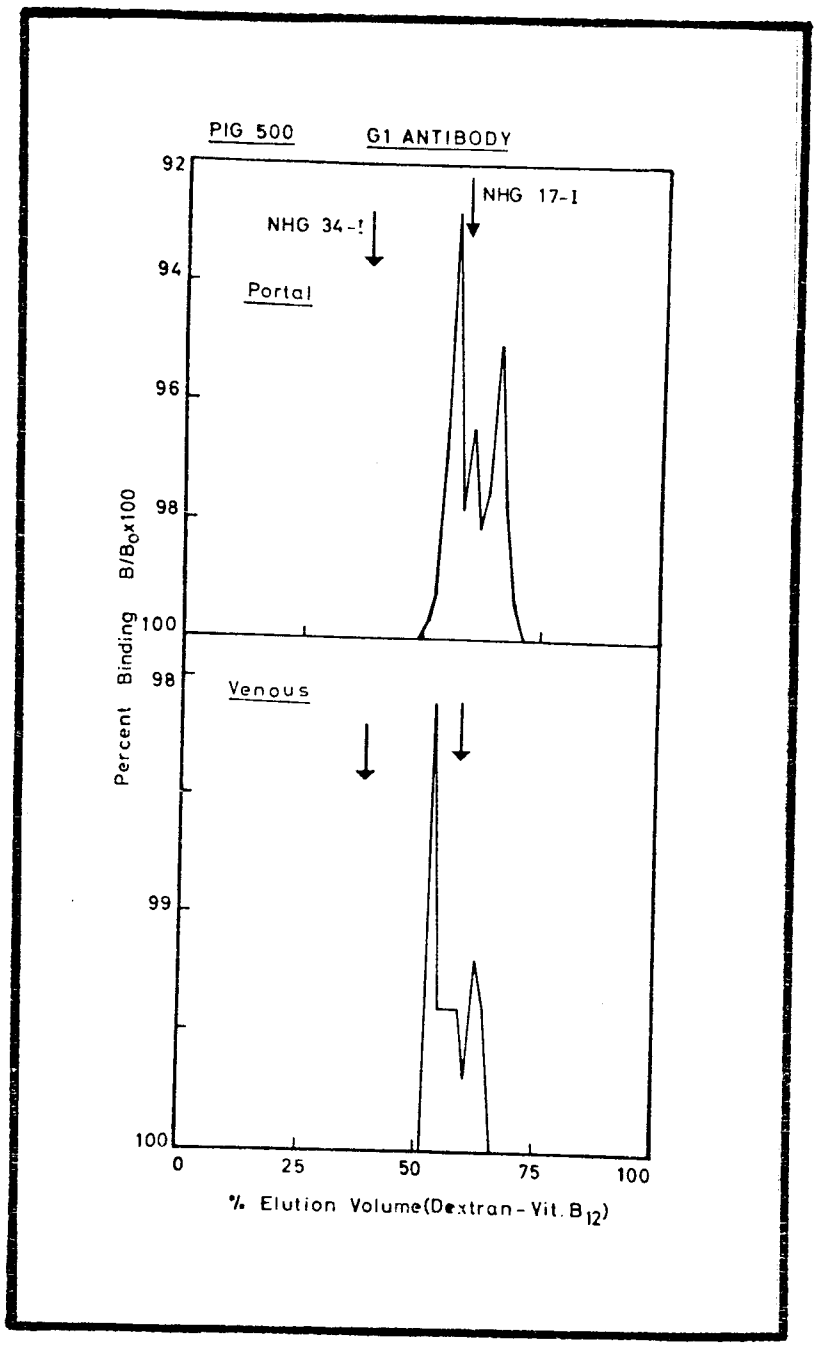


Fig. (4.IV.5.) Chromatographic elution profiles of gastrin in serum taken from the portal and hepatic venous circulations 120 minutes after commencement of the SHG-17 I gastrin infusion. See fig. (3.IV.2.) for protocol.

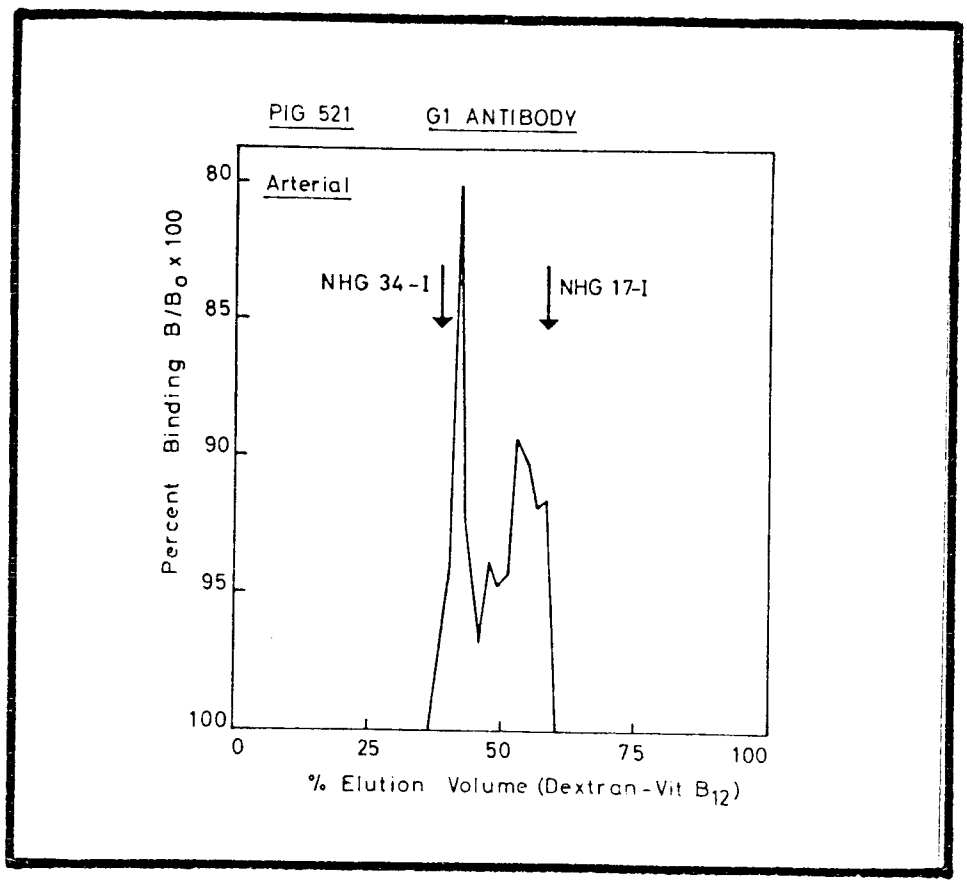


Fig. (4.IV.6.) Chromatographic elution profile of gastrin in serum taken from the arterial site 150 minutes after commencement of the SHG-17 I gastrin infusion. See fig. (3.IV.2.) for protocol.

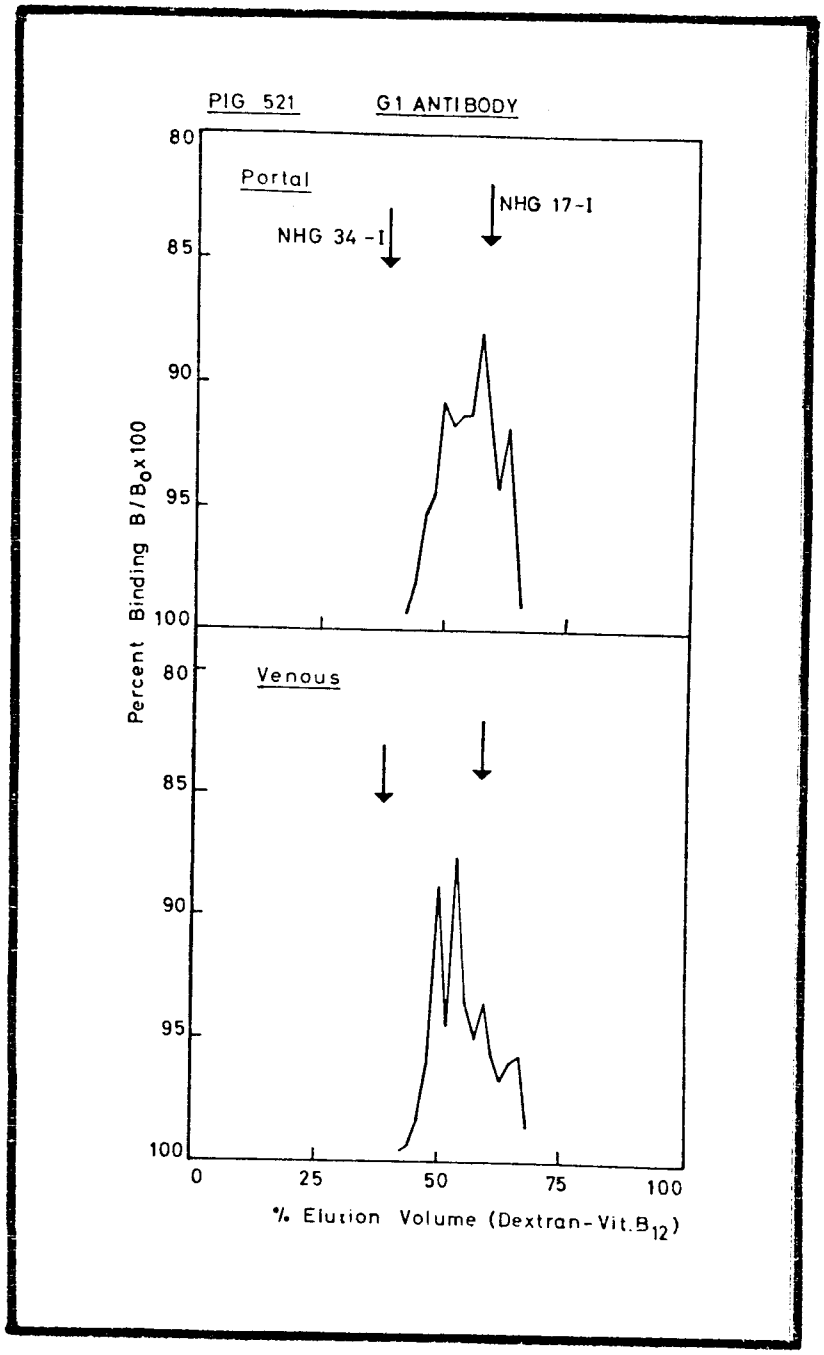


Fig. (4.IV.7.) Chromatographic elution profiles of gastrin in serum taken from the portal and hepatic venous circulations 165 minutes after commencement of the SHG-17 I gastrin infusion. See fig. (3.IV.2.) for protocol.

TABLE (4.IV.11)

Metabolic Clearance Rate of Heptadecapeptide Gastrin in Arterial, Portal and Venous Circulations in 7 Pigs, Calculated Using Three Infusion Doses and then Using the Two Highest Doses. Units are litres/minute.

Pig	Weight (kg)	Average of 3 Doses			Average of 2 Highest Doses		
		Arterial	Portal	Venous	Arterial	Portal	Venous
452	30	2,793	5,550	5,169	2,175	3,519	4,464
447	30	2,406	3,417	2,751	2,874	4,356	3,087
466	31	2,685	3,178	3,633	2,071	2,282	2,967
500	32	1,232	2,195	-	1,037	1,491	-
501	28	1,576	3,503	2,954	1,098	2,338	2,352
494	29	1,885	2,123	1,746	1,711	2,085	1,659
521	28	1,523	2,660	2,764	0,829	2,568	2,134
$\overline{X}$	29,7	2,014	3,232	3,170	1,685	2,663	2,777
S.E.M.	0,6	0,233	0,440	0,470	0,280	0,364	0,401
n	7	7	7	6	7	7	6

TABLE (4.IV.12)

Calculation of Daily Production of Gastrin from Metabolic Clearance Rate in Arterial Circulation, Using Three Infusion Doses and then Using the Two Highest Doses. Units are micrograms/day.

Pig	Average of 3 Doses				Average of 2 Highest Doses			
	Arterial M.C.R. l/min.	Arterial M.C.R. l/day	Arterial Gastrin pg/ml	Gastrin Production µg/day	Arterial M.C.R. l/min.	Arterial M.C.R. l/day	Arterial Gastrin pg/ml	Gastrin Production µg/day
452	2,793	4021,9	50	201,1	2,175	3132,0	50	156,6
447	2,406	3464,6	81	280,6	2,874	4138,6	81	335,2
466	2,685	3866,4	19	73,5	2,071	2982,2	19	56,7
500	1,232	1774,1	28	49,7	1,037	1493,3	28	41,8
501	1,576	2269,4	35	79,4	1,098	1581,1	35	55,3
494	1,885	2714,4	45	122,1	1,711	2463,8	45	110,9
521	1,523	2193,1	34	74,6	0,829	1193,8	34	40,6
$\overline{X}$	2,014	2900,6	41,7	125,86	1,685	2426,4	41,7	113,87
S.E.M.	0,233	334,9	7,6	32,0	0,280	403,7	7,6	40,3
n	7	7	7	7	7	7	7	7

Section V Studies with Rat Liver

4 V(i) Metabolism of Synthetic Human Heptadecapeptide Gastrin by the Isolated Perfused Rat Liver.

The flow rate during the perfusion was usually of the order of 10 ml/min with a range of between 8 and 12 ml/min in different perfusions. Liver weight varied between 3 and 3,5 grams, giving a flow rate of approximately 3 ml/min/g. Bile production during the perfusion ranged from 200 to 400 µl/hr. The residual volume in the system at the end of the perfusion was approximately 53 ml.

Although the high dose of gastrin added to the perfusion system was diluted to give 200 ng/ml, the actual gastrin concentrations detected in the assay after correction for dilution ranged from 85 to 236 ng/ml, giving a mean recovery of gastrin added to the system of 73,9% with a range of 42,5 to 118%. In the case of the low dose perfusion recovery of added gastrin ranged from 32 to 67,4%, with a mean of 49,7%.

Irrespective of the dose of gastrin added to the perfusion system the immunoreactive gastrin levels showed no statistically significant change for up to 60 minutes, as determined by analysis of variance. As can be seen in figure (4.V.(i).1.) insulin disappeared rapidly from the perfusate, in sharp contrast to gastrin, indicating the role of the liver in clearing insulin from the blood. In the control perfusions without the liver the levels of both hormones did not change significantly. The fluctuation of gastrin levels observed during the high dose perfusions both in the presence and absence of liver may be due to some gastrin becoming adsorbed to surfaces in the perfusion apparatus and then dislodged by the mechanical action of the perfusion. The actual gastrin concentrations recorded in these experiments are

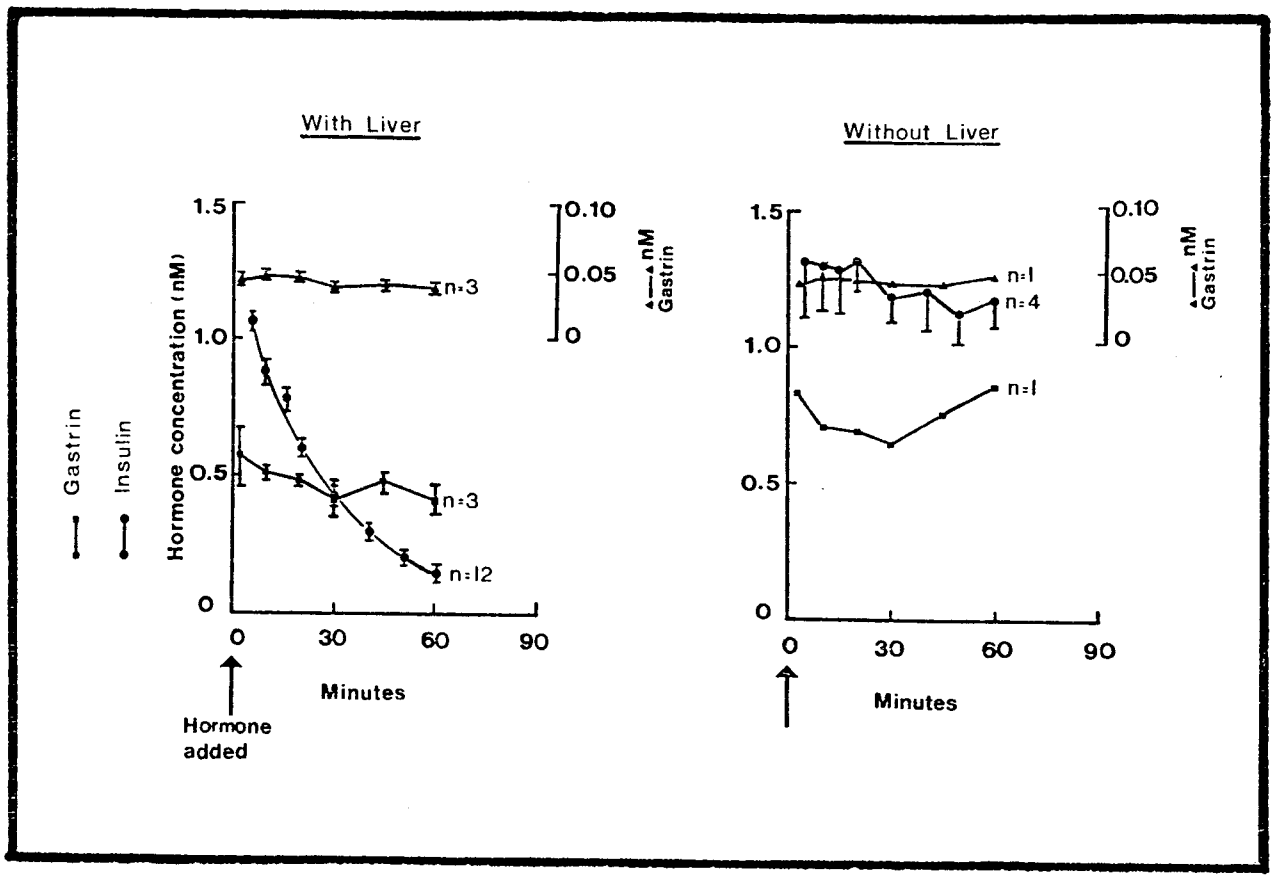


Fig. (4.V.(i).1.) Clearance of rat insulin and synthetic human heptadecapeptide gastrin from recycling perfusates with and without rat livers. Gastrin was perfused at high concentration (left hand ordinate scales) and at low or physiological concentration (right upper ordinate scales). Insulin was perfused at physiological concentration (left hand ordinate scales). Vertical bars indicate S.E.M.

shown in tables (4.V.(i).1) and (4.V.(i).2). There was no detectable gastrin in the samples taken 1 minute before addition of gastrin to the system.

The chromatographic elution profiles measured with antiserum G 1 obtained with samples collected at $2\frac{1}{2}$, 30 and 60 minutes after addition of the high dose of gastrin appear in figure (4.V.(i).2.). For the duration of the experiment gastrin remained as a single well-defined peak which corresponds with the elution volume of natural human heptadecapeptide gastrin type I. It is quite evident that no conversion to larger or smaller gastrin species occurred. The identical chromatographic profile was obtained when the procedure was repeated with samples collected during another perfusion. The recovery on the column of gastrin added was 75,8% for the $2\frac{1}{2}$ minute sample, 61,8% for the 30 minute sample and 44,2% for the 60 minute sample.

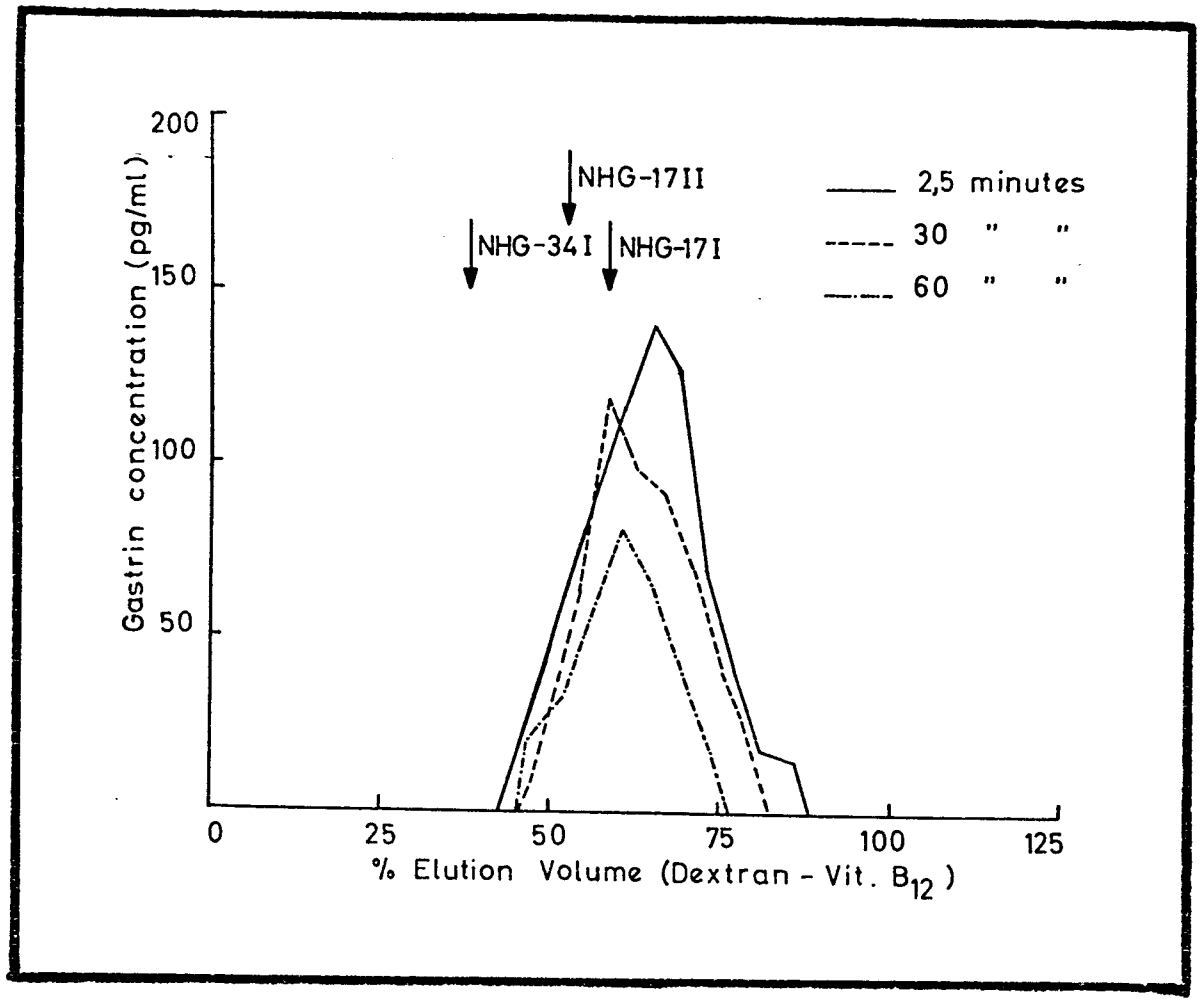


Fig. (4.V.(i).2.) Chromatographic elution profiles of gastrin in the perfusate of isolated rat liver at $2\frac{1}{2}$, 30 and 60 minutes after commencement of the perfusion, measured using antiserum G 1.

TABLE (4.V.(i).1)

Gastrin Levels During Supraphysiological Infusion

(a) With Liver

Time (min)		-1	2½	10	20	30	45	60
Expt. 1	pg/ml	U*	832	1040	1045	654	913	787
	nM		0,378	0,473	0,475	0,297	0,415	0,358
2	pg/ml	U	1650	1225	1150	1120	1078	863
	nM		0,750	0,557	0,523	0,509	0,490	0,392
3	pg/ml	U	1319	1123	1060	1000	1240	1167
	nM		0,600	0,510	0,482	0,455	0,564	0,530
pg/ml	$\bar{X}$	U	1267	1129	1085	925	1077	939
	S.E.M.		237,6	53,5	32,8	139,7	94,4	116,1
nM	$\bar{X}$	U	0,576	0,513	0,493	0,420	0,490	0,427
	S.E.M.		0,108	0,024	0,015	0,064	0,043	0,053

(b) Without Liver

Time (min)		-1	2½	10	20	30	45	60
	pg/ml	U	1841	1559	1538	1425	1670	1888
	nM		0,837	0,709	0,699	0,648	0,759	0,858

*U = undetectable

TABLE (4.V.(i).2)

Gastrin Levels During Physiological Infusion

(a) With Liver

Time (min)			-1	2½	10	20	30	45	60
Expt. 1	pg/ml		U*	80	98	94	88	80	65
	nM			0,036	0,045	0,043	0,040	0,036	0,030
2	pg/ml		U	106	107	94	71	90	85
	nM			0,048	0,049	0,043	0,032	0,041	0,039
3	pg/ml		U	102	100	100	90	94	100
	nM			0,046	0,045	0,045	0,041	0,043	0,045
pg/ml	$\bar{X}$		U	96	102	96	83	88	83
	S.E.M.			8,1	2,7	3,5	6,0	4,2	10,1
nM	$\bar{X}$		U	0,043	0,046	0,044	0,038	0,040	0,038
	S.E.M.			0,004	0,001	0,001	0,003	0,002	0,004

(b) Without Liver

Time (min)			-1	2½	10	20	30	45	60
	pg/ml		U	102	110	100	100	97	109
	nM			0,046	0,050	0,045	0,045	0,044	0,050

*U = undetectable

4 V(ii) In Vitro Binding of Gastrin to Rat Liver Ligandin

Figure (4.V.(ii).1.) shows the elution profile of rat liver supernate, BSP, and ^{125}I-albumen. The O.D.$_{280\ nm}$ profile shows three major protein peaks in the liver supernate, the middle one being known as the Y-peak. The Y-peak contains liver ligandin and its identity was confirmed by the binding of BSP to this fraction, as shown by the coincidence of the two peaks in the elution profile. Immunodiffusion studies against rabbit anti-rat liver ligandin confirmed the presence of ligandin in this peak (Fleischner, Mishkin et al., 1971).

Figure (4.V.(ii).2.) shows the elution profile obtained when ^{125}I-gastrin was added to the liver supernate and chromatographed. Approximately 80% of the radioactively-labelled gastrin was eluted in the peak containing ligandin (Kirsch, Vinik et al., 1975). This peak is clearly distinct from that obtained on chromatography of labelled gastrin alone, which eluted at approximately 3 times the void volume, and from ^{125}I-gastrin bound non-specifically to larger proteins, as shown by the small peaks preceding the main gastrin peak. The elution profile obtained when Na ^{125}Iodide was eluted with liver supernate showed some binding of radioactivity to the proteins in the large early peak, but none eluted in the region of the ligandin peak, confirming that it was actually the gastrin and not the ^{125}Iodine label that was bound by the ligandin.

Addition of excess unlabelled gastrin to liver supernate treated with ^{125}I-gastrin caused displacement of approximately half the labelled hormone from the ligandin peak, giving a recovery in this peak of less than 40% of the radioactivity applied. Measurement of gastrin content in the eluted fractions obtained on chromatography of liver supernate mixed with 15 ng cold gastrin produced a gastrin peak coinciding with the ligandin peak,

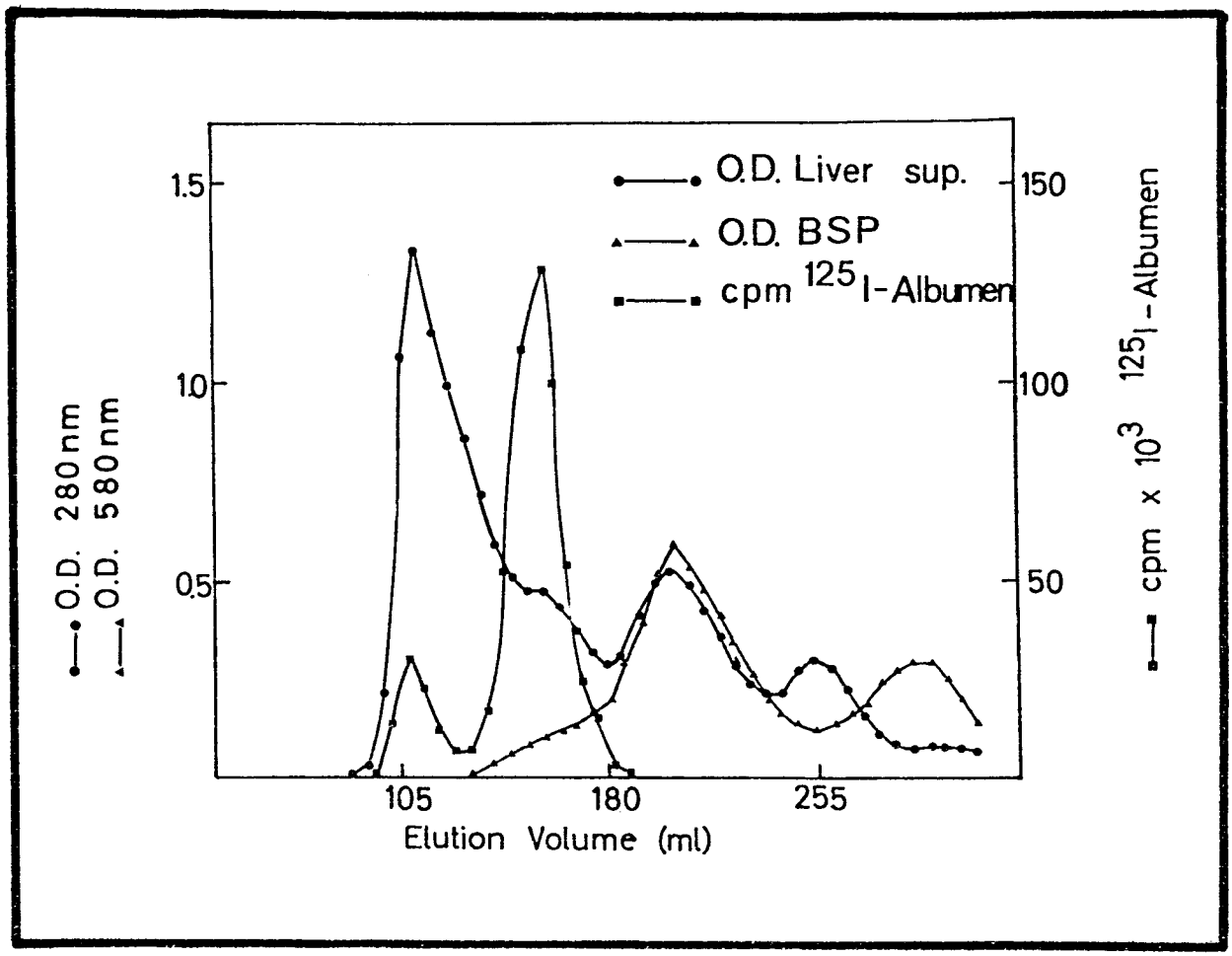


Fig. (4.V.(ii).1.) Sephadex (G-100) elution profile of 100 000 g supernate of a 25% homogenate of rat liver containing ^{125}Iodine - labelled albumen and Bromsulphthalein (BSP). For details of chromatographic procedure in this and subsequent 3 figures refer to section 3.V.(ii).in the text. In this and subsequent 3 figures O.D. represents optical density at the specified wavelength and cpm represents counts per minute of ^{125}Iodine in the eluted fractions.

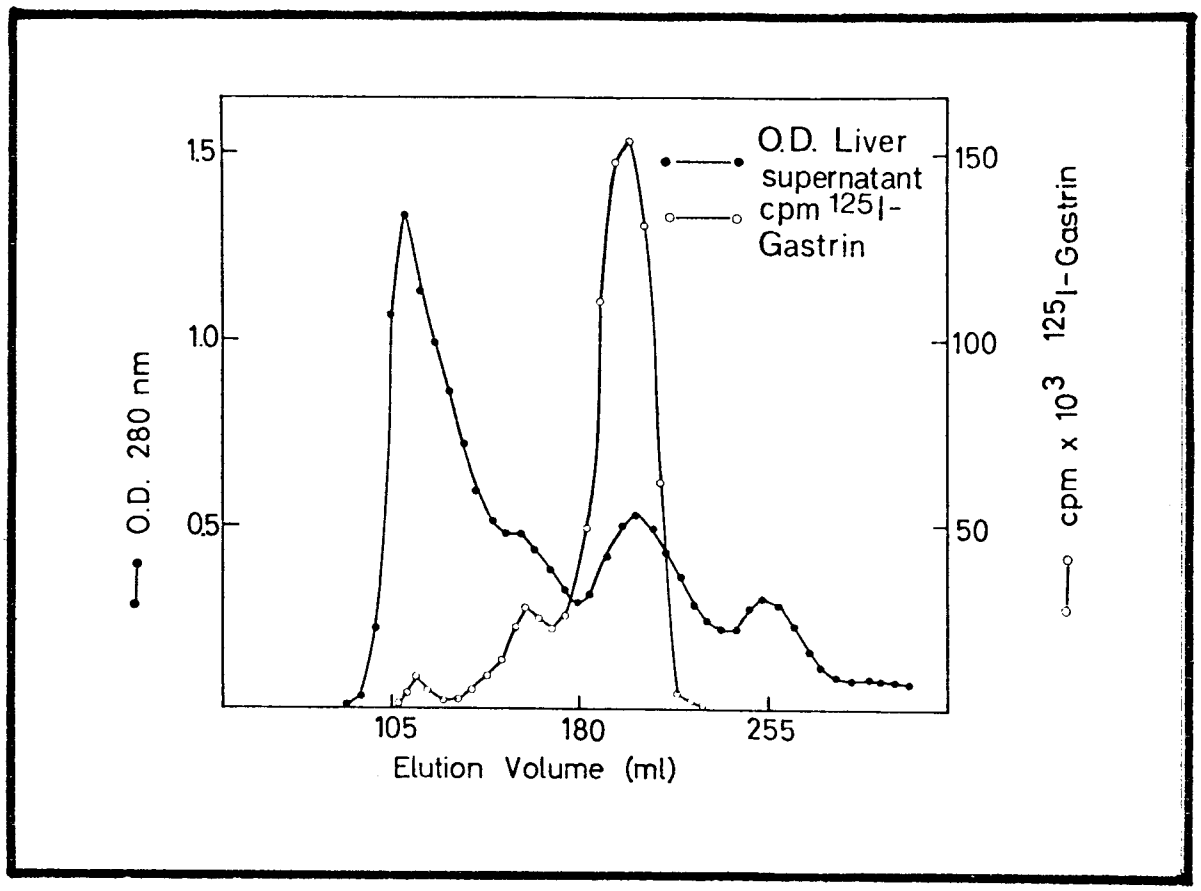


Fig. (4.V.(ii).2.) Sephadex (G-100) elution profile of a mixture of 100 000 g supernate of a 25% homogenate of rat liver and ^{125}Iodine - labelled heptadecapeptide gastrin I.

as shown in fig. (4.V.(ii).3.) (Kirsch, Vinik et al., 1975). The fact that it was possible to measure gastrin bound to ligandin by radioimmunoassay suggests that gastrin was bound to ligandin at a site other than the antigenic site on the heptadecapeptide molecule which is recognised by antiserum G 1. Since the antigenic determinant for antiserum G 1 lies in the (8-17) region of the heptadecapeptide molecule (Section 4 I(iv)), it is probable that ligandin binds to the N-terminal (1-8) region of heptadecapeptide gastrin.

Figure (4.V.(ii).4.) shows the chromatographic profiles obtained when ^{125}I-insulin and ^{125}I-glucagon were added to liver supernate and passed through the column at separate times. There was no evidence of binding of either of these hormones to ligandin. The small early peaks probably represent non-specific attachment of these labelled hormones to the larger proteins.

In summary, these studies show that rat liver ligandin binds radioactively labelled and non-labelled gastrin in vitro, the binding is to the gastrin itself and not to the ^{125}Iodine label, bound radioactive gastrin may be displaced by an excess of cold gastrin, and gastrin bound to rat liver ligandin may be measured by radioimmunoassay. This binding is specific for gastrin and was not observed with the other hormones tested in the same way, viz. insulin and glucagon.

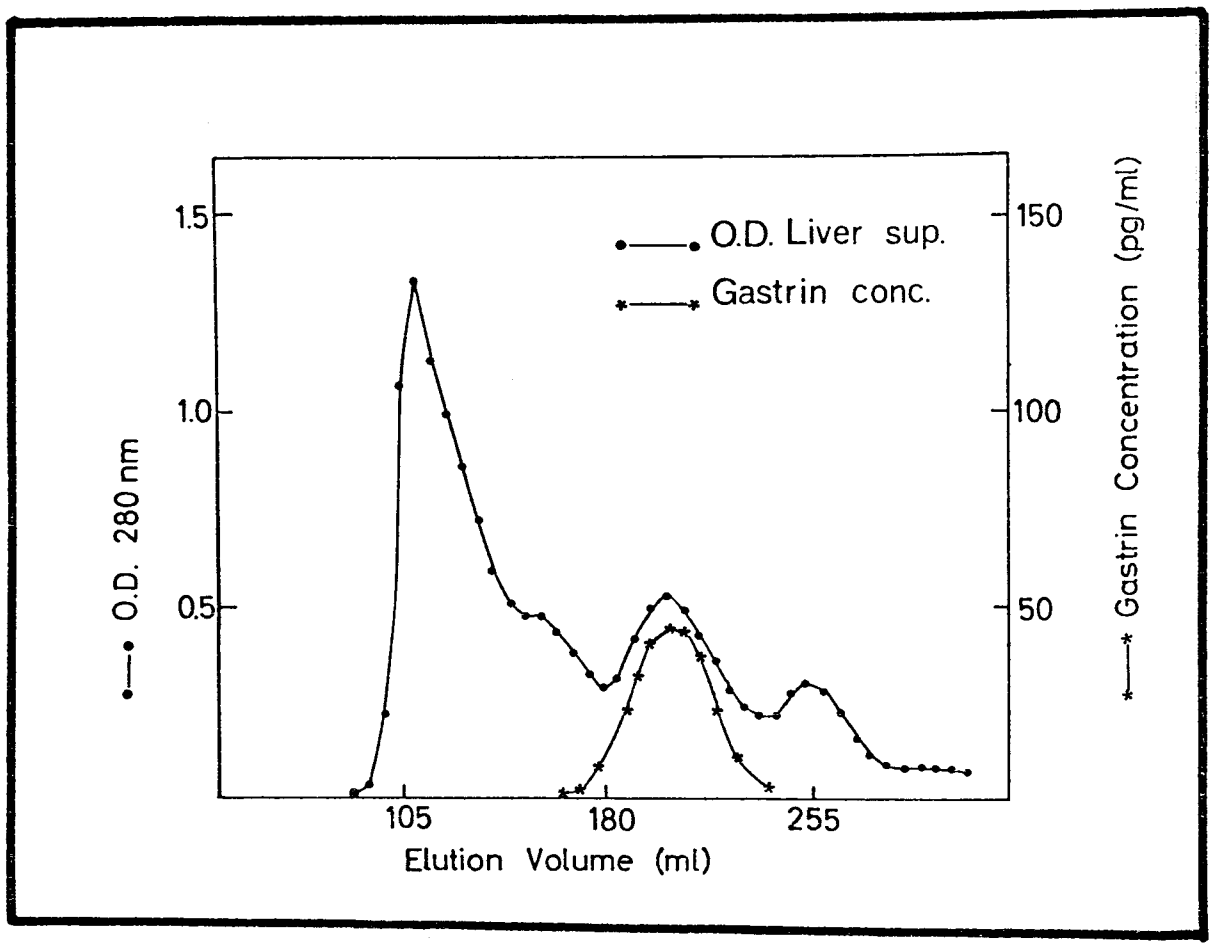


Fig. (4.V.(ii).3.) Sephadex (G-100) elution profile of 100 000 g supernate of a 25% homogenate of rat liver to which was added unlabelled heptadecapeptide gastrin I; gastrin in eluates was measured by radioimmunoassay using G 1 antiserum.

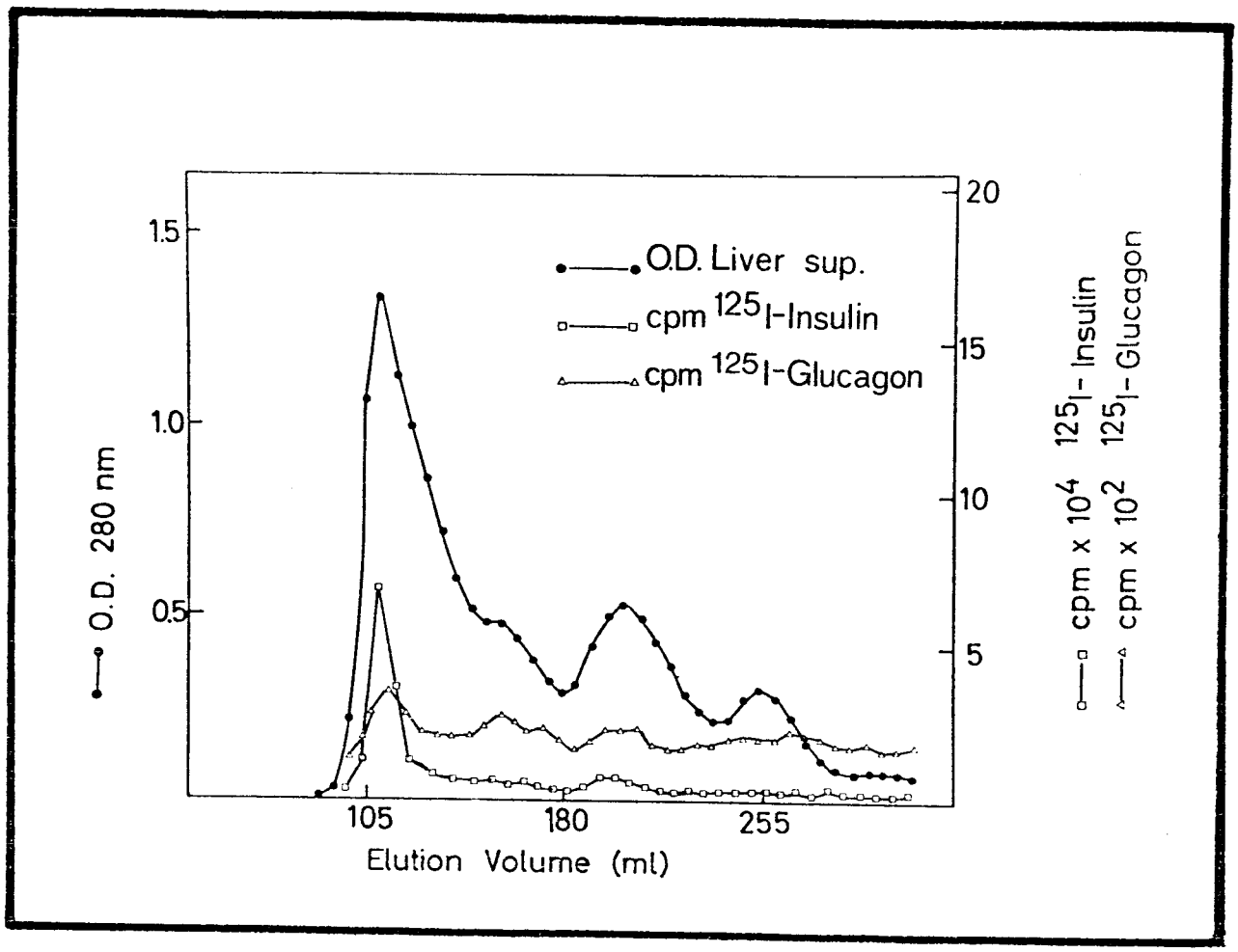


Fig. (4.V.(ii).4.) Sephadex (G-100) elution profile of a mixture of 100 000 g supernate of a 25% homogenate of rat liver, ^{125}Iodine-labelled insulin and ^{125}Iodine-labelled glucagon.

CHAPTER 5

DISCUSSION

Radioimmunoassay

There are several aspects of the radioimmunoassay which need to be discussed. These include the antisera, the iodinated hormone, validation of the assay and measurement of gastrin in biological fluids, which will be discussed in the sequence mentioned.

The Antisera

There does not appear to be one uniform immunization procedure that is suitable for all antigens and all species in which antibody production is attempted. Heptadecapeptide gastrin, being of small molecular size, is not very antigenic and must of necessity be linked to a larger carrier molecule, such as the haemocyanin used in this study, prior to injection. The coupled antigen was injected in an emulsion with Freund's complete adjuvant on each occasion via the subcutaneous route. Subcutaneous injections result in the formation of an emulsion depot at the site of injection with slow release of the antigen over a prolonged period, providing prolonged antigenic stimulation. Although it has been suggested that the subcutaneous route of injection should not be used for emulsions with Freund's complete adjuvant due to abscess formation (Weir, 1973), it was our experience that with careful treatment of the animals this regime led to the production of good antisera.

Raising antisera by immunizing laboratory animals is really a hit-and-miss affair and it is purely a matter of chance if an antiserum of sufficient titre and affinity for use in an immunoassay is obtained. It is necessary to

immunize several animals at a time in the hope that at least one will produce a suitable antiserum. This is upheld by the fact that Rehfeld, Stadil et al. (1972) obtained antibodies to gastrin in 23 animals out of a total of 113 immunized, and only three of these antisera had sufficiently high affinity to be used for measurement of circulating gastrin concentrations.

The antiserum obtained with each bleed from the same animal may vary in titre, affinity and capacity, which means that each bleed should be treated individually and characterised with regard to these parameters. The titre of an antiserum is a quantitative indication of the amount of antigen it can bind; it is determined as the final dilution of antiserum which binds 50% of the maximum of a constant dose of radioactive tracer hormone. The affinity refers to the avidity with which the antibody binds the antigen and is shown by the slope of the antibody-dilution curve or the curve of displacement of ^{125}I-labelled gastrin produced by increasing doses of unlabelled gastrin. The capacity of the antiserum is described by the concentration of binding sites, expressed in moles per litre, and is denoted by the percent binding of labelled hormone at a given dilution of antiserum. Given the optimum concentrations of labelled hormone and antiserum, the theoretical maximum binding obtainable would be 100%; however, such a situation is never realised due to steric factors relating to the three-dimensional conformational arrangement of the antibody and antigen, and to subtle changes induced in the antigen by the labelling process. The maximum binding obtained in the case of antiserum G 5 at 1:10 dilution was 63% and in the case of the third bleed of antiserum G 9 this value was 66% at 1:10 dilution.

The variation in titre, affinity and capacity between different bleeds was demonstrated in the case of antiserum G 9 in figure (4.1.1.). The increase in titre, capacity and affinity from the first to the second bleed is apparent. The fall-off in binding of ^{125}I-labelled gastrin, accompanied by a decrease in titre and affinity in the sixth and subsequent bleeds showed that the animal had become tolerant to the gastrin injections and was of no further use for antibody production.

The average affinity constants of antisera G 1, 2604-7 and G 5 were determined using the corresponding Scatchard plots as described in sections 3 I(iii) and 4 I(iii). As shown in table (4.1.2), antiserum 2604-7 had the highest value of K, the affinity constant, of $2,0 \times 10^{11}$ litres/mole, followed by antiserum G 1, with a K value of $4,0 \times 10^{10}$ litres/mole. The affinity constant of antiserum 2604-7 in our assay system was close to the values for this parameter determined by Rehfeld, Stadil et al. (1972) in the case of the third, fourth and fifth bleeds from this rabbit, which ranged between $1,79 \times 10^{11}$ and $6,67 \times 10^{11}$ litres/mole. Both antisera 2604-7 and G 1 were of sufficient affinity to enable measurement of basal levels of circulating gastrin as low as 8,2 and 16,5 pg/ml respectively.

Berson and Yalow (1959) obtained affinity constants for their antisera in the serum of insulin-treated human diabetic subjects in the range of 10^8 l/mol, which were used for detecting nanogram quantities of insulin in plasma. In the case of gastrin, the circulating levels are 1 000 fold lower; hence an antiserum with approximately a thousand times greater affinity than the insulin-binding antibodies of Berson and Yalow (1959) is required for sensitive measurement of basal values. Antisera 2604-7 and G 1 complied with these requirements.

Antiserum G 5 was found to be of low affinity, confirming that it was not entirely suitable for measurement of actual circulating gastrin levels. Hormone measurements with low affinity antisera such as G 5 lead to spuriously elevated values, a phenomenon which was encountered with the much higher serum gastrin values determined using antiserum G 5 in comparison with the values obtained using antisera G 1 and 2604-7. As shown in table (4.1.2), antiserum G 5 produces a much less sensitive radioimmunoassay than do the other two antisera and in fact it was the antiserum with the lowest titre as well. In view of these differences, antiserum G 5 was not used for measurement of gastrin concentrations in serum but was reserved for specificity studies investigating its cross-reaction with gastrin peptide fragments and related peptides.

The three antisera used in these studies, G 1, 2604-7 and G 5, were characterised with regard to their specificity for gastrin by extensive cross-reaction studies of these antisera with various peptides. Immunoreactivity studies using peptide fragments corresponding to portions of the heptadecapeptide gastrin molecule led to localisation of the antigenic site recognised by antiserum G 1 as lying in the (8-17) region, although a marked preference for the entire molecule was noted, whereas antiserum G 5 was specific for the (1-5) sequence of the heptadecapeptide.

Characterisation of antiserum G 1 with regard to its cross-reaction with other gut and gastrin-related peptides revealed minimal recognition of caerulein and the synthetic octapeptide of CCK-PZ, although at concentrations several orders of magnitude higher than the range of the assay. Failure of this antiserum to react with pentagastrin or CCK-PZ, coupled with the demonstration by Rehfeld, Stadil et al. (1972) that antiserum 2604 cross-reacted minimally with CCK-PZ and not at all with pentagastrin, meant that both antisera G 1

and 2604-7 could be used for measurement of immunoreactive gastrin in serum with the knowledge that any interference due to these related peptides was excluded. The cross-reaction of antiserum G 5 with these related peptides was not tested, which is an additional reason why this antiserum was not suitable for serum gastrin measurements.

Studies comparing the immunoreactivity of the natural gastrin preparations with that of synthetic human heptadecapeptide gastrin I revealed that w ith all three antisera synthetic human G-17 I was more reactive than natural human G-17 I. This may have been due to the fact that the natural preparations were not quite as pure as the synthetic ones. The same study revealed the specificity of G 1 antiserum for the non-sulphated heptadecapeptide gastrin species and the failure of this antiserum to recognise the sulphated heptadecapeptide, in contrast to the recognition of both sulphated and non-sulphated gastrin species by antiserum 2604-7. These cross-reaction studies were confirmed by the chromatographic studies using Sephadex G-25/G-50. The elution profiles obtained on gel filtration of NHG-34 I, NHG-17 I and NHG-17 II using antiserum 2604-7 revealed three peaks, one corresponding to each peptide chromatographed, whereas the profile measured with antiserum G 1 comprised only two peaks since the NHG-17 II peak was not detected.

An antiserum capable of distinguishing between the presence or absence of a sulphate group on the tyrosine residue of gastrin has been described previously. Hansky, Soveny et al. (1973) found that serum gastrin levels measured with their antiserum which was specific for both gastrins type I and II were significantly higher than levels measured using their antiserum that recognised only non-sulphated gastrin. Walsh, Trout et al. (1974) have

also described an antiserum, 1292, which distinguishes between sulphated and non-sulphated G-17.

The fact that an antiserum such as G 1 can discriminate between the presence or absence of a sulphate group on the tyrosine ring of a peptide gives some indication of how sensitive and specific the recognition of the antigenic site by an antibody must be. According to McGuigan (1968a) and McGuigan and Herbst (1975), the antibody-combining site on a peptide probably consists of a portion of the peptide backbone comprising approximately six amino acid residues. The localisation of the antibody-combining site of G 5 antibody to the N-terminal (1-5) sequence of the heptadecapeptide complies with this suggestion. The antigenic region recognised by antiserum G 1 was found to lie between residues 8 and 17; since it recognises the tyrosine in position 12 it follows that the antigenic site comprises a sequence which includes this residue.

Returning to the studies of cross-reaction of the natural gastrin peptides in the assay system, it was found that NHG-34 I was not recognised as strongly as NHG-17 I by either antisera G 1 or 2604-7, as shown in figures (4.1.10.) and (4.1.11.) and table (4.1.4). It appeared then that antiserum G 1 was specific for non-sulphated heptadecapeptide gastrin and it recognised non-sulphated big gastrin to a lesser degree. Antiserum 2604-7 reacted with both sulphated and non-sulphated heptadecapeptide gastrin and almost as strongly with non-sulphated big gastrin. This agrees with the report by Stadil and Rehfeld (1973a) that antiserum 2604 does not distinguish between the different serum gastrin components or between the different components and synthetic human gastrin.

With regard to antiserum G 5, a similar correlation of cross-reaction studies with the chromatographic data was found. The immunoreactivity studies showed failure of this antiserum to recognise NHG-34 I (figure (4.I.12.) and table (4.I.4)) and this was borne out by the elution profile obtained with this antiserum following chromatography of the three natural gastrin peptides (figure (4.II.4.)). Only two major peaks of immunoreactivity were found, corresponding to the elution positions of natural G-17 I and G-17 II.

Comparison of the specificity of our antisera with those in the literature suggests similarities between antiserum G 5 and antiserum 1295 described by Dockray (1975) and Walsh, Trout et al. (1974). Both appear to recognise the amino-terminal portion of heptadecapeptide gastrin, do not distinguish between sulphated and non-sulphated G-17, and do not detect the natural G-34 peptide.

Likewise, there is a similarity between the specificities of antiserum G 1 and antiserum L 6 described by Dockray and Taylor (1976), although not as striking as in the above instance. Both G 1 and L 6 are specific for heptadecapeptide gastrin, although G 1 recognises only non-sulphated G-17 whereas L 6 recognises both G-17 I and II. Although antiserum G 1 recognises G-17 I to a greater extent than G-34 I, it does show cross-reaction with big gastrin, whereas L 6 is absolutely specific for G-17.

Knowledge of the specificity of antisera permits manipulations such as subtraction of measurements of G-17, obtained using antiserum L 6, from "total" gastrin, estimated with antiserum 1296, to give an indirect estimation of big gastrin levels (Dockray and Taylor, 1976). Such estimations are the closest approximation to true measurement of the different gastrin forms that one can hope to achieve, short of chromatographic separation

of the gastrin components on a Sephadex column prior to measurement with antisera of known specificity. However, specific assays for these fragments are required for absolute quantitation of these fragments.

The two basic requirements for the establishment of a sensitive radioimmunoassay, capable of measuring basal circulating levels of a hormone are, firstly, an antiserum of high affinity, as has been mentioned, and, secondly, a labelled hormone preparation of high "specific activity".

The Iodinated Hormone

A tracer of high "specific activity" provides sufficient discharges per second to be detectable on a scintillation spectrometer with sufficient accuracy so that the content of hormone is lower than the levels one desires to measure in the assay, since the radioimmunoassay is basically competitive. This condition was satisfied in the assays performed for this study, in which case a discharge rate of 10 000 to 20 000 disintegrations per minute gave a count rate of 5 000 to 10 000 c.p.m. for 5 picograms of gastrin, assuming a 54% efficiency of counting. It was ideal to count 10 000 counts for each sample since this incurs a 1% error of counting.

Being a relatively stable molecule, it was not difficult to iodinate gastrin to a fairly high "specific activity", so that preparations with "specific activities" ranging from 700 to 1 800 μCi ^{125}Iodine per μg hormone were routinely prepared. The preparations with higher "specific activities" suffered from the disadvantage of being less intact, as determined by charcoal adsorption of intact labelled peptide. This "specific activity" was considerably higher than that used in radioimmunoassays described by other groups, as outlined in table (2.III.1).

Labelled preparations of high specific activity consist of a mixture of mono-iodinated and di-iodinated gastrins, from which it is possible to purify mono-iodinated ^{125}I-gastrin using AE-cellulose anion exchange chromatography as described by Dockray, Walsh and Grossman (1976). This separation of mono- and di-iodinated gastrins was not performed routinely for the purposes of this study. The "specific activity" of mono-iodinated ^{125}I-gastrin is between 900 and 1 000 μCi/μg according to the reports of Walsh (1974) and Ganguli and Hunter (1971), which suggests that preparations in this study with a "specific activity" of 1 500 μCi/μg consisted of approximately equal amounts of mono- and di-iodinated gastrin, since there is only one tyrosine residue in the heptadecapeptide gastrin molecule.

Four methods are generally available for testing the biochemical integrity of peptide hormones following labelling with radioactive iodine. These include: (i) chromatoelectrophoresis, (ii) trichloroacetic acid precipitation of the labelled peptide hormone, (iii) adsorption of the tracer with charcoal, and (iv) immunoprecipitation. For the purposes of this study methods (iii) and (iv) were found to be adequate for assessment of the biochemical and immunological integrity respectively, as described in section 3 I(ii). The technique of chromatoelectrophoresis on paper strips was found to be unsuitable for separation of intact and broken-down labelled gastrin, due to the paradoxical reversed behaviour of gastrin on paper strips. This phenomenon was also noted by Yalow and Berson (1970a) and is attributed to the highly acidic nature of the gastrin molecule.

A recurrent problem concerning preparations of iodinated hormones is whether the labelled product possesses biological activity, since this may be abolished during the iodination process in some instances. Although the biological activity of the labelled gastrin preparations used in this study

was not investigated, the demonstration of binding of ^{125}I-gastrin, with a "specific activity" of 1 440 μCi/μg, to the liver cytosol protein, ligandin (sections 3 V(ii) and 4 V(ii)) (Kirsch, Vinik et al., 1975) provides indirect evidence for the biological integrity of this labelled preparation.

The difficulty in preparing a biologically active preparation of ^{125}I-heptadecapeptide gastrin of high specific activity stems from the fact that during the iodination procedure with chloramine-T the methionine residue in position 15 becomes oxidized to the sulphoxide. Since methionine 15 is situated in the biologically active C-terminal tetrapeptide amide sequence (Tracy and Gregory, 1964), any change in this residue is likely to destroy the biological activity of the molecule. To circumvent this problem, preparations of 15-leucine gastrin have been synthesised (Wunsch) and used for iodination with ^{125}Iodine, as described by Dockray, Walsh et al. (1976).

Dockray, Walsh et al. (1976) compared the biological and immunochemical potency of mono- and di-iodinated natural human heptadecapeptide gastrin I and synthetic human 15-leucine gastrin which were purified by Sephadex G-10 chromatography, followed by gradient elution on AE cellulose. The ^{125}I-15-leucine G-17 I preparations were shown to be 50% more potent than the ^{125}I-G-17 I preparations in stimulation of acid secretion in dogs with gastric fistulae, although the immunochemical potency of the former was only 40% of that of G-17 I. However, there are several objections to this study, the most important being the dose of labelled gastrin which was used to produce the observed acid secretion. The dose was far in excess of the physiological range, and the measurements were made by bioassay, which has a sensitivity of approximately 15 ng (Smith, Lawrence et al., 1970). This is a million-fold greater than the dose of pentagastrin in the range of 5 femtograms/ml which has been shown to stimulate a biological

response in the parietal cell, using the cytochemical bioassay (Loveridge, Bloom et al., 1974).

Preparations of ^{125}I-gastrin have been used to demonstrate binding to receptors, as an indication of biological activity, in a couple of instances, such as in the study of Del Mazo and McGuigan (1976). Such studies of necessity involve labelled preparations with extremely high "specific activities", because a very small proportion of the total radioactivity is bound. As mentioned earlier, we have observed a decrease in integrity of the tracer with increased "specific activity".

Another group has resorted to the use of tritiated gastrin of high "specific activity" to demonstrate gastrin binding to receptors (Lewin, Soumarmon et al., 1976; Soumarmon, Cheret et al., 1977). No studies have yet been reported in which the binding of ^{125}I-15-leucine gastrin to receptors was studied; such an investigation may prove extremely useful in the field of gastrin-receptor interaction studies.

It is thus evident that the problem of obtaining labelled gastrin preparations of high "specific activity" which are biologically intact is not yet entirely resolved.

Having discussed the antiserum and tracer used in the radioimmunoassay, the question of validation of the assay will now be dealt with.

Validation of the Assay

With two high affinity antisera and a high "specific activity" tracer, the assay sensitivity obtained using antisera G 1 and 2604-7 was 16,5 pg/ml and 8,2 pg/ml respectively. This compares favourably with other reported gastrin radioimmunoassays. The lowest detectable gastrin

concentration which differed significantly from zero was of the order of 5 pg/ml in many cases (Hansky and Cain, 1969; Yalow and Berson, 1970a; Schrumpf and Sand, 1972; Feurle, Ketterer et al., 1972), while Stadil and Rehfeld (1971) obtained an assay sensitivity of 2 pg/ml.

By including internal standards in every assay the week-to-week assay variation could be monitored. The inter-assay coefficient of variation was high for assays involving all three antisera, ranging from 17,2% to 29,3%. Since this parameter is high in all cases it is likely that the variation is due to a factor common to assays involving all three antisera; variations in the quality of the commercially prepared ^{125}I-gastrin used in these assays is the probable explanation.

The intra-assay coefficient of variation, an indication of the precision of the assay, was high only in the case of assays involving antiserum 2604-7, in which case a value of 17,7% was obtained. Such variation could be attributed to pipetting errors or possibly the nature of the assay diluent buffer, but since these were common to assays involving all three antisera and yet the variation occurred in only one instance, this points to the fact that the variation may involve the antiserum. Perhaps the incubation period of 72-96 hours used for assays involving this antiserum was not long enough for the antibody-antigen reaction to reach equilibrium, in which case conditions in the assay would be unstable and could account for variation between samples in the same assay. However it was noted that Stadil and Rehfeld (1973a) used an incubation period of 48 hours with antiserum 2604 at 5°C. Since this group did not specify the bleed of this antiserum used in this study it may be that these differences can be attributed to differences between bleeds from the same animal. Alternatively it may be that we have not optimized the 2604-7 assay.

The variation in the standard curve produced by different incubation times and temperatures was illustrated in the case of antiserum G 1 as shown in figure (4.1.2.). Such variations may occur on a smaller scale during the separation of free and bound labelled hormone in an assay involving several hundred tubes, due to the time lag between addition of the separating reagent to tubes early and later in the assay. Assay tubes situated near the end of an assay may stand for 30 to 45 minutes at room temperature before addition of charcoal suspension, for example, during which time the antibody-antigen equilibrium may well be disturbed. With this in mind it is good practice to include a standard curve every 200 tubes in large assays and to read the results for the different tubes from the corresponding curves, to reduce intra-assay variation due to this phenomenon.

The index of precision, determined as the coefficient of variation of the ID_{50} value of each standard curve, gives an indication of how the mid-point of the standard curve varies from week to week. It is interesting that assays involving antiserum 2604-7 varied least from week to week, with an index of precision of 13,9%, although the highest within assay variation was encountered in assays involving this antiserum.

The best correlation between observed and expected values was found for assays involving antiserum G 5 with a value for r, the correlation coefficient, of 0,973. This good correlation was due to the fact that the standard curve obtained with G 5 antiserum is sensitive at high levels of added gastrin standard, under which conditions greater stability is obtained. This is borne out by the poorer correlation between observed and expected values found in the case of antisera G 1 and 2604-7, which measure lower concentrations of gastrin.

In keeping with reports in the literature of variations in immunochemical potency between different batches of synthetic human gastrin obtained from I.C.I. (Yalow and Berson, 1970a; Stadil and Rehfeld, 1972), such variations were noted in studies in this laboratory as well. In addition, it was shown that the I.C.I. preparation of SHG (1-17) was approximately half as potent immunochemically as the preparation obtained from CEA-IRE Sorin (Table (4.1.4)). In view of the variations in potency of I.C.I. preparations of SHG-17 I, this peptide was only used in the immunoreactivity studies specified. For the purposes of all regular assays the CEA-IRE Sorin preparation of SHG-17 I was used.

The establishment of this radioimmunoassay system enabled measurement of gastrin concentrations in several types of biological fluid, as discussed below.

Measurement of Gastrin in Biological Fluids

Measurements of gastrin concentrations were made by radioimmunoassay in various biological fluids during the course of this study. These included human and porcine serum as well as serum from other vertebrate species, aqueous extracts of gut tissues of various species, fractions eluted from Sephadex columns in 0,05 M - veronal buffer, and the perfusion medium with which the isolated rat liver was perfused in situ. An unsuccessful attempt was made to measure gastrin in pig bile which probably failed due to interference by bile salts in the assay.

No difference was found between measurements made in human plasma or serum, and in practically all cases serum was used. It was imperative to rule out any interference in the assay by factors in the sample, which may give rise to erroneous readings, as discussed by Berson and Yalow (1959)

in the case of insulin radioimmunoassay. In some instances enzymes present in plasma or serum may degrade the labelled or unlabelled hormone; glucagon is susceptible to such degradation, so in these cases the protease inhibitor, Trasylol, is included in the incubation mixture. This was not necessary in the gastrin assay. In all assays, control tubes in which antiserum was omitted, were included with serum samples from each patient or samples of the other biological fluids mentioned, to monitor non-specific binding of the tracer by factors in the sample and to allow correction for any damage to the labelled hormone on incubation.

Prior to separation of free and antibody-bound labelled gastrin by charcoal adsorption, the serum content of the standard curve was equalized with that of the rest of the serum samples in the assay by addition of an equivalent quantity of serum which had previously been treated with charcoal to remove all peptide hormones - such serum is referred to as "gastrin-free". In the same manner the serum content of samples containing column eluates, gut tissue extracts and perfusion medium was equalized with that of normal serum before commencing the separation procedure.

The preparation of serial dilutions of a serum sample or other unknown sample and comparison of the shape of the displacement curve produced by such dilutions with that obtained with the purified standard hormone preparation is the best method available for ascertaining how closely the hormone in the unknown sample resembles the standard. Parallelism of the two curves suggests that the material in the biological sample resembles the standard preparation, since they behave immunochemically identically. By coupling such observations on the immunological behaviour of a sample with the elution profile of the same sample obtained on Sephadex chromatography, which gives an indication of molecular size, a good indication of the

species of gastrin present in the sample is obtained. This procedure was used many times in this study, such as in the characterisation of the gastrin present in hypergastrinaemic serum and human tissue extracts, and of the material found in frog serum and skin extracts.

Although the procedure described above gives a good indication of the nature of the material in the unknown sample, only extraction of the material in sufficient quantity from serum or biological fluid for purification and determination of the amino acid sequence will provide the ultimate demonstration of identity of the material with the standard hormone preparation.

Turning to the recovery of gastrin in tissue extracts, it was shown that straight boiling of gastrin in water alone led to 77% recovery of added gastrin, whereas the recovery of gastrin following boiling in the presence of control tissue in the form of rat thigh muscle was 43%, as shown in table (4.1.6). Since the recovery of a known amount of gastrin added to individual tissues before boiling was not assessed, one had to assume that the recovery of extracted gastrin from the different tissue extracts was similar, so that comparison of the gastrin content of various tissues from different species could be made. Tissues such as pancreas contain proteolytic enzymes which would inactivate any hormone present in the tissue; consequently the tissues were boiled as soon as possible after removal from the animal in the hope of inactivating these enzymes. As was shown in section 4 I(vi), the addition of Trasylol (protease inhibitor) had no effect on the recovery of gastrin added to boiled muscle extracts, justifying its omission in the preparation of most tissue extracts.

In the next section, actual serum gastrin measurements in control subjects and patients with various disease conditions are discussed.

Serum Gastrin Measurements in Health and Disease

The mean fasting serum gastrin level in control subjects of 48,5 pg/ml is close to the values found by Schrumpf and Sand (1972), Feurle, Ketterer et al. (1972) and Walsh (1974) and agrees with the general finding of normal levels below 100 pg/ml.

In contrast to the findings of McGuigan and Trudeau (1970a) no direct relationship between increasing age and fasting serum gastrin levels was noted in this study. However, the population of patients investigated in this study included both normal subjects and patients suffering from various complaints, whereas the group investigated by McGuigan and Trudeau (1970a) comprised only normal subjects, which may account in part for the discrepancy. Neither Ganguli and Hunter (1972) nor Stadil and Rehfeld (1973a) could confirm the findings of McGuigan and Trudeau (1970a).

Significantly higher serum gastrin levels in females than in males were noted in this study, in contrast to the reports of Ganguli and Hunter (1972) and Stadil and Rehfeld (1973a). This discrepancy may be due to differences in specificity of the antisera used, as discussed below. The fact that serum gastrin levels in three ethnic groups were not significantly different in this laboratory suggests that serum gastrin is probably not related to the quality of dietary intake.

The rise in serum gastrin levels in control subjects after oral ingestion of Oxo with a maximum 45 minutes after eating agrees with the findings of peak postprandial gastrin levels between 30 and 60 minutes after eating reported by Ganguli (1970) and Korman, Soveny et al. (1971). The peak gastrin levels reported by these two groups were approximately five times basal, whereas the peak response to Oxo found here was only twice basal

(Vinik, Kalk et al., 1975). Since Oxo consists of hydrolyzed protein one may expect a slightly different response to that found with ordinary food. However, Forrester and Ganguli (1970) demonstrated postprandial gastrin levels of four to five times basal following oral administration of Oxo. The reason for the lower postprandial gastrin levels in this study may well lie with the specificity of antiserum G 1 used for these measurements. Since this antiserum measures only non-sulphated gastrins, chiefly of the heptadecapeptide or smaller size, any sulphated gastrins released in this situation would not have been detected by this antiserum.

The elevated serum gastrin levels found in patients with duodenal ulcer and the normal levels in gastric ulcer are at variance with most of the reports in the literature, in which the opposite situation was found, as outlined in section 2 III. The only group whose findings agree with ours is that of Byrnes, Young et al. (1970), but their normal levels are so high (400 pg/ml), probably due to a poor quality tracer or low affinity antiserum used in the assay, that these values do not form a good basis for comparison. The reason for the differences between our findings and those in the literature may be due to the specificity of G 1 antiserum, as previously mentioned. In support of this suggestion is the report of differences in serum gastrin levels measured in normal subjects and ulcer patients with two antisera showing different specificities for sulphated and non-sulphated gastrins by Hansky, Soveny et al. (1973).

We have no idea whether sulphated and non-sulphated gastrins are secreted in the same proportions under normal and pathologic conditions; the results of this study underline the importance of determining the specificity of the antiserum used for serum gastrin measurements under various conditions. Although it is known that the presence of a sulphated tyrosine residue on the CCK-PZ

molecule is essential for biological activity, whereas biologically active gastrin occurs naturally in both sulphated non-sulphated forms, the significance of sulphation of the tyrosine residue in type II gastrins is poorly understood at present.

Extremely elevated serum gastrin levels were found in patients with Zollinger-Ellison syndrome and pernicious anaemia, although the pattern of these levels differed from that found by other groups in that the levels in cases of pernicious anaemia were sometimes ten fold greater than those in Zollinger-Ellison syndrome. Serum gastrin levels measured in patients with Zollinger-Ellison syndrome in this study were only of the order of 300 pg/ml, whereas levels reported in the literature ranged from 1 ng/ml to 94 ng/ml (Schrumpf and Sand, 1972; Yalow and Berson, 1970a; Ganguli and Hunter, 1972).

Again the failure of antiserum G 1, used for these estimations, to measure sulphated gastrins may account for this discrepancy. Many circulating gastrin fragments in this syndrome may not be detected by antiserum G 1, which could account for the fact that only 4 cases of Zollinger-Ellison syndrome were detected in this hospital over a period of 3 years. This incidence is much lower than that generally found, and it is felt that had antiserum 2604-7 been used for these estimations a completely different picture may have emerged.

Serum gastrin estimations in patients with pernicious anaemia gave a mean value of approximately 3 000 pg/ml; this is in line with the values reported by other groups, which fell between 300 and 9 000 pg/ml, the range quoted by Yalow and Berson (1970a). This may indicate a difference between the forms of gastrin circulating in pernicious anaemia and Zollinger-Ellison syndrome, since a much greater proportion of non-sulphated heptadeca-

peptide gastrin was detected using antiserum G 1 in the former condition.

The finding that antrectomised patients had undetectable gastrin levels when measured with antiserum G 1 suggested that the gastrin secreted by the human antrum is chiefly the non-sulphated heptadecapeptide species and that the antrum appears to be the primary source of non-sulphated heptadecapeptide gastrin in the human. The former suggestion is borne out by the immunological identity of the material in the serum of a patient with isolated retained antrum with the G-17 I standard, as shown by the parallel displacement curves obtained in figure (4.1.13.).

The marked stimulation of gastrin release by intravenous arginine and the relatively poor response to oral arginine in normal subjects (Vinik, Kalk et al., 1975) suggests that a high concentration of arginine in the blood can stimulate gastrin release. The way in which arginine produces this effect has not been definitely established. Arginine may act as a general non-specific stimulant of hormone release, since it has been shown to stimulate the release of glucagon (Ohneda, Parada, Eisentraut and Unger, 1968), growth hormone (Merimee, Lillicrap and Rabinowitz, 1965), and insulin (Fajans, Floyd, Knopf and Conn, 1967). Alternatively, the release of gastrin may be brought about by electrolyte changes in the G-cell. It has been shown that intravenous arginine produced a significant rise in red blood cell potassium in subjects who secreted insulin in response to arginine, suggesting that a similar event may occur in pancreatic beta cells (Vinik and Jessop, 1974). Perhaps a similar event occurs in the G-cell in response to arginine.

The behaviour of serum from a patient whose gastrin levels had been stimulated by administration of intravenous arginine on dilution in the assay was identical with that of the standard G-17 I, as shown in figure (4.1.7.),

suggesting that intravenous arginine stimulated the release of heptadecapeptide gastrin I from the antrum.

Failure of antrectomised or vagotomised and partially antrectomised patients to respond to intravenous arginine, as measured with antiserum G 1, again supports the suggestion that heptadecapeptide gastrin is chiefly antral in origin. In addition, these findings suggest that the effect of arginine in stimulating antral gastrin release is mediated by the vagus.

An account of the findings relating to the heterogeneity of gastrin in human serum follows in the next section.

Gastrin Heterogeneity in Human Serum

The relative proportions of the various molecular gastrin species in serum may vary in normal subjects and patients with different hypergastrinaemic conditions. Even in the same individual, the total circulating gastrin content does not comprise the same proportions of the different gastrin types at all times; differences in turnover rates of the gastrin subtypes in the circulation, and the release of different components following stimuli such as ingestion of food, are factors affecting the relative distribution of gastrin components in the circulation. Hence the characterisation of gastrin subtypes in serum under various conditions reflects the situation at the time of collection of the blood sample.

Gastrin heterogeneity in human serum was studied using serum collected from a patient with pernicious anaemia in the basal state. This sample was chromatographed in the early stages of the study, before the preparations of natural human gastrins donated by Dr. M.Grossman had been received. The elution profiles in this instance and in the case of the human gut tissue extracts were plotted as actual elution volume in millilitres against $B/B_o \times 100$, as defined in section 3 I(iii), an indication of the relative peptide content of the eluted fractions. The molecular weight markers used were albumen and ^{125}I-SHG-17 I.

In later chromatographic procedures the abscissa was plotted as a percentage of the elution volume between blue dextran and Vitamin B_{12}, which were chromatographed with each sample to provide standardization of elution profiles performed on different occasions. Vitamin B_{12} is a small molecule with a molecular weight of 1 355 and is bright red in colour. It was more convenient to use this substance, which eluted in a volume almost as large

as the salt volume of the column, as shown in figure (4.II.1.), in preference to ^{22}Na as an index of total volume, since the position of Vitamin B_{12} in the eluted fractions could be determined by visual inspection as opposed to counting the radioactivity in the eluted samples. Likewise, the elution position of blue dextran was determined visually. Comparison of elution profiles plotted in this manner with the profiles of the natural human gastrins G-34 I, G-17 I and G-17 II chromatographed on the same Sephadex column allowed identification of the gastrin components found in the samples under investigation. Although such comparisons could not be made in the case of earlier chromatographic profiles, the fact that the position of the ^{125}I-SHG-17 I marker in the elution profiles in figures (4.II.2.), (4.II.3.) and (4.II.4.) corresponded exactly with that of the NHG-17 I peptide suggests that the ^{125}I-gastrin marker used in earlier fractionations was a reliable indicator of elution volume of heptadecapeptide gastrin type I.

As was mentioned in section 3 II, the chromatographic profiles in this study were obtained after one fractionation on a Sephadex column, which did not provide complete resolution of the different gastrin components. However, the separation was good enough to permit identification of the various gastrin types in chromatographic profiles which were compared with the natural gastrin standards.

Bearing these factors in mind, complete identification of the immunoreactive gastrin peaks in figure (4.II.1.) was not possible; their probable identities had to be inferred from their elution positions relative to the albumen, ^{125}I-SHG-17 I, Vitamin B_{12} and ^{22}Na markers. The elution profile in this figure was obtained on chromatography of fasting serum from a patient with pernicious anaemia, followed by estimation of the gastrin content of the eluted fractions using antiserum G 5. The paired peaks

are thought to represent sulphated and non-sulphated gastrins, since this antiserum recognised both these forms (section 4 I(iv)). A small amount of minigastrin appeared to be present, as well as some material eluting in the region of the void volume.

Calibration of the Sephadex column with the natural gastrin standards showed that the sulphated type II heptadecapeptide gastrin eluted before the non-sulphated species, and the degree of separation between the two was greater than would be expected on the basis of molecular weight alone. This may be due to a weak ion exchange effect between the charged sulphate group and the column matrix, as discussed in section 3 II. The emergence of sulphated G-17 before non-sulphated G-17 on Sephadex gel filtration has also been reported by Rehfeld, Stadil et al. (1974) and Dockray and Walsh (1975).

Although the primary structures of big, heptadecapeptide and minigastrin have been determined, component I and "big big" gastrin have not yet been chemically characterised. It has been established that tryptic digestion of component I liberates heptadecapeptide gastrin, and material apparently corresponding to serum component I has been identified and partially purified from extracts of porcine antral mucosa and gastrinoma tissue (Gregory, 1976). It thus appears that component I is a real entity.

The existence of "big big" gastrin is more doubtful in the light of the demonstrations by Rehfeld, Stadil et al. (1975) and Dockray (1975) that "big big" gastrin detected in serum may actually be due to association of smaller gastrin molecules with large serum proteins, producing a large complex which elutes in the void volume on Sephadex columns and behaves immunologically like gastrin. This is further substantiated by the detailed

study of Rehfeld, Schwartz and Stadil (1977), demonstrating the presence of macromolecular gastrin species in two out of four gastrinoma extracts, but not in normal serum or antral, duodenal or jejunal tissue extracts. The phenomenon of non-specific binding of smaller gastrins to plasma proteins may have produced the first peak of gastrin immunoreactivity in the elution profile shown in figure (4.II.1.), which had originally been thought to represent "big big" gastrin. This controversy will only be resolved if and when "big big" gastrin is isolated and characterised entirely.

Malagelada (1977) has proposed that a large form of gastrin, which is biologically inactive, occurs in the circulation of some patients from his findings of very elevated serum gastrin levels in 5 patients who, on the basis of their serum gastrin levels, would be classified as Zollinger-Ellison cases, although they did not present with the clinical features of the Zollinger-Ellison syndrome. These high serum gastrin measurements were obtained with only one antiserum, whereas measurements using another antiserum with a different region specificity for gastrin revealed normal levels. In addition, none of these patients responded with increased serum gastrin levels to infusions of calcium or secretin as normally happens in the case of patients with Zollinger-Ellison syndrome. On Sephadex G-50 chromatography this large gastrin component eluted as a single peak between the void volume and the position of G-34. Incubation with trypsin converted this component to a fragment with the elution profile of G-34 and in one case to a fragment which eluted in the region of G-17. It thus appears that this large species of gastrin described by Malagelada may be an inactive precursor form which contains gastrin fragments resembling G-17 and G-34 within its structure. It may or may not be a separate entity to "big big" gastrin and component I. This accidental finding may provide a clue to

the possible role of these big gastrins as precursors of the biologically active gastrins, similar to proinsulin and insulin.

It is thus apparent that the relationship between the different gastrin components in serum is far from well understood, as is the case for the relationship between serum and tissue gastrin components. Isolation and purification of different gastrin types from normal antral mucosa and tumour tissues has allowed chemical characterisation of these components. Only when the various gastrin components can be isolated from serum in sufficient amounts for determination of their primary structure can the identity of gastrins in serum and tissues be confirmed and their interrelationships better understood.

In the next section the heterogeneity of gastrins in tissue will be considered.

Gastrin Heterogeneity and Distribution in Human Tissues

Chromatographic profiles obtained on fractionation of aqueous extracts prepared from biopsies of human antral and duodenal mucosa differed, in that a higher proportion of the heptadecapeptide component was found in the antral extract. The peak eluting in the void volume on chromatography of both these extracts (figures(4.III.2.) and (4.III.3.))was tentatively referred to as "big big" gastrin. This agreed with the report of Rehfeld, Stadil et al. (1974), who identified a heterogeneous "big big" gastrin component in extracts of antral mucosa and gastrinoma tissue. However, the recent demonstration by Rehfeld, Schwartz et al. (1977) that a macromolecular gastrin species was present in gastrinoma tissue only and not in normal tissue extracts emphasises the fact that this aspect requires further clarification.

The immunoreactive gastrin in these extracts was resolved into peaks which eluted in a biphasic manner, as shown in figures (4.III.2.) and (4.III.3.). This reflects detection of both sulphated and non-sulphated gastrins by antiserum G 5. As was shown in sections 4 I(iv) and 4 II(ii), this antiserum recognises both gastrin types.

Confirmation of the difference between antral and duodenal gastrins was further demonstrated by their differences in immunoreactivity when measured with antisera G 1 and G 5. Both extracts produced parallel displacement curves when assayed with antiserum G 5, although measurement with antiserum G 1 revealed differences in immunoreactivity between the two extracts. The curve of displacement of ^{125}I-labelled gastrin from antiserum G 1 obtained with the duodenal extract was not parallel with that of the G-17 I standard, indicating non-identity of duodenal gastrin and pure

heptadecapeptide gastrin type I.

The higher proportion of immunoreactive heptadecapeptide gastrin in antral as opposed to duodenal extracts (Vinik, Grant et al., 1975) is in agreement with the findings of Berson and Yalow (1971) and was confirmed by Rehfeld, Stadil et al. (1975), Malmstrøm, Stadil et al. (1976) and Creutzfeldt, Arnold et al. (1976). The findings of Lai (1964b) and Elwin and Uvnäs (1966) that the biological activity of antral gastrin was greater than that of duodenal gastrin is in keeping with the demonstration that heptadecapeptide gastrin, which predominates at this site, is the most biologically potent form of gastrin (Walsh, Debas et al., 1974; Debas, Walsh et al., 1974).

The demonstration that the elevated serum gastrin levels produced in response to intravenous arginine infusion are due chiefly to an increase in the heptadecapeptide and smaller gastrin components (Kalk, Vinik et al., 1973), and that antrectomy abolished the response to arginine (Vinik, Kalk et al., 1975) provides additional evidence that the antrum is the primary source of heptadecapeptide gastrin. These findings suggest that the duodenum is a relatively unimportant source of heptadecapeptide gastrin released in response to arginine, but does not exclude the duodenum as a source of "gastrins" when other untested provocative substances are used.

The negligible levels of gastrin found in extracts of the cardia and body of the human stomach is in keeping with the findings of Malmstrøm, Stadil et al. (1976). The gastrin content of the body of the stomach was 1,3% of that in the antrum, which agrees closely with the value of 0,5-1% found by Malmstrøm, Stadil et al. (1976).

The actual tissue content of gastrin measured in human antral extract was 1,42 µg/g dry weight, or 2,034 µg/g total protein, as shown in table (4.III.1). These values are far lower than those found by Berson and Yalow (1971), Rehfeld, Stadil et al. (1975) and Creutzfeldt, Arnold et al. (1976), who found 30 µg/g, 28,38 µg/g and 15,9 µg/g frozen tissue respectively in human antral mucosa. Gastrin measurements in the proximal duodenum agreed more closely, since Rehfeld, Stadil et al. (1975) detected 3,96 µg/g and Creutzfeldt, Arnold et al. (1976) found 1,8 µg/g, in comparison with the values of 0,97 µg/g dry weight or 1,432 µg/g protein in the duodenal cap found in this study.

The duodenal gastrin content was 68-70% of that in the antrum in the duodenal cap, with a lower proportion, 27%, in the biopsy taken from the second part of the duodenum. These measurements reflect chiefly the non-sulphated gastrin content since the estimations were made with antiserum G 1. This high ratio of duodenal to antral gastrin content per unit weight of tissue contrasts with the findings of Malmstrøm, Stadil et al. (1976) who showed the proximal duodenal gastrin content to be 10% of that of the antrum, and that in the distal duodenum to be 1-2% of that in the antral mucosa. Creutzfeldt, Arnold et al. (1976) also found approximately 10% of the antral immunoreactive gastrin content in the proximal duodenal mucosa of control subjects. The biological gastrin activity in the duodenum was likewise found to be 10% of that in the antrum by Elwin and Uvnäs (1966), although the corresponding value found by Emås, Borg and Fyrö (1971) was approximately 33%.

In looking for a reason for the differences between absolute values of tissue gastrin content and the relative proportions of antral and duodenal immunoreactive gastrin content between this study and those of Malmstrøm,

Stadil et al. (1976) and Creutzfeldt, Arnold et al. (1976), several possibilities arise. The gradient of immunoreactive gastrin activity along the duodenum found in this study and that of Malmstrøm, Stadil et al. (1976) may help to explain the discrepancies, since the exact site of sampling may be different.

An alternative explanation derives from the fact that the estimations of gastrin content in this study were made relative to dry weight or total protein content of the tissue, whereas other groups expressed the gastrin content relative to the frozen weight of the tissue, although, if anything, one would expect the gastrin content per unit frozen tissue to be lower than that expressed per unit dry weight of tissue. In this study the gastrin content relative to the dry weight of tissue was always less than that expressed relative to the total protein (table (4.III.1)), as would be expected, and the relative gastrin content at different sites agreed very closely when expressed either way, indicating consistency in the results expressed in either manner.

No estimations of total antral or duodenal gastrin content were made in this study. The total mass of extractable immunoreactive gastrin in the human duodenum was 97% of that of the antrum according to Nilsson, Yalow et al. (1973) and the total extractable biologically active gastrin in the human duodenum was actually greater than that in the antrum as determined by Lai (1964b). This only applies in the case of the human, suggesting that duodenal gastrin in man may differ from that in other species. Although no estimations of total duodenal gastrin content were made for the purposes of this thesis, the relative duodenal gastrin content in man was found to be far higher than that in the rabbit, guinea pig, dog or pig, although the duodenal gastrin levels in the rat were similar to the value

found for the human.

Hence differences in extraction techniques could have resulted in marked differences in yield. The relatively lower yields of tissue gastrin content found here may be due to the fact that the tissues were not homogenised after boiling, in which case some of the gastrin may have remained in the tissue, although it was thought that cutting the tissue into small pieces before boiling in the case of the animal tissues (the human tissue biopsies were very small to start with) would have been sufficient to liberate the gastrin present. As shown in section 4 I(vi), the recovery of gastrin added to control muscle tissue treated in this manner was 43%. Even allowing for recovery of less than half of the gastrin in the tissues, the estimated gastrin content of the antrum was less than that observed by other authors.

Another factor contributing to the apparently lower yield found in this study compared with that found by other workers may be the fact that the tissues were not frozen as soon as they were removed from the animal, but were merely boiled within 30 minutes of this procedure. Both Berson and Yalow (1971) and Malmstrøm and Stadil (1975) showed that the yield of gastrin from tissues which were quick frozen following excision from the animal was higher than that in tissues which were left for up to an hour before extraction by boiling in water. Since the tissues in this study stood for up to 30 minutes at room temperature prior to extraction, this possibility is real. As the tissues were all boiled the same time after collection it was assumed that the relative yields for the different sites were the same. Unfortunately, the data of Berson and Yalow (1971) and Malmstrøm and Stadil (1975) do not allow an absolute comparison of the yield from tissues fast frozen and then extracted with tissues treated by

extraction alone, nor do I have the data to verify or exclude this possibility.

The lower number of tissue extracts investigated here (4) compared with 8 normal subjects studied by Malmstrøm, Stadil et al. (1976) and 21 subjects investigated by Creutzfeldt, Arnold et al. (1976) may also account in part for the discrepancy.

Another reason for the difference in the relative gastrin content of human antrum and duodenum between this study and that found by other workers could lie in the difference in specificities of antisera used in these studies. Measurements with antiserum G 1 reflect chiefly non-sulphated gastrin, and differences in the components of gastrin found at these two sites may well produce different values when measured with antisera of differing specificities. Malmstrøm, Stadil et al. (1976) made their estimations using antiserum 2604-7, which recognises both sulphated and non-sulphated gastrin components I, II and III, whereas Creutzfeldt, Arnold et al. (1976) used an antiserum that reacted with human G-34 with only one third of its affinity for equimolar amounts of G-17, although it did react with both sulphated and non-sulphated forms of gastrin. The antiserum used by Berson and Yalow (1971) and Nilsson, Yalow et al. (1973) detects a large gastrin component in serum (Yalow and Berson, 1972) which has not been detected by other workers. In addition, the antiserum employed by this group appears to cross-react with CCK-PZ or a CCK-PZ peptide to a certain extent (Straus, Yalow and Gainer, 1975), suggesting that the high values of tissue gastrin content reported by this group may not in fact represent only true gastrin.

It would seem therefore that, allowing for differences in extraction techniques and assay variables, the actual gastrin content of various tissues would probably be lower than those of earlier estimates. This is not

unusual in the immunoassay sphere, where with the advent of purer, better defined standards and improved labelling of the peptide, circulating and tissue levels have fallen.

The question of extra-gastric gastrin poses problems in the treatment of ulcer disease, since surgical antrectomy does not remove the only source of gastrin, and from the reports of Nilsson, Yalow et al. (1973), Lai (1964b), Stern and Walsh (1973) and Korman, Soveny and Hansky (1972b) it appears that in the human the duodenum is an important source of gastrin. The term "extra-gastric gastrin" also includes the "big big" gastrin found in extracts of human jejunum by Yalow and Berson (1972). Why this form of gastrin should be produced at a site so distant from the antrum is at present unexplained.

Reports of gastrin release in gastrectomised subjects from different groups should be viewed with caution, since the techniques of Billroth I and Billroth II gastrectomy may produce different results. Thus in the case of a Billroth II gastrectomy, which involves antrectomy and gastrojejunal anastomosis, failure to remove the entire antrum may result in the condition of isolated retained antrum, in which gastrin is secreted continuously from the antral remnant which has been removed from the influence of acid inhibition of gastrin secretion.

Total gastrectomy is the current surgical treatment of choice in cases of Zollinger-Ellison syndrome. Removal of the target organ of gastrin is more effective than removal of a gastrinoma, since these tumours are often so small that they are difficult to find, and since more than 90% of gastrinomas are malignant and have metastasised by the time of operation (Creutzfeldt, 1977). Thus the chances of complete removal of the gastrin secreting tumour at operation are very small.

The aspect of pancreatic gastrin was not investigated in the human, although negligible amounts of gastrin were detected in pancreatic extracts prepared from the rat, rabbit, guinea pig, dog or pig when measured with both antisera G 1 and 2604-7. The pancreatic gastrin detected by other groups in the human was below 10 ng/g tissue (Nilsson, Yalow et al., 1973; Rehfeld and Iversen, 1973; Gepts, 1973) except in the study reported by Greider and McGuigan (1971), who reported a value of 75 ng gastrin per gram human pancreas. However, although the human neonatal pancreas does contain gastrin, the data on the adult human pancreas are controversial. Pancreatic G-cells do occur in other species, but in man there has been no conclusive evidence for the presence of G-cells in the pancreas. In contrast, pancreatic gastrin has been localized by immunohistochemical techniques in the pancreatic D-cell in man (Erlandsen, Hegre et al., 1976), and it seems possible that pancreatic gastrin may differ from antral and duodenal gastrin, since at both the latter sites gastrin is secreted by the G-cell (McGuigan, 1968b; McGuigan and Greider, 1971; Pearse and Bussolati, 1972; Polak, Stagg and Pearse, 1972; Creutzfeldt, Creutzfeldt et al., 1974).

The demonstration of somatostatin and gastrin in the same pancreatic D-cell by Erlandsen, Hegre et al. (1976) is not the first instance of the concept of two hormones which are secreted by the same cell. This has also been shown in the case of two cells in the pituitary (Erlandsen, Hegre et al., 1976), one of which has been shown to produce melanocyte-stimulating hormone and adrenocorticotrophic hormone, while the other appears to produce both follicle-stimulating hormone and luteinizing hormone. Further studies are needed to substantiate and clarify these findings.

As was mentioned previously, measurement of the different gastrin components in the circulation at any given time represents an estimation of the balance between secretion into and removal of the various gastrin components from the circulation. The rate of removal from the circulation of the different gastrin components differs for each component and the half-lives of pure human big, heptadecapeptide and mini-gastrins in the canine circulation are respectively 9-15,8 minutes, 3-5 minutes and 1,8 minutes (Straus and Yalow, 1974; Walsh, Debas et al., 1974; Debas, Walsh et al., 1974). On the other hand little is known about the rate of secretion of these forms of gastrin. Whether the hypothetical biosynthetic sequence BBG → component I → big gastrin → heptadecapeptide gastrin → minigastrin will prove to be a reality, whether all these forms originate in the same cell type and tissue, and why so many different gastrin types are present in the circulation are all questions that need to be answered. It is known that oral ingestion of food (Stadil, Rehfeld et al., 1975) and intravenous administration of arginine (Kalk, Vinik et al., 1973) produce an increase in the levels of heptadecapeptide and smaller gastrins liberated by the antrum, but whether this release follows tryptic cleavage from big gastrin or a larger precursor form in the antral G-cell is not known.

It follows that the conditions under which the different components are detected, such as whether the individual was in the fasting or postprandial state when the blood was collected, are important in determining the relative proportions of the different components, and careful attention to these parameters may help in finally understanding the relationship between the different gastrin types. The biological potency of the various gastrin forms also varies, so that G-17 is roughly five times more potent than G-34 when the plateau level is measured endogenously after an infusion (Walsh,

Debas et al., 1974). Since the general concept of a precursor-hormone relationship involves a large precursor form which has low or no biological activity, as discussed in section 2 VII, the lower biological potency of G-34 relative to G-17, coupled with the fact that G-17 is liberated from G-34 by tryptic cleavage in vitro (Yalow and Berson, 1971a; Rehfeld, 1972) suggests that G-34 may well be a precursor of G-17. The demonstration of a macromolecular form of gastrin in serum which was devoid of biological activity (Malagelada, 1977), provides evidence for the existence of a large biologically inactive gastrin which may be a precursor.

The cleavage of G-17 from larger gastrin forms in vivo must still be demonstrated; it may occur via enzymes with trypsin-like specificity such as the enzymes responsible for the conversion of proinsulin to insulin (Steiner, Kemmler et al., 1974). If the hypothetical biosynthetic sequence progressing from the larger to the smaller gastrin forms can be demonstrated it will be of importance to determine whether all the conversion steps take place in the cell of origin and, if so, what the relation between the antral and duodenal G-cell is. Conversion of the larger to the smaller hormonal form has been demonstrated to take place by enzymatic cleavage in the cell of origin in the case of insulin (Steiner, Kemmler et al., 1974) and in vitro in the case of ACTH (Yalow, 1974a), glucagon (Rigopoulou, Valverde et al., 1970) and several other peptide hormones. A significant contribution to the gastrin story will arise when an antiserum to the amino-terminal heptadecapeptide fragment of G-34 can be raised, which will be useful for measuring circulating levels of this gastrin fragment. In addition it will be interesting to determine whether this fragment possesses biological activity, and how its potency compares with that of the other forms of gastrin.

The distribution of immunoreactive gastrin along the gastrointestinal tract of five mammalian species was studied by preparation of aqueous extracts of gut tissue in the same manner as for the human tissues. This is discussed in the next section.

Gastrin in Tissues of Mammals and Lower Species and Phylogenetic Considerations

The antral gastrin content in the pig was 10 to 30 times greater than that found in the human, in keeping with the finding of 11 times more antral gastrin in the pig compared with the human reported by Nilsson, Yalow et al. (1973), but at variance with the findings of Malmstrøm and Stadil (1975). The actual tissue gastrin content of 15,5 µg/g and 25,9 µg/g dry weight of tissue measured with antisera G 1 and 2604-7 is close to the porcine antral gastrin content of 22 µg/g found by Nilsson, Yalow et al. (1973). In the dog the antral gastrin content measured with antiserum G 1 was 4 to 11 times greater than in the human, while in the other species the antral gastrin content was similar to that of the human. The ratio of duodenal to antral gastrin of less than 1:200 found in this study in the case of the rabbit, guinea pig, dog and pig agrees closely with the corresponding ratio found in the dog, cat and pig by Nilsson, Yalow et al. (1973). This is in direct contrast to the much higher ratio of duodenal to antral gastrin found in the human, as discussed previously.

The high gastrin content of the fundic and duodenal mucosa of the rat is surprising and has not been described in the literature reviewed. The duodenal gastrin content relative to that of the antrum approximated that found in the human, while the high levels in the fundus are difficult to explain. The low antral gastrin content in the rat found in this study is in agreement with the findings of Thompson, Reeder et al. (1973) who reported a much lower antral gastrin content in the rat compared with other species.

The relatively high gastrin content found in rabbit colonic mucosa is also surprising. Perhaps further investigations would show this to be a type of macromolecular gastrin, as was found in the case of human gastrin extracted from tissues situated distally in the gastrointestinal tract (Yalow and Berson, 1972). The significance of this observation may relate to the fact that the rabbit is herbivorous, and it may be that the distribution of gastrin in the rabbit gut differs from that in carnivorous mammals.

Although gastrin was detected in the pancreas in a number of species, the quantities were of an extremely low order and cast some doubt on the validity of the observations. By the very nature of the measurement, radioimmunoassay is subject to a host of variables which could yield spurious results at low detection limits, which could be erroneously interpreted as "true gastrin". The absence of G-cells in the pancreas of most species including adult man would support this notion. Nilsson, Yalow et al. (1973) failed to detect gastrin in the pancreas of the cat, dog or pig, whereas Thompson, Reeder et al. (1973) reported high concentrations (2 020 and 2 800 ng/g) in the rat and cat pancreas, as well as in the frog. However, these values may in fact be a printing error, since in the same paper these authors quote values in the units "pg/g" as opposed to the units "ng/g" shown in their table. If in fact the true values are 2 020 and 2 800 pg/g, then this brings the pancreatic gastrin content of the rat and the cat, found by Thompson, Reeder et al. (1973), in line with the very low content of gastrin in mammalian pancreatic tissue extracts found in this study, as shown in table (4.III.1), in which instance the units are"pg/mg" tissue.

The demonstration of immunoreactive gastrin in the serum of the dozen mammals tested, coupled with the higher levels detected by antiserum 2604-7

compared with antiserum G 1, indicates the presence of both sulphated and non-sulphated gastrin in these species. In the chicken, demonstration of immunoreactive gastrin in the serum is in keeping with the findings of immunoreactive gastrin in extracts of the gizzard-duodenal junction of the chicken by Larsson, Sundler et al. (1974), the demonstration of biologically active gastrin in extracts of chicken gut by Olowo-Okorun and Amure (1973), and the immunohistochemical demonstration of gastrin cells in the chicken gut by Polak, Pearse et al. (1974).

Both sulphated and non-sulphated gastrin were found in the serum of the reptile, Dasypeltis scabra scabra, and extracts of various gut tissues showed that the highest tissue gastrin content was in the intestine, in which case only sulphated gastrin was detected. Extracts of the stomach of this species contained approximately one tenth the amount of sulphated gastrin found in the intestine. The only other report of reptilian gastrin comes from Olowo-Okorun (1975b) who demonstrated the presence of antral gastrin by bioassay of an extract prepared from the python, Python molurus.

In three species of teleost and one elasmobranch, only sulphated gastrin was found in the serum. In a conclusive and well-controlled study, Hansen (1975) demonstrated the presence of biologically active gastrin in the antrum of an elasmobranch or cartilaginous fish, Rhinobatus productus, confirming the presence of this hormone in the lowest form of vertebrate investigated in this study.

If one disregards the amphibia, the logical sequence of the evolution of gastrin in the species investigated in this study suggests that in the vertebrates gastrin first arose only in the sulphated form, as was found in the fishes investigated. In the reptile both species of gastrin were detected in the serum, whereas extracts of the gut of the snake investigated contained only

sulphated gastrin, since antiserum G 1 failed to detect any immunoreactive material in these extracts. In all the species situated higher on the evolutionary tree which were investigated, both sulphated and non-sulphated gastrin were detected in the serum. Thus it is tempting to speculate that gastrin was present in the fishes in the sulphated form, and only in later evolutionary development did the non-sulphated species of gastrin appear. This would imply that the sulphated molecule was the more stable, and resisted evolutionary change.

The failure to detect immunoreactive gastrin in the invertebrate species studied using antiserum G 1, which measures primarily non-sulphated heptadecapeptide gastrin, could be explained in the light of this hypothesis. Since the invertebrate extracts were not measured with antiserum 2604-7 as well, it was not established whether sulphated gastrin was present in these species, which included representatives of the phyla Coelenterata, Mollusca, Crustacea and Echinodermata. However, it is felt that these results would have been negative.

The finding of a negligible amount of non-sulphated gastrin in extracts of the snail, Helix sp. which amounted to only 0,0171% of that of the human antrum is upheld by the failure of Fritsch, Van Noorden et al. (1976) to demonstrate gastrin-containing cells in the intestine of another molluscan species, Mytilus edulis, using the technique of immunofluorescence.

The findings in this study are difficult to reconcile with those of Straus, Yalow et al. (1975), who measured gastrin levels in the blood of two species of mollusc, one being the land snail, Otala lactea, which were comparable to the levels found in the human circulation. The immunoreactive gastrin content of the intestinal tissues investigated by Straus, Yalow et al. (1975) was roughly one hundred fold greater than the gastrin content of the total

body tissues extracted in the case of Helix sp. in this study. In addition, Straus, Yalow et al. (1975) demonstrated molecular heterogeneity of their molluscan gastrin using Sephadex G-50 chromatography. However, certain objections can be raised against this study. Firstly, the antiserum used by Straus, Yalow et al. (1975) was raised to crude porcine gastrin, which means that it may not be entirely specific for gastrin only but may measure additional peptides such as CCK-PZ. Secondly, the exact position of the smaller gastrin species in the elution profile on Sephadex G-50 was a considerable distance from the ^{125}I-porcine heptadecapeptide gastrin I marker, suggesting that this molecular species may in fact represent a small CCK-PZ peptide and not G-17 gastrin. However, this is a criticism which could be levelled at some of the elution profiles in this study as well, and is a reminder that the technique of Sephadex gel filtration only permits inference of the nature of the molecular species fractionated from its molecular size, but does not provide information on the precise chemical nature of the molecule.

No other studies concerning gastrin in invertebrates have been reported. Gastrin-containing cells were demonstrated by immunofluorescence in the intestinal epithelium of the hagfish, Myxine glutinosa, (Östberg, Van Noorden et al., 1976) confirming the presence of this hormone in the cyclostomes. The cyclostomata are the only living representatives of the class Agnatha (Romer, 1971), the earliest class in the subphylum Vertebrata, having arisen in the Ordovician period, some 480 million years ago.

Coupling the findings of gastrin distribution in this investigation, which failed to demonstrate gastrin in invertebrate tissues, with the immunohistochemical demonstration of gastrin-containing cells in the cyclostomata (Östberg, Van Noorden et al., 1976) makes it tempting to suggest that gastrin arose

for the first time in the lowest class of vertebrates, the Agnatha. One could postulate that the sulphated gastrin species arose at this time and persisted until some time in the Carboniferous Period, 300 million years ago, when the reptiles emerged, and non-sulphated gastrin appeared for the first time. This is postulated on the basis of the finding of non-sulphated gastrin for the first time in the species of reptile examined in this study.

The evolutionary origins of the pancreatic peptide hormones, insulin and glucagon, have been fairly well established. Immunoreactive insulin has been demonstrated in the intestines of molluscs, echinoderms, crustaceans, tunicates and many vertebrates (Wilson and Falkmer, 1965; Davidson, Falkmer et al., 1971). Glucagon immunoreactivity has been convincingly demonstrated in higher vertebrates and there is evidence, although less concrete, for the presence of immunoreactive glucagon in the mollusca and the crustacea (Assan, Tchobroutsky et al., 1969).

Makhlouf (1974) has suggested that secretin and insulin were present in invertebrate species and that glucagon and gastrin arose for the first time in the gnathostomes, coincidentally with the development of jaws and a stomach. Biological activity suggesting the presence of the structurally related hormone, CCK-PZ, as well as secretin, was found in the intestine of the lowest form of agnathan vertebrates, two species of the order cyclostomata, by Barrington and Dockray (1970). Makhlouf (1974) proposed that CCK-PZ and secretin are the molecular ancestors of gastrin and glucagon respectively.

Thus the demonstration of gastrin-containing cells in the gut of an agnathan vertebrate by Östberg, Van Noorden et al. (1976) dates the appearance of gastrin earlier than suggested by Makhlouf (1974). The theory of origin of the gut peptide hormones proposed by Makhlouf (1974)

is, however, a very attractive one, and it would be unwise to discount it on the basis of one report, especially a report based on immunohistochemical observations, which may be subject to sources of error based on cross-reaction between related hormones. It is probably fair to assume that gastrin appeared for the first time either just before or coincident with the appearance of the gnathostomes. It will be difficult to obtain much information on this aspect because most of the agnathan species are extinct, but confirmation of the presence of gastrin in the two living species of cyclostome by other workers will be useful. In addition, it would be of value to determine whether immunoreactive gastrin is present in extracts of the tunicate, Ciona sp., which belongs to the subphylum Urochordata, a division of the phylum Chordata. This is an accessible species which arose in evolution after the invertebrates, but before the subphylum Vertebrata, and would thus form an additional link in such investigations.

The arrangement of hormone producing cells in these early species differs from that of present day mammals, in which the endocrine cells have become grouped into more discrete areas and in some instances into hormone producing glands. The gastrin-containing cells in the hagfish, described by Östberg, Van Noorden et al. (1976) were found scattered amongst the intestinal epithelial cells, as was the case for cells containing pentagastrin, caerulein and glucagon. The insulin-containing beta cells in this species were found in islet tissue parenchyma located around the bile duct and intestine (Östberg, Van Noorden et al., 1976). The gastrin-containing cells in the hagfish differed from those of mammals in that the APUD characteristics could not be demonstrated in these cells. However, the cytochemical architecture of the gastrin-containing cell in the hagfish was of the "open type", extending from the basal membrane to the lumenal

surface of the gut, a primitive arrangement which has been retained in the mammalian antral G-cell today.

The functions subserved by these hormones found in primitive vertebrate species almost certainly differ from their functions in mammals to a certain extent, because the digestive systems of these animals are not as complex as those of higher mammals. For example, Barrington and Dockray (1970) suggested that the substances with secretin and CCK-PZ activity found in the river and marine lamprey may act locally as tissue hormones, released for instance by secretagogues in the food. Steiner, Kemmler et al. (1974) suggested that many peptide hormones evolved from more primitive enzymes such as secreted hydrolases, and Adelson (1971) also proposed that many of the secreted proteins of vertebrates originated among the digestive enzymes of the gut of pre-vertebrate species.

The current concept that many gut peptide hormones may also have a neurotransmitter or regulatory function, as has been suggested in the case of somatostatin (Polak, Pearse et al., 1975), is upheld by the recent findings of gut-related peptides in the brain and neural tissue of several species. A brain gastrin peptide (BGP) has been described in the cerebral cortex and other parts of the brain of the human, dog, pigeon, frog and trout by Vanderhaegen, Signeau et al. (1975). Thus there is evidence for this peptide, which is immunologically similar to but not identical with gastrin (2-17), in species dating back to the fishes. CCK-PZ-like peptides have been described in the brain of the rat, pig and dog (Dockray, 1976). Straus, Muller et al. (1977) confirmed these findings by describing a peptide resembling the C-terminal octapeptide of CCK-PZ in the rat cerebral cortex, and two CCK-PZ-like peptides were found in extracts of pig cerebral cortex by Muller, Straus et al. (1977). Vasoactive intestinal polypeptide (VIP)

has been demonstrated in brain and intestinal tissue of the human and the pig by Said and Rosenberg (1976) and Bryant, Bloom et al. (1976). Further studies on the distribution of these "neurotransmitter" peptides in lower vertebrate species and possibly invertebrates should help clarify the physiological significance and interrelationships of these neural and gut related peptides.

Since much of the foregoing discussion on the evolutionary origin of the gastrin molecule embraces the aspect of the presence or absence of a sulphate group on the tyrosine residue situated six positions from the C-terminus, the significance of sulphation in the related hormones, CCK-PZ and caerulein should be mentioned. Both sulphated and non-sulphated forms of heptadecapeptide gastrin are biologically active (Gregory and Tracy, 1964), and both sulphated and non-sulphated versions of the larger and smaller gastrin peptides have been described, although the relative proportions of sulphated to non-sulphated gastrins differ in man and hog (Gregory and Tracy, 1975). In the case of CCK-PZ and caerulein, both have the identical C-terminal octapeptide sequence except for one residue, and both possess a sulphated tyrosine residue in the seventh position from the C-terminus (Ondetti, Plusec et al., 1970; Anastasi, Erspamer et al., 1967). Unlike gastrin, the presence of this sulphate group is essential for biological activity in the case of caerulein (Johnson, Stening and Grossman, 1970) and CCK-PZ (Erspamer, 1973). The overlap in actions produced by pharmacologic doses of the three peptides caerulein, CCK-PZ and gastrin II is a result of the structural similarities between the molecules and the fact that they all contain the identical pentapeptide sequence.

Since it has been suggested that CCK-PZ is the molecular ancestor of gastrin (Makhlouf, 1974), and CCK-PZ is only active in the sulphated form,

the observations in this study that gastrin arose for the first time in the sulphated form, and is present in higher species in both sulphated and non-sulphated forms suggest that in more recent times the gastrin molecule lost the prerequisite of a sulphated tyrosine for biological activity. This is confirmed by the finding of biologically active gastrin in both sulphated and non-sulphated forms today (Gregory and Tracy, 1964; 1975).

These studies demonstrated that the gastrin-like material found in the serum and skin extracts of the frog, Xenopus laevis, differed considerably from heptadecapeptide gastrin. The material found may well be caerulein, since the majority of immunoreactive material obtained on Sephadex G-25/G-50 chromatography was of a molecular weight smaller than the G-17 I marker, and caerulein is composed of 10 amino acids (Erspamer, 1973). This suggestion seems likely in view of the demonstration by Anastasi, Bertaccini et al. (1970) of the presence of caerulein in extracts of the skin of the same species of frog. Measurement of this gastrin-like material in tissue extracts of Xenopus laevis here showed higher concentrations in the skin than at any other site, except in the brain tissue extract. However, this high ratio is thought to be artefactual due to the low protein content of brain tissue, resulting in a spuriously elevated "gastrin" to total protein ratio in this instance. The readings obtained with antiserum G 1 in the skin and stomach extracts of this species were greater than those found using antiserum 2604-7. This is the only instance where readings with 2604-7 antiserum were not greater than those obtained with G 1 antiserum, and again points to the fact that material different from pure heptadecapeptide gastrin is present in these tissues.

Fractionation of the serum of Xenopus laevis produced one peak eluting in the void volume and a broad poorly-defined peak in the molecular weight region which may correspond to caerulein. This pattern differed from that found on G-50 Sephadex chromatography of serum from the frog Rana catesbeiana by Gibson, Mihas et al. (1976) in which case two well-defined peaks, one in the void volume and one coincident with the natural G-34 marker, were obtained. Rana and Xenopus, although both anuran amphibia, may differ considerably in their way of life; whereas Rana can survive on land for long periods of time, Xenopus is more dependent on an aquatic habitat for survival. It would thus appear logical to find a molecular heterogeneity of gastrin in Rana which resembles that in man more closely than did that found in Xenopus. Unfortunately, the report of Gibson, Mihas et al. (1976) does not allow comparison of the nature of immunoreactive gastrin in Rana skin extracts with that of Xenopus described in this study.

It is obvious that the "gastrin" immunoreactivity found in the serum and skin extracts of Xenopus laevis does not fall in with the proposed hypothesis on the evolutionary origin of gastrin based on measurements of gastrin in other vertebrate species described in this study. One needs to ask what the significance of this gastrin-like peptide in this species is, and whether in fact it was actually caerulein that was being measured. A reason for the presence of high concentrations of this material in the skin of Xenopus must be found.

The true story, when it emerges, may turn out to be far more complex than imagined. According to Erspamer (1973) seven new polypeptides have been identified in and purified from amphibian skin. These include physalaemin, phyllokinin, phyllomedusin, caerulein, phyllocaerulein, alytesin and bombesin. All these polypeptides have been synthesised subsequent to their

purification (Erspamer, 1973) and many contain sequences common to mammalian neural and endocrine peptides (Pearse, 1976). Recently the presence of the mammalian tripeptide, thyrotropin-releasing hormone in the skin of the frog Rana pipiens has been demonstrated (Jackson and Reichlin, 1977). This peptide occurred in the frog skin in concentrations double that found in the hypothalamus of this species, and it was shown to be biologically active. These findings also point to the fact that frog skin may be a huge endocrine organ (Jackson and Reichlin, 1977).

A logical explanation for these findings has been proposed by Pearse (1976). He points out that anuran cutaneous glands arise from specialised ectoderm which originates in the embryonic region adjacent to the neural plate and neural crest. It is thus easy to see that these glands have a common origin with cells producing the brain and intestinal peptides. Pearse (1976) has suggested that the cutaneous glands and skin peptides of the frog evolved in parallel with the nervous system in response to the hostile external environment.

Several possible roles of the gastrin-like peptide found in the skin extracts and serum of Xenopus laevis in this study may be suggested. Since this amphibian species is mainly aquatic in nature, this peptide may perhaps subserve a function connected with regulation of passage of water and electrolytes through the skin, as has been suggested by Erspamer (1973). Along the same lines, one could suggest that this material plays a role regarding external secretion of the skin, as a pheromone or even as a sexual attractant. It may even transpire that this peptide is important as a growth factor, influencing the development of young tadpoles. This suggestion is substantiated by the demonstration that gastrin possesses a trophic action in mammals; for example, it has been shown to affect the tissues of the gastrointestinal

tract in the rat by increasing the synthesis and total tissue content of DNA (Johnson, 1977).

The following section deals with studies investigating aspects of the porcine and rat liver in relation to heptadecapeptide gastrin, as well as measurement of kinetic parameters of infused synthetic human heptadecapeptide gastrin I in the porcine circulation.

The Role of the Liver in Relation to Circulating Gastrin and Kinetics of G-17 I Gastrin in the Porcine Circulation

The half-life value of 3,19 minutes for synthetic human G-17 gastrin in the porcine circulation found in this study using samples drawn at the carotid arterial site is in good agreement with the values found in the dog following infusion of natural or synthetic gastrin preparations (Reeder, Jackson et al., 1972; Thompson, Reeder et al., 1973; Schrumpf and Semb, 1973; Walsh, Debas et al., 1974; Strunz, Walsh et al., 1978) as shown in table (2.IX.1). The half-life of heptadecapeptide gastrin in the circulation is rapid in comparison with the clearance rate of pancreatic peptide hormones from the circulation. The half-life time of insulin in the circulation has been shown to lie between 13,8 and 50 minutes, as determined by infusion of ^{131}I-labelled insulin into man, stimulation of endogenous insulin levels by glucose infusion, and perfusion of the isolated rat liver (Berson, Yalow et al., 1956; Berson and Yalow, 1966; Rubenstein, Pottenger, Mako, Getz and Steiner, 1972). The removal of proinsulin from the circulation was ten to fifteen times slower than that of insulin (Rubenstein, Pottenger et al., 1972). The half-life time for clearance of endogenous glucagon in the pig was 42-55 minutes, and 2-4 and 36-57 minutes for the first and second disappearance phases of exogenous glucagon from the porcine circulation (Van Hoorn, Vinik and Van Hoorn-Hickman, 1978). Pancreatic polypeptide (P.P.) had an average half-life time of 34 minutes in the circulation, comprising first and second disappearance components of 6,8 and 60 minutes respectively (Sive, Lund, Van Tonder and Vinik, 1977; Sive, 1978).

In contrast to the pancreatic peptide hormones, the gut peptide hormones appear to be more rapidly removed from the circulation. Recent reports have shown a half-life time of 4,06 minutes for removal of exogenous secretin from the circulation in man (Kolts and McGuigan, 1977); 3,1 minutes for the clearance of exogenous porcine VIP from the canine circulation (Strunz, Walsh, Bloom, Thompson and Grossman, 1977); and half-life values of 4,36 minutes following infusion of exogenous synthetic 13-norleucine motilin in man, and 4,56 minutes for the clearance of endogenous motilin from the human circulation (Mitznegg, Bloom, Domschke, Domschke, Wuensch and Demling, 1977).

Since the half-life of gastrin in the circulation is very short, a study was designed to investigate whether the liver was responsible for metabolism and clearance of gastrin from the circulation. This was deemed possible in the light of reports of hepatic inactivation of the peptide hormones insulin (Samols and Ryder, 1961; Kaden, Harding et al., 1973), glucagon (Kenny, 1956) and secretin (Skillman, Silen et al., 1962). In addition, it seemed likely that the liver may affect circulating gastrin because all gastrin produced by the gut passes through the liver before it reaches its primary site of action, the parietal cells of the gastric fundus.

The study showed that the pig liver had no effect on circulating levels of immunoreactive endogenous gastrin or on elevated levels simulating postprandial levels and greater, produced by infusion of synthetic human heptadecapeptide gastrin I (Vinik, Hickman et al., 1978). Following stepwise infusions an increased mass of gastrin was presented to the liver, which did not differ significantly from the corresponding increased gastrin mass leaving the liver at each stage of the infusion. These findings confirm the reports that the liver did not remove immunoreactive G-17 from the

circulation (McGuigan, Jaffe et al., 1970; Reeder, Brandt et al., 1972; Dencker, Håkanson et al., 1973), and the demonstration that the liver did not reduce the biological activity of gastrin by Gillespie and Grossman (1962). In contrast, the findings do not support the report by Thompson, Reeder et al. (1969) that the liver inactivates 46% of supraphysiological levels of G-17 gastrin presented to it, but this assay was too insensitive to measure basal levels of circulating gastrin.

It was further evident that even if one considered variations in hepatic arterial, portal and venous blood flow and liver mass, quantitatively the liver had no effect on gastrin transit.

In keeping with these findings that the liver did not remove immunoreactive G-17 from the circulation is the observation by Le Roith, Vinik et al. (1975) that immunoreactive gastrin levels were not elevated in patients with chronic liver disease. In other words, an alternative site(s) for the removal of gastrin must exist. The finding of elevated gastrin levels in renal disease reported by Dent, Hirsch et al. (1972), Korman, Laver et al. (1972), Le Roith, Vinik et al. (1975), Sullivan, Tustanoff et al. (1976) and many others suggests that the kidney may be important in this respect.

Although this study showed that G-17 gastrin was not removed by the pig liver (Vinik, Hickman et al., 1978) or the rat liver (Sacks, Vinik et al., 1978) these measurements were of immunoreactive gastrin, and no estimations of biological gastrin activity were made. Both these studies involved measurements made with G 1 antiserum, which has been shown to react equally well with human and porcine G-17 and to recognise the (8-17) sequence of non-sulphated heptadecapeptide gastrin, as described in section 4 I(iv). Since this antigenic region includes the C-terminal pentapeptide amide sequence of gastrin, which is responsible for its biological

activity (Tracy and Gregory, 1964), it is likely that measurements with this antiserum reflect biological activity as well as immunoreactivity. This is substantiated by the demonstration that antiserum G 1 failed to cross-react with heptadecapeptide gastrin fragments which did not include the C-terminal 4 amino acids, such as the (6-13) and (9-13) sequences, as shown in section 4 I(iv). Thus if cleavage of the biologically active portion from the rest of the molecule had occurred it is unlikely that any remaining inactive fragments would have been detected with antiserum G 1. However, such a situation was described by Lewin, Hunziker et al. (1971), who showed biological inactivation of gastrin by the liver with no apparent effect on immunoreactive measurements of gastrin in the same study.

It may mean that antiserum G 1 continues to detect the carboxyl-terminal portion of gastrin despite alterations which would reduce biological potency. Hence changes in the molecular form during hepatic transit could account for qualitative rather than quantitative effects.

No changes in the molecular form of gastrin for up to 60 minutes of perfusion of the isolated rat liver in situ with synthetic human G-17 I were detected using Sephadex G-25/G-50 chromatography, as shown in figure (4.V.(i).2.). In the case of the pig liver, chromatography of serum samples collected during the second and third hours of stepwise infusion of synthetic human G-17 I into the pig suggested changes in the molecular size of gastrin in the porcine circulation. Samples drawn from the venous and portal sites in two pigs revealed broadening of the peaks in the chromatographic profile with a suggestion of the presence of gastrin species smaller than the heptadecapeptide (figures (4.IV.5.) and (4.IV.7.)). Chromatography of a serum sample drawn from the arterial site in one pig suggested the presence of a larger form of gastrin in addition to the heptadecapeptide, as shown in

figure (4.IV.6.).

The discrepancy in the chromatographic profiles of gastrin obtained in the rat and pig transhepatic studies may be due to differences in the hepatic handling of G-17 gastrin by these two species. Alternatively the differences could be explained by the longer time of circulation of the exogenous gastrin in the pig as opposed to the rat, or by the fact that the study on the pig was performed in vivo, whereas that on the rat was performed in situ, so that the former study reflects measurements of immunoreactive gastrin in blood, whereas the latter involved measurements in perfusion medium. It may be that the smaller peptides detected in the venous and portal samples of the pig are artefacts or breakdown products produced as a result of prolonged circulation in vivo. It is difficult to explain the appearance of a larger gastrin form in the arterial circulation after prolonged circulation, as this does not correlate with the hypothetical biosynthetic sequence of gastrin leading from larger to smaller forms as discussed in section 2 VI(iii).

Neither is this sequence supported by the observation in vivo that infusion of pure human G-34 into a human subject produced a clear cut peak of G-34 on gel chromatography, with no evidence of conversion to G-17 or other smaller gastrin species (Walsh, 1975b). However, infusion of supraphysiological doses of a hormone does not necessarily reflect the normal physiological situation, and it may well be that in the physiological situation such conversion from larger to smaller forms does occur. In the same study Walsh (1975b) showed that pure human G-17 I eluted as a single well-defined peak on gel chromatography following infusion of this material into a human. Walsh (1975b) did not specify how long after commencement of the infusion the samples were collected for chromatography. Possibly they were collected earlier than the samples used for

chromatography in this study, and had our samples been collected earlier during the gastrin infusion a similar clean peak of G-17 gastrin might have been obtained.

The failure of the rat liver to alter the molecular form of gastrin during in situ perfusion, as revealed by Sephadex chromatography, found in this study is in agreement with the demonstration of Dencker, Håkanson et al. (1973) of no difference in the immunoreactive gastrin components in human serum drawn from portal and peripheral sites.

It thus appears from these studies and those reported by Gillespie and Grossman (1962); McGuigan, Jaffe et al. (1970); Reeder, Brandt et al. (1972) and Dencker, Håkanson et al. (1973) that the liver does not inactivate heptadecapeptide gastrin. That the liver appears to inactivate gastrin peptides shorter than the heptadecapeptide has been conclusively demonstrated by Temperley, Stagg and Wyllie (1971), Stagg, Temperley et al. (1971) and Debas and Grossman, 1974) in studies in the dog and rat.

The data on ligandin binding of gastrin add to the conflict of whether or not the liver inactivates gastrin. If the data on altered molecular size after hepatic transit in the pig can be confirmed, it may be that gastrin is bound to ligandin in which form it circulates. This may represent inactivation, since ostensibly the active site(s) could be masked by binding to the protein. Further studies are needed to establish that this postulate is indeed true. Alternatively, the binding could represent merely an artefactual situation created by the experimental protocol. Indeed, the studies with isolated rat liver tend to support the latter suggestion. Because of the possibility of species differences it would be premature to draw any firm conclusions.

The sites of inactivation of heptadecapeptide gastrin include the gastric fundus (Evans, Reeder et al., 1974), small bowel (Becker, Reeder et al., 1973) and kidney, as suggested by the studies of Dent, Hirsch et al. (1972), Korman, Laver et al. (1972), Clendinnen, Reeder et al. (1973), Sullivan, Tustanoff et al. (1976) and others, as outlined in section 2 IX(i). The renal clearance of gastrin of 80 ml/minute amounts to a small fraction of the total clearance of gastrin of approximately 2 litres/minute determined in this study. Thus it appears that although elevated gastrin levels have been reported in many instances of renal disease (Dent, Hirsch et al., 1972; Korman, Laver et al., 1972; Clendinnen, Reeder et al., 1973; Le Roith, Vinik et al., 1975; Sullivan, Tustanoff et al., 1976), the actual contribution of the kidneys to removal of gastrin from the circulation may not be as large as is sometimes thought. The major site of inactivation of gastrin has not been definitely established, but it is likely that the gastric fundus may play a large role, and the possibility that gastrin is inactivated locally at the site of its target organ, the parietal cell, is real. This suggestion is borne out by the report of binding and inactivation of ^{125}I-labelled gastrin by isolated gastric mucosal cells by Del Mazo and McGuigan (1976) and the localisation of gastrin receptors on isolated rat parietal cells by Soumarmon, Cheret et al. (1977).

The metabolic clearance rate of 2,014 litres/minute or 67,8 ml/kg/min found in this study using samples collected from the arterial site is in good agreement with the value of 83 ml/kg/min found in the dog following infusion of NHG-17 I by Debas, Walsh et al. (1974) and the value of 70 ml/kg/min following infusion of SHG into the dog by Schrumpf and Semb (1973). The high M.C.R. coupled with the short half-life of 3,19 min for SHG-17 I in the pig found in this study agrees well with the corresponding values of

70 ml/kg/min and 3,7 min found by Schrumpf and Semb (1973), 3,2 min for $T\frac{1}{2}$ found by Walsh, Debas et al. (1974) and 83 ml/kg/min found by Debas, Walsh et al. (1974) following infusion of synthetic and natural G-17 gastrins into dogs. This suggests that human G-17 gastrin is cleared from the porcine and canine circulations in a similar manner, and that the clearance of heterologous gastrin in this study was more rapid than the clearance of homologous gastrin. This was illustrated by the findings of longer half-life values and lower metabolic clearance rates of human gastrin following infusion into human subjects by Ganguli, Elder et al. (1970) ($T\frac{1}{2}$ = 5,5 - 10,5 min), Schrumpf, Semb et al. (1973) ($T\frac{1}{2}$ = 7,5 min and 12,8 min in first and second disappearance phases, M.C.R. = 9,1 ml/kg/min) and Walsh, Maxwell et al. (1975) ($T\frac{1}{2}$ = 5 min, M.C.R. = 16,5 ml/min).

The higher metabolic clearance rate found in the portal circulation (3,232 l/min) as opposed to the value found in the arterial circulation (2,014 l/min) may be due to extraction of gastrin from the circulation by the gastric fundus. If this were the only reason for the differences found at this site, it would be expected that the $T\frac{1}{2}$ value would be shorter in the portal than in the arterial circulation. In fact the opposite was found, with $T\frac{1}{2}$ values of 3,19; 4,23 and 4,32 minutes determined in the arterial, portal and venous circulations respectively during the first ten minutes after cessation of the gastrin infusion (table (4.IV.9)). This deviation from the expected findings could be due to the production of gastrin at one or more sites in the gut, which would result in a longer disappearance time of gastrin measured at the portal site. Thus measurements in the portal circulation represent a balance between secretion of gastrin into the circulation and extraction of gastrin by the gastric fundus.

The fact that the M.C.R. found in the hepatic venous circulation (3,170 l/min) was similar to that measured in the portal suggests that passage through the liver had no effect on this parameter, in keeping with the findings in this study that the liver had no effect on gastrin concentrations in the circulation, even at supraphysiologic doses of exogenous gastrin.

As shown in table (4.IV.11), when the M.C.R. was determined using the two highest dose levels of infused gastrin the values obtained at the arterial, portal and venous sites were lower than the mean M.C.R. values calculated using the mean of values obtained at all three dose levels of gastrin in each case. This discrepancy, found by expressing the findings in the same study in two different ways, emphasises how variation between values for the same parameter can occur based merely on the manner in which the results of the calculations are expressed. Naturally, further differences arise between studies in which the infusion is given in a step-wise manner as opposed to a constant dose over a prolonged period of time, and in instances where studies are commenced by administration of a loading dose before the infusion. Further variation in values for these parameters may be due to sampling of blood at different sites, as illustrated by the differences in M.C.R. values found using samples drawn from the arterial, portal and hepatic venous circulations in the pig.

Similar variation was found in the values of V, the space of distribution of human G-17 gastrin, determined from samples drawn at these sites in the pig. As shown in table (4.IV.10), the space of distribution of G-17 determined using arterial samples was 6,401 litres or 0,216 l/kg, which amounts to 21,6% of body weight, or roughly equal to the extracellular fluid volume. However, the same parameter determined using portal and venous samples was more than double this value in both cases, giving spaces of distribution of

46% and 56% of body weight respectively. This distortion in the estimations of volume of distribution of infused gastrin may be due to the endogenous gastrin secreted into the portal circulation, which correspondingly affected estimations in hepatic venous blood. The space of distribution of 21,6% of body weight determined using the arterial samples is in good agreement with the value of 0,24 l/kg for the space of distribution of NHG-17 I in the dog, found by Walsh, Debas et al. (1974), and is close to the values of 19,2% and 28,5% of body weight for the distribution of SHG-17 I in the dog reported by McGuigan, Isaza et al. (1971). The finding that SHG-17 I distributed itself in a volume roughly equivalent to the extracellular fluid volume in this study in the pig is not supported by the reports of spaces of distribution of infused gastrin equal to plasma volume in the dog (Strunz, Walsh et al., 1978), or twice plasma volume in the human (Walsh, Maxwell et al., 1975), or twice plasma volume following pulse injection in the dog, as reported by Straus and Yalow (1974).

This great variation in values determined for these various parameters between different groups almost certainly reflects differences in experimental design and technique, as already discussed, as well as differences in sites of sampling in the experimental animals. As has been shown in this study, the values of $T\frac{1}{2}$, M.C.R. and V all varied according to the sampling site. Very few of the studies reviewed in section 2 IX(iii) actually specified where the samples were collected and in those that did, the sampling site involved a peripheral vein (McGuigan, Isaza et al., 1971; Straus and Yalow,1974; Le Roith, Vinik et al., 1975). It seems reasonable to assume that when the sampling site is not specified it was in fact a peripheral venous site. Considering the various possibilities as regards experimental determination of these different parameters, it is hardly surprising that such great variation

between the work of different groups occurs. Most of the studies on gastrin kinetics have been reported in the human or the dog. It would appear that, bearing in mind the discrepancies due to experimental technique, the kinetics of SHG-17 I in the pig are similar to those found by other workers in the dog under similar conditions.

As shown in table (4.IV.12), the daily production of gastrin in the pig was approximately 120 μg/day as determined using the M.C.R. estimations made at the arterial site. The portal gastrin production was estimated to be approximately 19 μg/day as determined in a separate group of pigs. Bearing in mind that these two estimations were made in two separate groups of pigs and that these figures were arrived at by different calculations in the two instances, the discrepancy between the two estimations is still remarkable. One cannot escape the obvious conclusion that there is an alternate site or sites of gastrin production, in the pig at least. In looking for possible candidates for sites of extra-gastric gastrin secretion, the recent reports of the presence of gastrin-like peptides in the brain by Vanderhaegen, Signeau et al. (1975), Dockray (1976), Muller, Straus et al. (1977) and Straus, Muller et al. (1977) as discussed in section 2 VI(i) suggest the possibility that brain and neural tissue may be far more important sites of gastrin production than previously believed. The validity of these observations and suggestions would be substantiated by further work in this exciting field.

CHAPTER 6

SUMMARY AND CONCLUSIONS

In this chapter a summary of the most important findings of the studies for this thesis is presented. This is followed by suggestions for lines of future research arising from the findings documented in this thesis.

A sensitive gastrin radioimmunoassay, capable of detecting basal levels of circulating gastrin as low as 8,2 pg/ml was established and the assay criteria of validity and reliability were fulfilled. Three antisera used in the radioimmunoassay system were characterised with respect to their affinities for gastrin and their degree of cross-reaction with synthetic gastrin fragments, various natural and synthetic gastrin peptides and related gut peptides. The identity of gastrin in normal and hypergastrinaemic serum with the synthetic human heptadecapeptide standard was confirmed by dilution curves in the assay, and the recovery of gastrin extracted from tissues by boiling in water combined with various treatments was determined. The behaviour of pig serum on dilution in the assay was identical with that of the gastrin standard, confirming that porcine serum gastrin could be measured with this assay system.

Using the radioimmunoassay system incorporating antiserum G 1, a normal range of basal circulating gastrin levels in the human was determined. Serum gastrin measurements in a large group of patients showed no correlation between age or ethnic group and circulating gastrin levels, although significantly higher levels of gastrin were found in females than in males. Elevated serum gastrin levels were recorded in disease conditions such as Zollinger-Ellison syndrome, pernicious anaemia, duodenal ulcer and gastric carcinoma.

In normal subjects elevated levels of serum gastrin were produced by administration of oral Oxo and intravenous arginine.

The heterogeneity of gastrin in serum from a hypergastrinaemic patient was investigated using Sephadex G-25/G-50 gel chromatography. The chromatographic profile revealed at least four and possibly five components of gastrin, in agreement with the findings of other workers in this field. The elution profiles of natural human big and heptadecapeptide gastrins in the chromatographic system were established.

The gastrin content of extracts prepared from human antral and duodenal tissue biopsies was measured and characterised immunochemically and the heterogeneity of the gastrin in these extracts was studied. The tissues were found to contain the same gastrin components as did serum, with a greater proportion of the heptadecapeptide species in the antral as opposed to the duodenal extracts.

Measurement of the gastrin content of tissue extracts prepared from biopsies collected at various sites in the gastrointestinal tract of five mammalian species showed that the highest levels of gastrin occurred in the antral extracts. Unlike the human, the duodenal gastrin content of these species was small relative to that of the antrum. Other sites where gastrin levels were found to be higher than expected included the rat fundus and the rabbit colon.

Measurement of serum gastrin concentrations in nineteen vertebrate species with two antisera of known specificity revealed the presence of both sulphated and non-sulphated gastrin types in all animals investigated, with the exception of three species of bony fish and one cartilaginous fish,

in which cases only sulphated gastrin was detected in the serum. This suggested the possibility that sulphated gastrin arose before non-sulphated gastrin in evolution.

Inordinately high levels of a peptide displaying gastrin-like immunoreactivity were measured in the serum and skin extract of the frog Xenopus laevis. An attempt to characterise this material by investigating its behaviour on dilution in the assay and on Sephadex gel filtration suggested the possibility that this material is similar to the decapeptide, caerulein, which has been isolated from the skin of the Australian bullfrog, Hyla caerulia, by Anastasi, Erspamer et al. (1967). The nature of the gastrin-like material in frog skin and frog serum was different, as shown by the different elution profiles obtained on Sephadex chromatography of the two samples.

Measurements of the content of immunoreactive "gastrin" in various tissue extracts prepared from the frog, Xenopus laevis, with two antisera of known specificity suggested that the immunoreactive material differed from gastrin, since higher levels were measured with antiserum G 1 than with antiserum 2604-7, in contrast to the situation found in plasma and tissues from higher species.

No remarkably high levels of gastrin-like immunoreactivity were recorded in the tissue extracts prepared from the snake, Dasypeltis scabra scabra. These findings, coupled with the "normal" levels of serum gastrin in the snake, were very different from the findings in the frog, Xenopus laevis. Suggestions relating to the significance of these observations were discussed.

No immunoreactive gastrin was detected in any tissue extracts prepared from invertebrates, representing four different invertebrate phyla. The absence of gastrin at this stage in evolutionary development, coupled with the findings of only sulphated gastrin in the fishes and both sulphated and non-sulphated gastrin in higher vertebrate species, suggested that the gastrin molecule arose in evolution at approximately the same time as the appearance of the gnathostomes. The evolutionary development of related gut hormones was discussed.

Transhepatic measurements of circulating gastrin in the pig revealed that the liver had no effect on basal endogenous or elevated levels of exogenous gastrin produced by prolonged infusion of synthetic human hepta-decapeptide gastrin.

The daily production rate of gastrin at the portal site in the pig was estimated to be approximately 20 micrograms.

The half-life for the disappearance of infused SHG-17 I from the porcine circulation was 3,19 minutes, as determined using samples drawn from the hepatic arterial circulation.

Longer half-life values of 4,23 and 4,32 minutes, calculated using hepatic portal and hepatic venous samples are thought to be due to endogenous gastrin production into the portal circulation.

The metabolic clearance rate of the infused gastrin was 2,014 litres/minute or 67,8 ml/kg/min, when estimated using hepatic arterial samples. The corresponding values determined using samples obtained at the portal and venous sites were higher, reflecting the longer half-life values at these sites.

The space of distribution of infused gastrin in the pig calculated from the arterial samples was 6,401 litres or 21,6% of body weight, which corresponds with the extracellular fluid volume.

The total daily production of gastrin in the pig, calculated from the metabolic clearance rate, was of the order of 120 micrograms. Since the calculated portal production of gastrin was only 20 µg/day, an alternative site or sites of gastrin production is suggested. The possibility that neural tissue is a source of gastrin production appears feasible from these observations.

Sephadex G-25/G-50 chromatography of serum samples collected during the infusion suggested the appearance of a gastrin species larger than the heptadecapeptide at the arterial site during the 3rd hour of infusion of SHG-17 I, whereas at the portal and venous sites the presence of a smaller gastrin species in addition to the heptadecapeptide was detected after two hours of infusion. The significance of these observations is poorly understood.

Perfusion of the isolated rat liver in situ with a plasma-free medium containing synthetic human heptadecapeptide gastrin showed that the rat liver did not change the levels of circulating gastrin either at physiologic or supraphysiologic dose levels. This is in agreement with the findings that the pig liver had no effect on basal or stimulated levels of circulating gastrin.

Gel filtration of samples of perfusate collected at different times during the perfusion of the isolated rat liver revealed no change in the chromatographic profile of the heptadecapeptide gastrin during the 60 minutes of perfusion. This is at variance with the appearance of smaller and larger gastrin species in the porcine circulation following prolonged infusion of SHG-17 I in vivo. The additional peaks found in the porcine circulation may in part be due to artefacts produced by prolonged circulation in vivo.

The binding of ^{125}I-labelled and unlabelled SHG-17 I to the rat liver cytosol protein, ligandin, *in vitro* was demonstrated. This binding was specific for gastrin and was not found with the gut hormones insulin or glucagon. Bound ^{125}I-gastrin could be displaced by addition of excess unlabelled gastrin and it was shown that the binding involved the gastrin molecule itself and not the ^{125}Iodine label.

This phenomenon of binding of gastrin by a rat liver protein does not correlate with the finding that the pig liver did not take up gastrin at high delivery rates. Whether the binding of gastrin to rat liver ligandin is the result of a non-specific ionic attraction due to the highly anionic nature of the gastrin molecule, or whether it represents a role of the liver in relation to gastrin which is not yet understood, remains to be explained.

As is the case with most research, when one sets out to answer one question several more avenues open up during the investigation. This was experienced many times during the studies described here, so that many of the newly-posed questions remain unanswered. The following is an outline of possible lines of future research which stem from the findings reported in this thesis.

The successful production of an antiserum directed towards the N-terminal (1-17) portion of the big gastrin molecule, followed by measurement of the circulating levels of this gastrin fragment in normal subjects under basal and post-prandial conditions, and in hypergastrinaemic conditions would be invaluable for investigation of the possible biosynthetic precursor relationship of the G-34 and G-17 molecules. Measurement of gastrin at duodenal and antral sites *in vivo* and of gastrin secretion by *in vitro* oriented sheets of

antral or duodenal mucosa with such an antiserum would bring us a long way towards understanding the significance of the different gastrin components at these two sites.

Characterisation of the high levels of gastrin found in the rat fundus and rabbit colon by gel chromatography and dilution in the assay, using antisera of known specificity would help clarify the significance of these findings.

The gastrin content of neural tissue should be systematically investigated by preparing extracts of various parts of the brain and perhaps large nerves, such as the vagus, from different species. Such a study would possibly substantiate the sprinkling of reports of the presence of gastrin or gastrin-like peptides in brain and neural tissue which have appeared recently. In addition, such an investigation would confirm or refute the suggestion that gastrin production by neural tissue may in part account for the difference in daily production of gastrin found in the whole body of the pig as opposed to the production at the portal site. Should such studies not be rewarding, alternative sites for gastrin production, in the pig at least, could be investigated.

In order to verify the hypothesis put forward in this study concerning the evolutionary origin of the gastrin molecule, a study of the occurrence of gastrin measured with antisera of known specificity in other species situated on the evolutionary tree at the point where the vertebrates arose would be invaluable. Such an investigation would, however, be limited by the number of species from this era which are living today. Collection of serum samples from a large number of teleosts and chondrichthyes for measurement with antisera specific for both sulphated and non-sulphated

types of gastrin would help verify the findings reported here.

The immunoreactive gastrin-like material found in the serum and skin extracts of the frog, Xenopus laevis, could be extracted in large amounts and purified, followed by testing of the immunological and biological activity of the pure peptide. Determination of the amino acid sequence of such a purified peptide would confirm whether it was in fact identical with caerulein.

The significance of the gastrin-like material found in frog skin may relate to the stimulation of growth of tadpoles or possibly act as an ionic regulator or sexual attractant, and could be investigated. It may be that "gastrin" is a pheromone in the frog.

If sufficient amounts of the purified natural and synthetic gastrin peptides were available, the response of the pig liver to infusion of increasing doses of these preparations could be investigated. The kinetics of these preparations in the porcine circulation could be studied in the same way as the kinetics of SHG-17 I in the pig were followed. Gel filtration of serum samples collected during the infusion would reveal any changes in the molecular forms in vivo, possibly throwing some light on the interconversion of the different gastrin components in vivo.

Repetition of the study of the transhepatic response to basal and stimulated levels of circulating gastrin accompanied by measurements of both immunoreactive gastrin and measurements of gastrin bioactivity using a bioassay would confirm the assumption that the liver had no effect on the biological activity of gastrin.

Further studies on the binding of gastrin to rat liver ligandin in vitro, such as displacement of bound ^{125}I-labelled gastrin with increasing concentrations of unlabelled gastrin, as in a radioimmunoassay system, would help to characterise the nature of the binding observed in these studies and possibly throw some light on its biological significance.

These are some of the many unanswered questions raised by the studies in this thesis. Gastrin attracted me as a simple, well-defined peptide which would be easy to study. The ever increasing complexities as the studies progressed were exasperating at times, but some of the observations may prove useful as a basis for further study for such diverse disciplines as gastroenterology, endocrinology, neurology, dermatology and genetics. If I have succeeded in this small respect, I will feel vindicated for the sufferings and forbearance of my husband, supervisor and colleagues.

CHAPTER 7

APPENDIX

7 (i) Outline of McGuigan's Conjugation and Immunisation Procedures

(McGuigan J.E., Gastroenterology 54 (6) : 1005-1111 (1968))

Conjugation Procedure

Six milligrams of SHG 2-17 were dissolved in 0,4 ml N,N-dimethyl formamide, to which was added 0,6 ml 0,05M-potassium phosphate, pH 7,4. Twelve milligrams bovine serum albumen in 0,4 ml 0,05M-potassium phosphate, pH 7,4, and 50 mg EDAP-carbodiimide in 0,2 ml 0,05M-potassium phosphate, pH 7,4, were added. The reaction mixture was gently stirred for 20 hours at 20°C, and then dialysed for 48 hours against 2 litres of 0,15M-NaCl -0,1M-potassium phosphate, pH 7,4, at 4°C.

Immunisation

The conjugate was emulsified with an equal volume of 4:1 mineral oil - Arlacel A. Eight hundred µl, containing 2 mg gastrin, was injected into the foot pads of New Zealand white rabbits, so that an equal volume was given via each foot pad. Immunisation was repeated 2 months and 5 months after the first injection. Fourteen days after each injection 30-45 ml blood was collected from each rabbit by cardiac puncture. Sera were individually precipitated by dropwise addition of saturated ammonium sulphate to a final concentration of 40% to isolate the gamma-globulin fraction.

7 (ii) Folin-Lowry Protein Determination

(Work T.S. and Work E. Laboratory Techniques in Biochemistry and Molecular Biology. Vol. 1 : 366 (1970). North Holland, Amsterdam, London.)

The principle of the method is that a copper-tartrate complex is allowed to react with the protein in alkaline solution. The protein-copper complex can reduce phosphomolybdate to form a blue substance with a broad absorption peak around 750 nm. The procedure described here is a modification of the original method described by Lowry, Rosebrough, Farr et al. in 1951, and detailed in Work and Work (1970).

Reagents

Reagent A : 2% anhydrous Na_2CO_3 in 0,1 N-NaOH

Reagent B : 0,5% $CuSO_4.5H_2O$ in 1% Na Tartrate

Reagent C : 1 ml reagent B mixed with 50 ml reagent A, made up fresh daily.

Reagent D : Commercially available Folin-Ciocalteu reagent (Analar), 1,8 N, diluted 1:1 with water.

Protein standards were prepared using Highland Control Serum I. One hundred µl of the stock solution containing 6g/100 ml was diluted to 6 ml with distilled deionized water to give a solution containing 1 mg/ml, or 100 µg/100 µl. A series of six standards ranging from 100 µg/100 µl down to 10 µg/100 µl was prepared by dilution of this solution with 0,1 N-NaOH. Unknowns were diluted to fall within the range of these standards. The degree of dilution for the unknowns was determined by trial and error.

To 100 µl of standard or unknown, 100 µl of 0,1 N-NaOH was added. The blank tubes contained 200 µl 0,1 N-NaOH. Two ml reagent C were added to each tube, which were then shaken and allowed to stand for 10

minutes at room temperature. Two hundred μl of reagent D, the diluted Folin reagent, was added and the tubes were mixed rapidly. After leaving the tubes to stand for at least 30 minutes to allow the blue end product to stabilize, the solutions were read spectrophotometrically at 750 nm. If the protein concentrations were too high, the solutions were read at 500 nm, where the absorbancy is lower. The standard curve was constructed and the unknowns were read off and corrected for dilution.

7 (iii) Details of Operative Preparation of Pigs and Blood Flow Measurement Technique

Anaesthesia was maintained with oxygen and nitrous oxide given via a Magill-type circuit. The animals were allowed to breathe spontaneously to avoid possible interference by intermittent positive pressure ventilation with portal flow (Rabinovici, 1968).

The catheter introduced via the right external jugular vein was manipulated into the left hepatic vein by intra-abdominal palpation, and could almost always be felt within the liver substance when small pigs were used. The position of the catheter was confirmed by a bloodless patch in the liver upon rapid infusion of saline. The cather was then withdrawn 1-2 cm to prevent wedging.

The portal catheters were inserted via a small tributary draining the pancreas in order not to disturb portal circulation. No pancreatic oedema or infarction was noted.

Total operating time was between 60 and 90 minutes in all cases. All animals were given 20 m Eq. sodium bicarbonate to counteract the mild metabolic acidosis (base deficit 5-7 m Eq/l) which occurred after pentothal induction, and heparin 2 mg/kg to prevent clotting in the catheters.

The priming dose of BSP was 300 mg and infusion was continued at the rate of 5 mg/min. The priming dose of ICG was 2,5 mg followed by infusion at 0,156 mg/min. Portal flow was measured with PAH using a priming dose of 12,5 mg and subsequent infusion at 72 mg/min.

Individual flow values for each experiment were a mean of the three samples taken during the second 30-minute period, and for each group a mean of these individual values was taken.

7 (iv) Technique of Perfusion of the Isolated Rat Liver in Situ

(Hems, R., Ross, B.D., Berry, M.N., Krebs, H.A., Biochem. J. 101 : 284-292 (1966))

For each perfusion 62 ml of medium were used, which consisted of: (i) Krebs-Ringer Bicarbonate buffer (KRB)*; (ii) 4-5 week old donor human erythrocytes, thrice washed with 2-3 volumes KRB, with a haematocrit of 8% and a haemoglobin concentration of 2,7 g/100 ml; (iii) 4,5 mM D+ glucose, and (iv) 2,5% (w/v) bovine serum albumen, fraction V (Miles Laboratories, Cape Town). The final pH of the medium was 7,38 - 7,50.

Rats were lightly anaesthetised with diethyl ether and the bile duct cannulated. During portal and inferior vena caval cannulation the liver was perfused with plasmalyte B (35-38°C) containing in millimoles/litre, Na^+ 130, K^+ 4, Mg^{2+} 1,5, Cl^- 109, and HCO_3^- 28. The usual time from portal venous ligation to establishment of perfusate flow was $3\frac{1}{2}$ - 4 minutes. The first 2 or 3 ml effluent were discarded. The perfusion pressure was adjusted to 15-20 cm water to provide flow rates of 3,0 - 5,0 ml/min/g wet weight of liver, which would satisfy maximal oxygen requirements without liver swelling or surface exudation. Flow rates, monitored by timing 10 ml vena caval effluent every $\frac{1}{2}$ hour, were uniform 30 minutes after the start and for a further 90 minutes of perfusion. Adjustments were made to maintain flow rates where necessary. Liver temperature was monitored by a telethermometer probe placed between adjacent lobes, and was maintained at 37-38°C. Under the conditions of the perfusion the metabolic integrity of the liver, assessed in separate preliminary experiments, was confirmed by satisfactory maximal rates of lactate gluconeogenesis from 30-120 minutes, time 0 minutes being taken as the start of the

perfusion. The values, 0,85 ± 0,05 μmoles glucose/min/g liver, (mean ± S.E.M., n=9) accord closely with those reported by Hems et al. (1966) and Exton and Park (1967). Bile production ranged from 100-160 μl/g wet liver between 30 and 120 minutes perfusion.

* The KRB buffer was made up as follows:

0,9%	NaCl	100 parts by volume
1,15%	KCl	4 parts by volume
1,22%	$CaCl_2$	3 parts by volume
2,11%	KH_2PO_4	1 part by volume
3,82%	$MgSO_4.7H_2O$	1 part by volume
1,3%	$NaHCO_3$	21 parts by volume

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