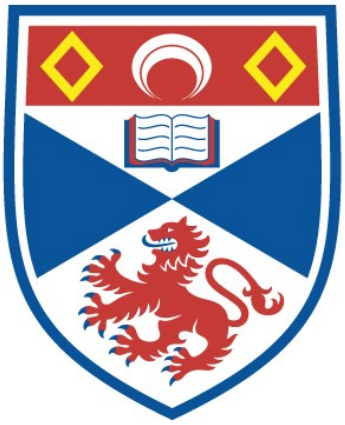
CHOLECYSTOKININ: MEASUREMENT, BIOLOGICAL ACTION AND CLINICAL SIGNIFICANCE

Christopher Edwin Marshall

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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CHOLECYSTOKININ: MEASUREMENT, BIOLOGICAL ACTION AND CLINICAL SIGNIFICANCE

by

Christopher Edwin Marshall

A Thesis submitted to the University of St Andrews in application for the Degree of Doctor of Philosophy

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MARCH, 1979

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Professorial Department of Surgery, Charing Cross Hospital Medical School, London, under the supervision of Dr M.W.Steward, Mr A.G.Johnson and Dr S.Bayne.

C.E.MARSHALL

CERTIFICATE

We hereby certify that Christopher Edwin Marshall has spent ten terms engaged in research work under our direction and that he has fulfilled the conditions of Ordinance General No. 12 and the Resolutions of the University Court 1967, No. 1, and that he is qualified to submit this thesis for the Degree of Doctor of Philosophy.

M. W. STEWARD

A. G. JOHNSON

S. BAYNE

CAREER

I first matriculated as a part-time external student in the Faculty of Science (Department of Biochemistry and Microbiology), University of St Andrews in October 1974 to work under the supervision of Dr M.W.Steward, Mr A.G.Johnson and Dr S.Bayne following a four year professional career in research; this included three and a half years industrial research with Brooke Bond Liebig Ltd., and one year, more biochemically and clinically orientated with the Professorial Department of Surgery at Charing Cross Hospital Medical School, London.

I have remained at Charing Cross Hospital and the work described in this thesis was performed there during my employment as a research Biochemist.

ACKNOWLEDGMENTS

I would like to express my grateful thanks to Dr. M.W. Steward and Mr. A. G. Johnson for all their help, encouragement and advice throughout this work, and to Dr. S. Bayne for his assistance in preparing this thesis.

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Thanks are also due to the Charing Cross Hospital Clinical Research Committee and the North West Thames Regional Health Authority for the financial support of this project, and to Professor A.J.H. Rains and my parents for personal financial support.

I must also thank Mr. S.Ganley and Mr. C. Burgess of the Department of Medical Illustration, Charing Cross Hospital Medical School for preparing the illustrations.

Finally, I must thank Mr. C.J.C. Kirk, Dr. S. L. Howell and all other members of the Professorial Department of Surgery for their active support in discussions and for their tolerance of my frequent demands for blood donations.

ABBREVIATIONS

With the following exceptions all abbreviations in this thesis are those recommended by the IUPAC - IUB commission on Biochemical Nomenclature as published in the Biochemical Journal Instructions to Authors (revised) 1978 (1).

The following exceptions are for the most part defined the first time that they appear in the text but are collected together here:

ab	antibody
ACTH	Adrenocorticotropic hormone
ag	antigen
CCK	Cholecystokinin
CCK-V	Cholecystokinin varient
CHR units	Crick, Harper Raper units
CU	Clinical Unit (of secretin)
GI-tract	Gastrointestinal tract
G.I.H.	Gastrointestinal Hormone (Research Institute, Karolinska)
G.I.P.	Gastric Inhibitory Peptide
5-HT	5-hydroxytryptamine
(m)IDU	(milli) Ivy Dog Unit
KIU	Kallikrein Inactivator Unit
P.V.P .	Polyvinylpyrrolidone
PZ	Pancreozymin
V.I.P.	Vasoactive Intestinal Peptide

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Summary

This thesis describes in full the development of a biological method of estimating cholecystokinin (CCK) in human serum by superfusion of rabbit gall-bladder strips, from the early stages of the manual technique, which would estimate approximately twelve samples per day, to the more sophisticated and clinically useful automated technique of today which can estimate up to forty eight sample solutions in duplicate each day.

In developing this bioassay for CCK it was necessary to determine whether the gall-bladder preparation was stable for the duration of the experiment, and also whether or not there were other factors influencing the response of the gall-bladder strips to a given stimulus. It was also necessary to determine whether the assay was entirely specific for CCK in human serum and to counteract any possible degradation of CCK during processing and storage of serum.

In the light of the findings during the above experiments the technique was modified to eliminate errors that would otherwise be made in estimating the CCK content of a serum sample.

Since our attempts to set up a radioimmunoassay were unsuccessful a comparison of this bioassay was made with

another groups' radioimmunoassay. Although no direct comparison could be made as the two assays measure CCK in totally different units, nevertheless a straight line relationship between the two very different methods was found, and this experiment led directly to the discovery by bioassay of CCK mimicking substances in serum.

The action of trypsin on CCK has been clearly demonstrated and its ability to release the C-terminal octa and more probably the dodeca peptide, both of which are considerably more active than the full molecule, is beyond question.

It is therefore important that an enzyme inhibitor is added to blood samples to prevent this spontaneous breakdown of more active fragments and prevent false readings of serum cholecystokinin activity.

During chromatographic investigations a new molecule in human serum which possessed cholecystokinetic activity but had a molecular weight in excess of 30,000 (c/f 3,900 for normal CCK) was discovered. This molecule appeared to predominate in the fasting state but to decrease in concentration during the response to a meal.

The practical use of measuring serum CCK is demonstrated in some clinical trials in which CCK's target organs are removed or the stimulation for its release is modified by various surgical procedures. Some interesting changes are noted.

INTRODUCTION

Cholecystokinin (CCK) is a 33 amino acid peptide hormone found in the upper part of the gastro-intestinal tract (GI tract). Classified with the kinins by virtue of its action on smooth muscle, it is released from the duodenum on stimulus with fat or acid and is now known to have at least two main functions:

1. It causes the gall-bladder to contract thereby mixing the duodenal contents with bile, emulsifying the fat and facilitating digestion.

2. It stimulates the release of enzymes from the pancreas. In addition there is evidence that it exerts some control over the pyloric sphincter and has some effect on gut motility.

The physiology and biochemistry of CCK are not well understood. This appears to be due to:

a) difficulties in estimating the concentration of CCK, and,

b) the lack of commercial source of CCK of sufficient purity and guaranteed activity to allow research work to proceed without constant doubts about the reliability of the results.

This project set out to:

1. Establish at least one readily acceptable form of routine assay for CCK.

2. To compare the results obtained by bioassay with those obtained simultaneously by radioimmunoassay - if possible.

3. To use the assays to study the changes in blood concentrations of CCK following meals after different gastric surgery procedures, and note any changes related to pathological conditions, e.g. pancreatitis.

4. Tp determine the active form or forms in which CCK normally exists in blood serum.

5. To study the relative potencies of the commercial preparations of CCK available and assess their values as standards.

SECTION A

HISTORICAL REVIEW

Chapter A1

A1.1 The Very Beginning

It is difficult to assess the exact moment when interest in the function of the pancreas and the origin and function of bile began to pave the way for the discovery of the numerous peptide hormones of the upper gastro-intestinal tract known today.

The pancreatic duct was first discovered in the turkey by Moritz Hoffman in 1641. One year later the existence of a duct system in the human pancreas was published by Wirsung to whom Hoffman had communicated his findings in the turkey (Wirsung, 1642). Then began the argument as to whether the function of the duct was secretory or excretory. In 1671 Regnier de Graaf published the first report of the collection by cannulation of pancreatic juice.

Interest in the pancreas was mainly centred on the role it played in digestion, and little seems to have been learnt through the 18th and early 19th centuries. In 1826 Tiedemann and Gmelin quoted both Haller and Magendie as saying effectively that despite numerous hypotheses nothing was really known about the function of this enigmatic gland.

However, it was in 1825, in work that has been largely ignored outside France, that the first hint that hormones - a term then

unknown - could be involved in digestion was found by the two Frenchmen Leuret and Lassaigne (1825). This was published in their book which was entered for the same competition as that of Tiedmann and Gmelin. Leuret and Lassaigne (1825) found that the introduction of vinegar into the duodenum stimulated bile flow and secretion of pancreatic juice together with a dilation of the orifices of the pancreatic and bile ducts in the duodenum. They also noted that the contents of the stomach being acid, their observation was clearly important in the understanding of digestion, since the movement of the acid contents of the stomach into the duodenum could be expected to have a similar effect.

The next major observation was by Bernstein in 1869 who showed that pancreatic secretion was not inhibited if all the nerves to the pancreas were cut; this led to the postulation by Bayliss and Starling in 1902, of a 'chemical reflux' - a change in the function of one organ consequent on manipulation of a remote structure without the involvement of any nervous pathway.

A1.2 The First Gastrointestinal Hormones

Bayliss and Starling's postulation (Bayliss and Starling 1902, 1902a) was based on an experiment with the denervated

jejunum as the chemical source and the exocrine pancreas as the target organ. They noted that a stimulus of 0.4% hydrochloric acid in the duodenum produced a flow of pancreatic juice. They were then surprised to see the same response to the same stimulus in an isolated denervated loop of jejunum. Starling then scraped some mucosa from the duodenum, shook it with 0.4% hydrochloric acid, filtered, and injected the filtrate into the jugular vein of the dog used in the experiement. An immediate response in the form of pancreatic output was noted, thus chemical transmitters became an established fact. Bayliss and Starling gave the name of Secretin to the particular chemical transmitter they assumed to have been released at this time. The term 'hormone' (from Greek meaning 'I arouse to activity' or ' I excite') was first used by Starling in the fourth of a series of lectures on the 'Chemical Correlations of the Functions of the Body' (Starling, 1905).

This was the spark that started the fire of interest in the upper gastro-intestinal hormones, the study of nervous reflexes being temporarily forgotten.

This research led eventually to the discovery of Insulin by Banting and Best in 1922 and to the real beginning of the Ch olecystokinin story in 1928 with the demonstration of gall-bladder contraction following the injection of a purified extract of intestinal mucosa.

Chapter A2

A2.1 The Discovery and Naming of Cholecystokinin and Pancreozymin

Contraction of the guinea pig gall-bladder following an injection of a purified extract of intestinal mucosa was first demonstrated by Ivy and Oldberg in 1928, the active substance was not histamine or choline and was not thought to be secretin. Ivy and Oldberg proposed the name 'Cholecystokinin', classifying the unknown hormone with the kinins by virtue of its action on the smooth muscle of the gall-bladder, no action on blood vessels or nociceptive nerve endings being demonstrated.

Over the next decade several methods for the preparation of cholecystokinin (CCK) were reported (e.g. Ivy, Kloster, Lueth and Drewyer, 1929; Gunnar, 1939) and the presence of cholecystokinin in the human and rabbit intestine was also demonstrated (Drewyer and Ivy, 1930).

Early experiments (Lueth, Ivy and Kloster, 1929) showed that while injection of a preparation of cholecystokinin caused contraction of the gall-bladder, it was without effect on liver volume or gastric motility, although variable action on intestinal motility was noted. The reduced response of the diseased gall-bladder was also demonstrated at this time (Ivy, Drewyer and Orndoff, 1930), and on comparison with pituitrin and pilocarpine cholecystokinin was found to be

the only substance causing gall-bladder contraction without altering blood pressure or causing a general parasympathetic stimulation (Voegtlin and Ivy, 1934).

A new hormone came on the scene in 1943 when Harper and Raper showed that the injection of intestinal mucosa extracts increased the output of pancreatic amylase and trypsinogen but not the total volume of the pancreatic output. Its action was not affected by severing the splanchnic or vagus nerves, nor by atropine; it was stable in acid solution and at 100°C, but was destroyed by alkali. This discovery was confirmed by Greengard, Grossman, Woolley and Ivy (1944), and the name pancreozymin originally conferred on th is hormone by Harper and Raper (1943) was accepted.

A.2.2 Purification of Cholecystokinin and Pancreozymin

The initial stages for the preparation of cholecystokinin have hardly changed in the fifty years of the hormone's history. Harper and Raper (1943) began their preparation of pancreozymin (PZ) by following the procedure of Mellanby (1932) for the preparation of secretin. The first metre of pig intestine was extracted with alcohol, calcium chloride was added to the extract which was then filtered and concentrated. Bile salt (tauroglycocholate)(in dilute acetic acid) was added to the concentrate. The precipitate contained secretin and was collected by centrifugation, then purified by extraction with alcohol and reprecipitation with acetone.

Pancreozymin was not adsorbed by the bile acid and remained in solution. The supernatant was saturated with sodium chloride and left to stand for 2 - 3 days in the dark. A sticky precipitate containing pancreozymin formed; this was collected, dissolved in water and dried in a desiccator. Variations in the properties of commercial bile salts resulted in low yields, however, and the technique was later modified (Crick, Harper and Raper, 1949), the secretin being adsorbed on to a bile-charcoal complex. This bile-charcoal complex was prepared by adding Norite charcoal to bile, mixing and evaporating to dryness. The solid material was extracted with methylated spirit on a boiling water bath, the suspension filtered and ether added to the filtrate. A white precipitate was allowed to separate at $0^{\circ}C$. The supernatant was removed and the precipitate washed with ether and dried. Yield: 2.14g bile salts/100ml bile. It was also found necessary to mix pig and ox bile to obtain the best adsorption of secretin.

Perhaps it was because of the difficulties in preparation that cholecystokinin and pancreozymin were virtually ignored through the 1950's until Professors Jorpes and Mutt turned their attention from secretin in the latter part of the decade. In 1959 Jorpes and Mutt (1959) while reviewing the current clinical aspects of secretin, cholecystokinin and pancreozymin, outlined their procedure for the preparation of cholecystokinin.

Again starting with the first metre of hog intestine, they extracted this material with acetic acid for one hour at 100°C, this gave a less bulky crude material than hydrochloric acid. The hormones were then adsorbed on to ion exchangers of non-crosslinked polymers, of which alginic acid (polymannuronic acid) was found to be the best. The hormones were eluted from the ion exchanger with hydrochloric acid and precipitated with sodium chloride.

Later using the commercial product 'Cecekin' (Vitrum Co.,) as their starting material Dhariwal, Schally, Meyer, Sun, Jorpes and Mutt (1964) obtained a 30 fold purification of both cholecystokinin and pancreozymin activity in just two steps on DEAE and CM cellulose columns. In a continuation of these experiments and finding that cholecystokinin and pancreozymin activities increased proportionally through all purification stages using both acidic and basic ion exchangers Jorpes and Mutt (1966)suggested that the two very different types of activity were in fact due to the same substance. This work resulted in cholecystokinin preparations with an activity of 3000 Ivy Dog Units per milligram (IDU/mg), although one preparation had an activity of 6000 IDU/mg. (An Ivy Dog Unit of cholecystokinin is that amount of dry material which when dissolved in isotonic

saline solution and injected intravenously to a dog over 10 - 15s causes a 1cm rise in the bile pressure within 1 - 5 minutes (Ivy and Janacek, 1959). Bile pressure is measured by the height of bile maintained in a cannula inserted into the fundus of the gall-bladder.)

Chapter A3

A3.1 Characteristics of Cholecystokinin

In the early days of secretin preparation Bayliss and Starling (1902) showed that the active substance was non-volatile, thermostable, soluble in aqueous ethanol even at high ethanol concentrations, but was insoluble in absolute ethanol and in ether, it was dialyzable and easily destroyed by proteolytic enzymes, the salts of mercury, lead and iron and by oxidation with dilute solutions of potassium permanganate. These properties were strong indications of a peptide compound.

Professors Jorpes and Mutt with Dr Jelling began work on secretin in 1949 in response to a request for a clinical preparation (Mutt, 1959). They quickly established that, despite claims to the contrary, secretin had not at that time been prepared in pure form. By the end of the 1950's Jorpes and Mutt (1959) were routinely preparing secretin in pure form, and while continuing studies on the amino acid sequence of secretin, began to turn their attention to the related hormones Cholecystokinin and Pancreozymin.

Isolation of cholecystokinin and pancreozymin began with the preparation of a crude concentrate; the upper intestines of hogs were immersed in boiling water to inactivate the proteolytic enzymes, after a few minutes these were minced and extracted with 0.5M acetic

acid. The active material was adsorbed onto alginic acid, eluted with 0.2M hydrochloric acid and precipitated by saturation with sodium chloride. This precipitate contained secretin, cholecystokinin and pancreozymin. (Jorpes and Mutt, 1959).

The precipitate was dissolved in water and fractionated with ethanol, the fraction with the highest secretin activity being recovered in aqueous solution and precipitated at pH4.5 with sodium chloride. At this point there was no marked separation of secretin from pancreozymin and cholecystokinin. However in the next step when the precipitated material was extracted with methanol it was found that the bulk of the secretin was methanol-soluble, whereas the bulk of cholecystokinin and pancreozymin was in the methanol-insoluble fraction.

Attempts to purify further pancreozymin from cholecystokinin resulted in the observation that every fractionation leading to an increase in pancreozymin activity also resulted in a parallel increase in cholecystokinin activity. With these results before them Jorpes and Mutt (1966) proposed that cholecystokinin and pancreozymin might be two different effects of the same hormone.

Five final purification steps (Jorpes, Mutt and Toczko, 1964) at last produced an apparently homogeneous polypeptide with both cholecystokinin and pancreozymin activities. Then began the painstaking work of elucidating the amino acid sequence.

It was first observed that cholecystokinin (having been discovered first it was deemed reasonable to call this polypeptide cholecystokinin and denote it as having a pancreozymin effect) did not contain threonine or cystine/cysteine but did contain methionine. Cleavage at the methionyl residues resulted in several fragments, one of which was identified as aspartylphenylalanine amide (Mutt and Jorpes, 1967), suggesting a C-terminal sequence identical to gastrin. Following a final purification step (chromatography at pH8 on CMC (Carboxy Methyl Cellulose)) the elucidation of the amino acid sequence was perfectly straightforward.

Cleavage with thrombin and trypsin (Mutt and Jorpes, 1968) whose specificities are known produced five tryptic peptides. The amino acid sequence of these peptides was determined and the final full amino acid sequence of cholecystokinin (Mutt and Jorpes, 1970) was shown to be:

Lys-Ala-Pro-Ser-Gly-Arg---Val-Ser-Met-Ile-Lys---Asn-Leu-Glu-
Ser-Leu-Asp-Pro-Ser-His-
Arg---Ile-Ser-Asp-Arg---Asp-Tyr(SO_3)-Met-Gly-Trp-Met-Asp-Phe-NH_2

The cholecystokinin C-terminal octapeptide amide was synthesised almost immediately and shown to have a considerably greater activity on a molar basis than the whole molecule (Ondetti, Rubin, Engel, Pluscec and Sheehan, 1970), and in the same paper it was shown that the C-terminal portion of the molecule was the active part, and that

at least seven amino acids, including the sulphated tyrosine, were necessary for biological action. The amide grouping at the C-terminus is also essential for gastrin activity (Morley, Tracy and Gregory, 1965) and, since no synthetic fragment has been synthesised without it (Ondetti et al, 1970) although no specific reference can be found, it would seem certain that this amide grouping is essential for cholecystokinetic activity. We, (in this work) and others (Ondetti et al, 1970) have observed that the octapeptide without the sulphate on the tyrosine is inactive and may exert an inhibitory effect on the natural molecule.

More recently Mutt (1976) has isolated a form of cholecystokinin having 39 amino acids, it is identical to the 33 amino acid form but has an additional six amino acids at the N-terminal end in the form of the peptide Tyr-Ile-Gln-Gln-Ala-Arg: the activity also appears to be equivalent to the 33 amino acid form.

A3.2 Assay of Cholecystokinin

From Ivy's original in vivo experiments with dogs (Ivy and Oldberg, 1928) to the automated superfusion techniques of today (Marshall, 1976; Marshall, Egberts and Johnson, 1978) the majority of assays for cholecystokinin have been biologically based. While other peptide hormones have yielded readily to radioimmunoassays, such assays for cholecystokinin have been rare, and all have given conflicting results (Reeder, Becker, Smith, Rayford and Thompson, 1973; Go, Ryan and Summerskill, 1971; Young, Lazarus and Chis holm, 1969; Harvey, Dowsett, Hartog and Read, 1974; Schlegel and Raptis, 1976).

With only occasional excursions into other species, such as the frog (Seager, 1939, 1941), cat (Ivy and Janecek, 1959), and rat (Lin and Alphin, 1962) all bioassays until 1969 for cholecystokinin appear to have been carried out on anaethetised dogs; cholecystokinin activity being measured by the increase in intragall-bladder pressure, and pancreozymin by the increased output of amylase in the pancreatic juice.

In the late 1960's advances in electronics and the increased use of organ baths brought the first in vitro assay for cholecystokinin (Amer and Becvar, 1969), where strips of rabbit gall-bladder were attached to isometric transducers, although the sensitivity was low - only 0.05 IDU (50m IDU) could be detected.

Two years later came the first use of superfusion over isolated rabbit gall-bladder using whole blood from an anaethetised cat (Berry and Flower, 1971).

This technique was then applied to human serum and the first sensitive bioassay* for cholecystokinin in human serum (Johnson and McDermott, 1973) soon followed; this used isolated rabbit gall-bladder strips superfused with Krebs balanced salts solution. It is essentially this technique, its extensive modification (Marshall, 1976; Marshall, Egberts and Johnson, 1978) and the results obtained using this technique that form the basis of this thesis together with reports of the problems involved in developing a radioimmunoassay.

* It would be more correct to say'biological method of estimating' but the word 'bioassay' is used through this thesis for convenience.

Chapter A4

A4.1 Known Functions, Uses and Potential Uses of Cholecystokinin (Pancreozymin)

A4.1.1 Introduction

From the original experiments of Ivy and Oldberg (1928) the first function assigned to cholecystokinin was the contraction of the gall-bladder. Some fifteen years later stimulation of enzyme secretion by the pancreas (Harper and Raper, 1943) was discovered, but attributed to another hormone, pancreozymin, until the two were shown to be identical (Jorpes and Mutt, 1966). Since stimulation of gall-bladder contraction was discovered first the hormone is now generally known as cholecystokinin.

In addition to these two main physiological functions, cholecystokinin has also been shown under experimental conditions, mainly on animal models, to have a number of effects on other body organs.

A4.1.2 Release Mechanisms

Cholecystokinin has been shown to elicit bicarbonate (Rayford, Miller and Thompson, 1976) and insulin (Unger, Ketterer, Dupre and Eisentrout, 1967) release from the pancreas. It has however since been shown that insulin was not released by CCK but by impurities in the CCK preparation used in that study (Hedner, Persson and Ursing, 1975).

A4.1.3 Stimulatory Effects

Cholecystokin has been shown to stimulate: intestinal motility (Hedner, Persson and Rorsman, 1967) contraction of the quiet - i.e. non-contracting stomach - (Cameron, Phillips and

Summerskill, 1967), the pyloric sphincter (Grossman, 1973), the flow of hepatic bile (Jones and Grossman, 1970), the secretion of Brunner's glands (Stenning and Grossman, 1969), blood flow in the superior mesenteric artery (Bowen, Pawlik, Fang and Jacobson, 1974), and pancreatic growth (Barrowman and Mayston, 1974).

A4.1.4 Inhibitory effects

Cholecystokinin has been shown to inhibit; contraction of the lower oesophageal sphincter (Grossman, 1973); contraction of the sphincter of Oddi (Ondetti et al, 1970); absorption of fluid, sodium, potassium and chloride from the jejunum and ileum (Johnson, 1974); motility of the active stomach (Cameron, Phillips and Summerskill, 1967).

A4.1.5 Action on arterial pressure

Cholecystokinin has been shown to decrease the pressure in the systemic artery (Post and Hanson, 1975).

A4.1.6 Proven Physiological Effects in Man

To date very few of the effects of cholecystokinin shown in animals have been demonstrated to hold true for human tissue. It is however beyond doubt that cholecystokinin causes gall-bladder contraction (Goldstein, Grant and Margulies, 1974), pancreatic enzyme secretion (Hanscom, Jacobson and Littman, 1967) and has some effect on gastric acid secretion or can act as a competitive inh ibitor of gastrin (Grossman, 1970). More recently (Hedner and Rorsman, 1972) cholecystokinin has been shown to affect human gut motility and to decrease small gut transit times.

A4.1.7 More recent developments

It has been suggested that cholecystokinin may also be a satiety signal, and, by negative feedback, instruct the animal to stop feeding. After an overnight fast, rats with chronic gastric fistulae will continuously sham feed a liquid meal for several hours (Young, Gibbs, Antim, Holt and Smith, 1974), a quantity of the liquid meal introduced directly into the small intestine quickly stops sham feeding and initiates the behavioural sequence associated with satiety.

Of several gut hormones injected into the peritoneal cavity only cholecystokinin and the C-terminal octapeptide of cholecystokinin inhibited sham feeding (Lorenz, Gibbs and Smith, 1978) although the dosage given (5-160 IDU/Kg of CCK, or 20-640 IDU/Kg of the octapeptide) would appear to be well outside the normal circulating level of this hormone. Although cholecystokinin has not yet been shown to inhibit feeding in man (Goetz and Sturdevent, 1975) the possibility that some forms of obesity are due to a hormone deficiency or a failure of the receptor sites on the brain stem must be investigated.

A4.1.8 Recent use in clinical practice

It has also been shown (Hedner and Rorsham, 1972; Levant, Kun, Jachna, Sturdevant and Isenberg, 1974) that cholecystokinin and its C-terminal octapeptide are both powerful stimulants of small bowel motility. Thus patients with prolonged intestinal ileus

may be relieved by treatment with cholecystokinin, but this still needs evaluation. Cholecystokinin has also been used as the stimulant for cholecystogram investigations (Goldstein, Grant and Margulies, 1974) and pancreatic function tests, (Hanscom, Jacobson and Littman, 1967).

A4.2 Release of Cholecystokinin

Cholecystokinin is released into the blood stream in response to the presence of food in the duodenum and jejunum. The amount of hormone released is related to the rate at which food enters the upper intestine and the length of intestinal mucosa that is stimulated (Johnson, 1974).

The efficiency with which cholecystokinin is released varies considerably with the stimulus given; some foods and digestive products are very effective, others release virtually no cholecystokinin at all. In the first group are; L-amino acids, fatty acids greater than nine carbons in length and hydrochloric acid (Meyer, 1974). In particular the amino acids phenylalanine, valine and methionine were very active and tryptophane rather less active (Go, Hoffman and Summerskill, 1970) while acid is only a weak releaser of cholecystokinin (Meyer and Grossman, 1971) however, Berry and Flower (1971) found hydrochloric acid to be the strongest stimulant for cholecystokinin release in the cat, although if there was no response at 0.01N the acid had to be 0.025 - 0.1N to evoke this response. In contrast hydrolytic products of carbohydrates and glycerol have

either no effect or are weak releasers of cholecystokinin (Go, 1978). Perfusion of the duodenum with a 6 - 12mM calcium solution stimulated pancreatic enzyme output and gall-bladder contraction in a manner comparable with exogenous cholecystokinin (Holtemuller, Malagelada, McCall and Go, 1976).

A4.3 Location of Cholecystokinin

From the beginning (Ivy and Oldberg, 1928) it has been known that cholecystokinin originates in the mucosa of the upper intestine, but it is only in the last decade, with the advent of immunocytochemical techniques, that the cell responsible for releasing, and probably manufacturing, cholecystokinin has been identified.

Following the work of Pearse (1966, 1966a) a number of peptide hormone-producing cells were grouped together on the basis of their cytochemical characteristics. In 1968 Pearse produced a much larger series of cells with similar cytochemical properties: from the pancreas, pituitary, thyroid, intestine and stomach, cells were found which had (I) high α- glycerophasphate menadione reductase, (II) high non-specific esterase and/or cholinesterase, (III) fluorogenic amine content (presumed 5-HT), (a) primary, (b) secondary, (IV) amine precursor uptake, (V) amino acid decarboxylase (presumed).

Since no single term could be found to cover properties III, IV and V above, the letters APUD were used to indicate Amine content

and Amine and Precursor Uptake and Decarboxylation. Cells containing the three characteristics together with one or other of the associated enzyme systems have since been referred to as belonging to the APUD series, the peptide hormone-producing cells being classified by letter within this series.

The cholecystokinin-producing cell has been shown to correspond closely to the intestinal I cell (Burra, Solcia and Go, 1976; Polak, Pearse, Bloom, Buchan, Rayford and Thompson, 1975) and is now generally accepted as being the I cell.

A4.4 Catabolism of Cholecystokinin

Studies on the catabolism of cholecystokinin are at present incomplete. It has been shown that the disappearance half life of exogenously administered cholecystokinin was 2.4 min. in man and 2.6 min. in dogs (Rayford, Miller and Thompson, 1976).

Use of ^{125}I-CCK has shown the sites of tissue uptake and catabolism of cholecystokinin to be the kidneys, liver, stomach, intestine, pancreas, gall-bladder, lungs and spleen, with a circulating half life of 3 minutes (Go, 1978; Owyang, Ng and Go, 1976). Eight minutes after injection the kidneys had concentrated about half the labelled cholecystokinin injected. This suggests the kidneys play a major role in cholecystokinin catabolism and it is not therefore surprising that Go (1978) and Owyang, Go, DiMagno, Miller and Brennan, (1977), using their radioimmunoassay found elevated serum levels of cholecystokinin in patients entering renal failure.

Chapter A5

Relationship of Cholecystokinin with other Gastrointestinal Hormones

A5.1 The Cholecystokinin Family

Structural similarities between the peptide hormones of the gastrointestinal tract divide them indisputably into families. The cholecystokinin family at present consists of only four naturally occurring peptides (excluding variants and fragments); cholecystokinin gastrin, caerulein and phyllocaerulein. Of these four only cholecystokinin and gastrin have been shown to occur in the human, caerulein and phyllocaerulein being extracted from the skin of the Australian frog Hyla caerulea (DeCaro, Endean, Erspamer and Roseghini, 1968) and the South American amphibian Phyllomedusa Sauvagei (Anastasi, Bertaccini, Cei, De Caro, Erspamer and Impicciatore, 1969) respectively.

The structural similarity is in the carboxy-terminal end of the molecule, the last five amino acids at this end of the molecule being identical and the resemblance continuing with only minor variations to the residue: see Table 1.

A5.2 Structure, activity relationships

Many biological actions of both gastrin and cholecystokinin are produced by the C-terminal tetrapeptide amide (Tracey and Gregory, 1964; Morley, 1970), however Ondetti et al (1970) found the shortest sequence with a significant amount of cholecystokinin activity to be the heptapeptide. The loss of the sulphate group from the

TABLE 1

Structural similarities between peptides of the cholecystokinin family

Cholecystokinin	Ile-Ser-Asp-Arg-Asp-Tyr (SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2
Caerulein	Pyr-Gln-Asp-Tyr (SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2
Phyllocaerulein	Pyr-Glu-Tyr (SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2
Gastrin II	Glu-Glu-Glu-Glu-Glu-Ala-Tyr (SO_3H)-Gly-Trp-Met-Asp-Phe-NH_2

heptapeptide was accompanied by almost total loss of activity, but this tyrosine sulphate group alone and with its surrounding amino acids is virtually devoid of gall-bladder contracting ability. Maximum activity on a weight basis seems to be reached around the C-terminal decapeptide which is 10-15 times as active as the whole molecule, with the C-terminal octapeptide being 10 times as active on a weight basis and 2.5 times as active on a molar basis as the full molecule (Ondetti et al 1970).

The decapeptide caerulein also showed a similar potency while the movement of the sulphated tyrosine by one amino acid in either direction i.e. to positions C_6 or C_8 (from C-terminus) resulted in almost total loss of activity.

A5.3 The Secretin Family

The Secretin family consists of four structurally similar established polypeptide hormones; Secretin, Vasoactive Intestinal Polypeptide (V.I.P.), Glucagon and Gastric Inhibitory Polypeptide (G.I.P.) (Hacki, Greenberg and Bloom, 1978) see Table 2 where points of identity are highlighted by boxes.

TABLE 2

Structural similarities between Secretin, Glucagon, G.I.P. and V.I.P.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
SECRETIN	His	Ser	Asp	Gly	Thr	Phe	Thr	Ser	Glu	Leu	Ser	Arg	Leu	Arg	Asp	Ser	Ala
GLUCAGON	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg
G.I.P.	Tyr	Ala	Glu	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser	Ile	Ala	Met	Asp	Lys	Ile
V.I.P.	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln	Met

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
SECRETIN	Arg	Leu	Gln	Arg	Leu	Leu	Gln	Gly	Leu	Val-(NH_2)																
GLUCAGON	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr														
G.I.P.	Arg	Gln	Gln	Asp	Phe	Val	Asn	Trp	Leu	Leu	Ala	Gln	Gln	Lys	Gly	Lys	Lys	Ser	Asp	Trp	Lys	His	Asn	ILeu	Thr	Gln
V.I.P.	Ala	Val	Lys	Lys	Tyr	Leu	Asn	Ser	Ile	Leu	Asn-(NH_2)															

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A5.4 Interreaction of Gastrointestinal Polypeptide Hormones

A5.4.1 Cholecystokinin and Secretin

Since these two hormones share a common target organ, the pancreas, it is not surprising to find evidence for their interaction: it appears that cholecystokinin potentiates the action of secretin on pancreatic bicarbonate output (Meyer, Spingola and Grossman, 1971) for example an infusion of 0.25 CU/kg/hr of secretin in man produces half the maximum secretory rate, but when the same is given together with cholecystokinin maximum bicarbonate secretion can be achieved (Wormsley, 1969). (1 CU (Clinical Unit) of secretin is approximately equal and equivalent to 4 Crick, Harper, Raper Units of 4 Ivy Dog Units of secretin; 1 IDU secretin is defined as: that amount of dried material in normal (0.9%) saline solution which, when injected intravenously during 10 or 15s, will cause a 10 drop (0.4 ml) increase in the rate of flow during a period of 10 min, the control or basal flow not being more than 1 drop in 2 min (Ivy and Janecek, 1959; Harper, 1967)).

Although secretin itself has no effect on gall-bladder contraction (Berry and Flower, 1971), there is some evidence (Stening and Grossman, 1969a) that it may potentiate gall-bladder contraction stimulated by cholecystokinin, this effect, however may be pharmacological rather than physiological.

A5.4.2 Cholecystokinin and Gastrin

In the cat (Way, 1971) and the rat (Chey, Sivasomboom, Hendricks, and Lorber, 1970) both gastrin and cholecystokinin are able to elicit a maximal output of gastric acid, and although the molar concentration of cholecystokinin required was about six times that of gastrin, both hormones have full efficacy or are full agonists. However, in the dog (Johnson and Grossman, 1971) and probably also in man the maximal output of gastric acid that could be stimulated by cholecystokinin was only one fifth of that produced by gastrin. In these cases cholecystokinin is only a partial agonist for gastrin and has been shown (Grossman, 1971; Johnson and Grossman, 1971), to be a competitive inhibitor of gastrin, although it is unlikely that inhibition occurs at physiological concentrations.

A5.4.3 Cholecystokinin and Insulin

Many studies based on the use of impure preparation of cholecystokinin have suggested that cholecystokinin stimulates insulin release thus contributing to the greater rise in blood insulin following an oral load of glucose than the rise following an intravenous load. These observations have been well reviewed (Pfeiffer, Raptis and Fussganger, 1973).

However a more recent study by Hedner, Persson and Ursing (1975) found insulin to rise following injection of an impure preparation of cholecystokinin but not after injection of the purer

hormone or the synthetic octapeptide. This, together with Harvey and Read's (1973) observation that an oral load of magnesium sulphate elevated blood cholecystokinin but not blood insulin, suggests that cholecystokinin is not the powerful insulin releaser it was once thought, and that this action is far more likely to be due to the presence of G.I.P. in the impure preparations (Dupre, Ross, Watson and Brown, 1973).

SECTION B

BIOASSAY OF CHOLECYSTOKININ

Chapter B1

Background and initial manual technique

B1.1 The development of the superfusion technique

Superfusion of isolated tissues was first used by Finkleman (1930) to study the nature of inhibition in the intestine. His crude apparatus was heated by an external carbon filament lamp, and the muscle strips were attached to levers thus recording their contractions directly on to smoked drums. This was improved by Caddum (1953) when measuring the effect of drugs on rat uterus, and the sophistication of a peristaltic pump to ensure a steady flow of superfusing solution was added eleven years later by Vane (1964).

B1.2 Application of superfusion to cholecystokinin bioassay

Sensitive bioassays for cholecystokinin began to appear in the late 1960's, when the introduction of electronic transducers made this possible. The first (Amer and Becvar, 1969) made use of an organ bath; cholecystokinin was injected into the bath in increasing concentrations and a linear response noted up to a concentration of 40 milli Ivy Dog Units per millilitre of solution (m IDU/ml).
(An Ivy Dog Unit of cholecystokinin is that amount of dry material which when dissolved in isotonic saline solution and injected intravenously in to a dog over 10 - 15s causes a 1cm rise in the bile pressure within 1 - 5 min (Ivy and Janacek, 1959).)

This was quickly followed by the first superfusion technique for cholecystokinin (Berry and Flower, 1971), where isolated strips of rabbit gall-bladder were superfused with whole blood from the portal vein of a cat, the blood was returned to the animal via the femoral vein. Apart from cholecystokinin a number of other substances were infused into the cat and the response of the rabbit gall-bladder strips noted. All the following were found to be without effect upon the gall-bladder strips: histamine, 5-hydroxy-tryptamine, bradykinin, prostaglandins, E_1, E_2 and F_2, angiotensin, noradrenaline and secretin, at a concentration of 1-5ng/ml. Adrenaline produced a relaxation of the tissue.

As a direct development from Berry and Flower's (1971) work came the first truly in vitro superfusion assay for cholecystokinin capable of measuring cholecystokinin concentrations in human serum (Johnson and McDermott, 1973). This assay tested pentagastrin and the synthetic hexadecapeptide of gastrin and found no response by the gall-bladder, nor any potentiation or inhibition in conjunction with CCK.

B1.3. Early manual superfusion assay for cholecystokinin in human serum

B1.3.1 Apparatus

Some of the results to be presented later were obtained using this original technique. Basically the same as that used by Berry and Flower (1971) the apparatus (Fig. B1.1) consisted of two water baths

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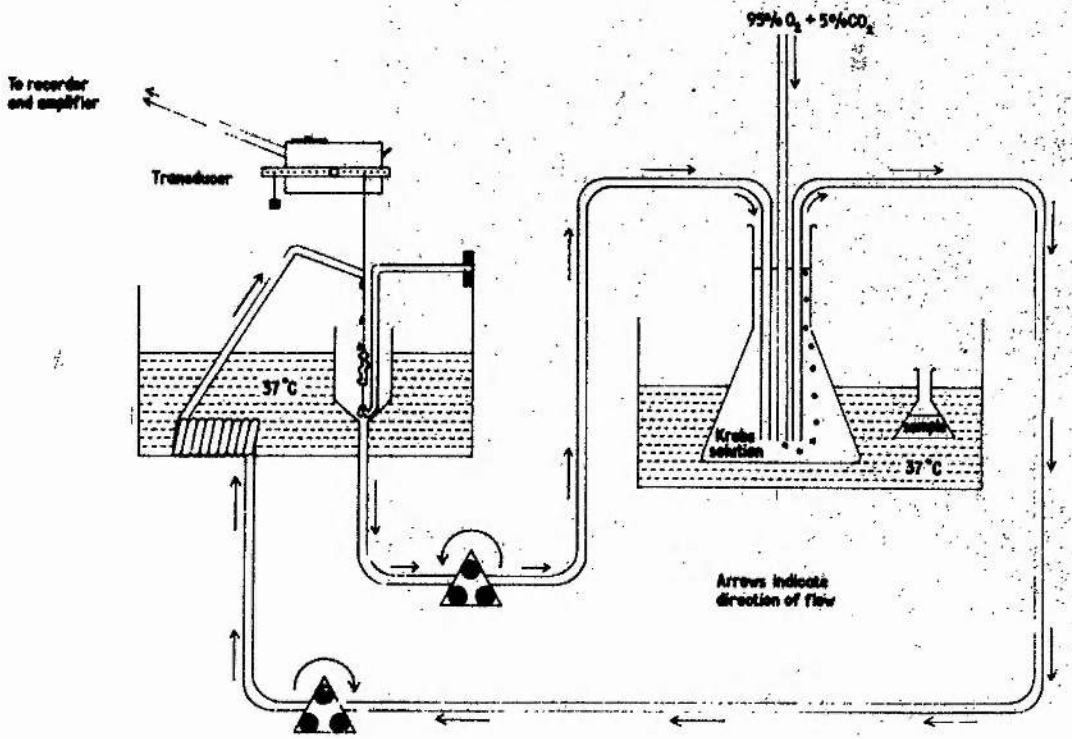

Fig B1.1 Apparatus for the original manual technique for the bioassay of cholecystokinin.

maintained at 37°C, a peristaltic pump (Watson Marlow MHRE 22 with Delta head), an isotonic transducer and associated amplifier and chart recorder.

One of the water baths held the organ chamber in which was suspended a strip of rabbit gall-bladder. One end of the gall-bladder was attached to a bent glass rod firmly anchored to the side of the bath, the other end of the gall-bladder was attached to the isotonic transducer (C.F.Palmer (Searle Diagnostics) High Wycombe) fixed directly over the organ chamber so that the tissue in the organ chamber was below the level of the warming water outside and the lever on the transducer was held horizontally or very slightly inclined at the point of attachment (see Fig. B1.1).

From a 250ml reservoir in the second water bath Krebs balanced salts solution (see appendix A for composition), saturated with oxygen by the continuous bubbling through it of a mixture of oxygen (95%) and carbon dioxide (5%), was superfused over the rabbit gall-bladder strip in a closed circuit at a rate of 8-10ml per minute. Heat losses during passage of solution between the reservoir and the pump were compensated for by the inclusion of a short glass coil contained in the same water bath as the organ chamber and situated just before the nozzle applying the solution to the suspending cotton above the tissue. All the connecting tubing was approximately 2mm internal diameter.

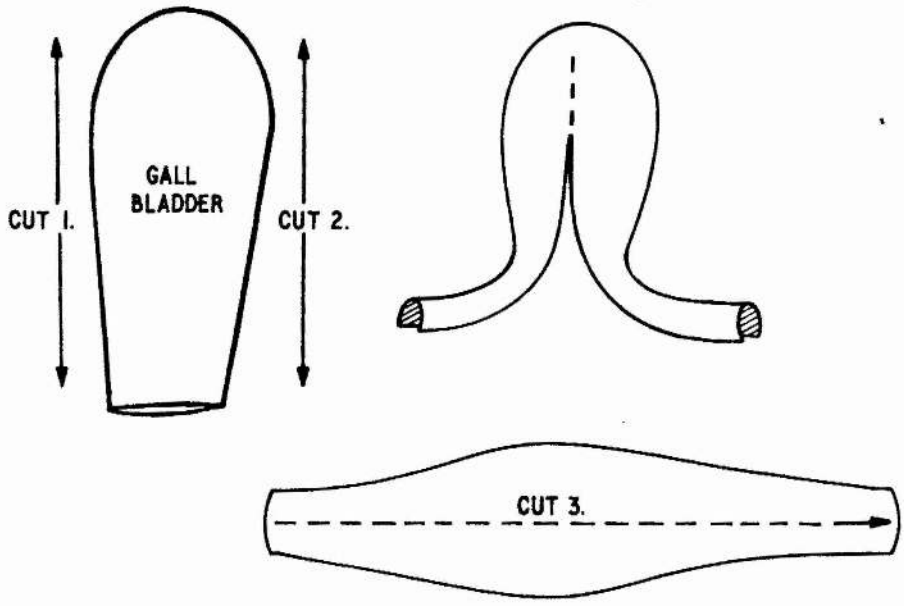

Fig B1.2 Preparation of rabbit gall-bladder for superfusion

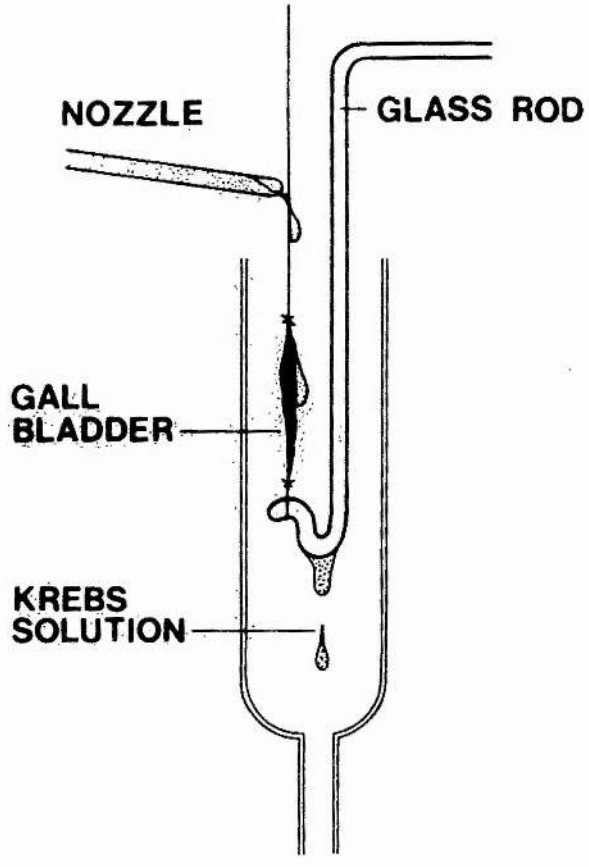

Fig B1.3 Close-up of tissue undergoing superfusion

B1.3.2 Preparation of rabbit gall-bladder

A male New Zealand White rabbit weighing approximately 2.5Kg (Useful range 2-3.5Kg) was killed by injection into the ear vein of 2.5ml 'Expirol' (200mg/ml Phenobarbitone Sodium, Abbott Laboratories Ltd., Kent). The gall-bladder was immediately removed, drained of bile and immersed in oxygenated Krebs solution. While still immersed the gall-bladder was cut from the neck to the apex on both sides, opened out, scraped free of clinging bile and cut longitudinally into two strips (see Fig. B1.2). One strip was placed into fresh oxygenated Krebs solution, covered, and stored at 4°C for use next day.

Lengths of cotton (approx 4" and 15") were attached to the ends of the remaining gall-bladder strip. The short length was attached to the end of the bent glass rod and pulled through to leave only ¼" of cotton between the tissue and the glass rod; the excess cotton was removed and the glass rod attached to the water bath. The other thread was carefully passed through the V of the superfusing nozzle (see Fig. B1.3) and attached to the arm of the isotonic transducer, leaving the gall-bladder strip suspended in the organ chamber so that it was in no danger of catching on the sides of the chamber or its supporting glass rod, and was being evenly superfused with warm Krebs solution.

The first 30ml of superfusing solution to pass over the gall-bladder strip was allowed to run to waste; thereafter the tissue was

allowed to equilibrate with the recirculating solution for a minimum period of ninety minutes.

B.1.3.3 Preparation of Standards

Four preparations of cholecystokinin were available for use as standards: (1) Pancreozymin (Boots Pure Drug Co.) in vials of approximately 100 Crick Harper Raper Units (25 IDU), (ii) Cholecystokinin (Gastrointestinal Hormone Research Institute, Karolinska, Sweden) in vials of 75 IDU, (these preparations contain approximately 0.1% and 10% cholecystokinin respectively), (iii) 99% pure cholecystokinin (gift of Professor V. Mutt, Medicinska Nobel Institutet Biokemiska Avdelningen, Karolinska Institutet, Solnavagen 1, Stockholm, 104-01-60), 500 ug (rated activity 3000 IDU/mg) redispensed in vials of 1 ug and 10 ug, lyophilised and vacuum sealed in this laboratory, (iv) the synthetic octapeptide of cholecystokinin (gift of Dr. M. Ondetti, Squibb & Sons, Inc., Princeton, New Jersey), 2 mg with declared activity of 22,000 IDU/mg, reduced by serial dilution and stored lyophilised in vacuum sealed vials containing 625, 62.5, 62.5 IDU cholecystokinin activity.

In practice most assays were performed using either Boots Pancreozymin or the synthetic octapeptide as standard. However at some time all four preparations were used for the assay and vials of each were diluted with Krebs solution in the following manner to give stock solutions containing 125 mIDU/mg;

1) Pancreozymin: vial containing 100 CHR units pancreozymin activity contents dissolved in 20ml Krebs solution; 5ml of this solution diluted to 50 ml giving a standard solution of 125 mIDU/ml.

ii) Cholecystokinin: 75 IDU dissolved in 25ml Krebs solution; 2ml of this solution diluted to 50ml giving a standard solution of 120mIDU/ml.

iii) 1µg 99% pure cholecystokinin was diluted to 25ml with Krebs solution, giving a standard solution of 120mIDU/ml.

iv) 570µg synthetic C-terminal octapeptide of cholecystokinin dissolved in 20ml distilled water.

1ml portions of this solution dispensed into snapules lyophilised and sealed under vacuum. Each vial contained approximately 625 IDU cholecystokinin activity.

vi) The contents of one of these vials dissolved in 10ml distilled water.

vii) 1ml portions lyophilised and sealed as before, each vial contained 62.5 IDU cholecystokinin activity.

viii) The contents of one of these vials further subdivided into 10 vials each containing 6.25 IDU cholecystokinin activity.

ix) The contents of one of these final vials dissolved in 50ml Krebs solution gave a standard solution containing 125mIDU cholecystokinin activity per millilitre.

All the vials were stored at $-20^{o}C$ until required.

For calibration of the gall-bladder strips 0.1, 0.2, 0.4, 0.6, 0.8 and 1ml of the standard solutions (i) and (iv) diluted to 25ml gave solutions containing 0.5, 1, 2, 3, 4 and 5mIDU/ml. Using solutions (ii) and (iii) slightly more of the original standard solution was needed for the same effect.

The conversion factor used throughout this thesis for changing Crick Harper Raper units into Ivy Dog Units was 4 CHR units $\equiv$ 1 I.D. Unit, based on data in Table 2 of the paper by Professor Jorpes (Jorpes. 1968). This despite the statement in Martindale : The Extra Pharnacopoeia p.474 that the British manufacturers state that in the case of their product 1 CHR $\equiv$ 1 I.D Unit. This would seem to explain fully the findings in Chapter B4, but it would seem more likely that the methods of assessing the biological activity of each batch of cholecystokinin or pancreozymin by the two commercial manufacturers are in fact incompatible and one of their fundamental definitions is wrong.

B1.3.4 The Standard Meal; Preparation of patients and sampling times

In order to study the release of cholecystokinin and to determine any differences in levels of serum cholecystokinin in different clinical conditions and to note any changes following surgical correction of these conditions a standard procedure for blood sampling was laid down.

Patients were fasted overnight (nothing to eat, drink or smoke from 10 p.m.) for test at 9 a.m. the following day. Samples of venous blood were withdrawn from suitable arm veins with tourniquet application through a 21 gauge needle.

An initial fasting sample of blood was taken. The patient was then fed a standard fatty breakfast consisting of: two rashers of bacon, fried; one egg, fried; two slices of bread; butter and marmaladeaand one or two cups of tea or coffee with sugar to taste (Fig. B1.4). Patients were asked to consume this meal within ten minutes if possible. Most finished the meal; some were not able to do so but were not eliminated from the series.

Timing began with completion of the solid portion of the meal and further blood samples were taken 10, 20, 30, 60 and 120 minutes after completion of the meal.

In many cases the whole experiment was repeated approximately four weeks after the patient's operation after the patient's system had fully adjusted to the new conditions.

B1.3.5 Blood treatment

Initially all blood samples were 10ml in volume, in later experiments the initial fasting sample was increased to 20ml, other specific experiments took different amounts.

The blood was dispensed in 10ml amounts into plain glass tubes (in later experiments these tubes contained 10,000 Kallikrein Inactivator Units (K.I.U.) of (Trasylol) aprotinin. Once the blood had clotted the tubes were centrifuged at 1000g for 10 mins. and the serum removed and stored at -20°C until required for cholecystokinin measurement.

B1.3.6 Calibration and operation of the assay

Calibration of each gall-bladder strip began after it had been superfused with the recirculating Krebs solution for a minimum of ninety minutes and, with a steady base line on the recorder, was judged to be in equilibrium with the system. Standard solutions were prepared immediately before use from the stock solutions described above; in most instances this was the Boot's Pancreozymin.

1ml of the stock solution (125mIDU/ml) was diluted to 25ml with Krebs solution; final concentration of cholecystokinin was 5mIDU/ml. This was warmed to 37°C alongside the reservoir of Krebs solution for 4 mins during which time the oxygen (95%)/ carbon dioxide (5%) mixture was bubbled through it. The supply tube was then transferred from the Krebs reservoir to the standard solution. When the system was full of standard solution the return tube was also transferred to the vessel

Fig B1.4 Standard meal given to patients

holding the standard solution. Care was taken to see that no standard contaminated the main reservoir and that the standard was not diluted further by premature transfer of the return tube, a little of each solution being allowed to run to waste.

The standard solution was recirculated over the gall-bladder until the contraction reached a maximum (plateau on the recorder), the delivery tube was then replaced in the main Krebs reservoir and the return tube moved to a waste receptacle. Solutions were allowed to run to waste for 4 mins and Krebs solution was then recirculated for a further 4 mins or until base line was firmly re-established. The next standard was then introduced and a six point calibration line obtained by use of standards of 0.5, 1,22, 3, 4 and 5mIDU/ml. With some relatively insensitive gall-bladders the line was obtained with standards of 0.5, 1, 3, 5, 7 and 10mIDU/ml.

The average height (i.e. original base line to peak, and final base line to peak heights average) of each contraction due to each standard solution was plotted as a dose-response curve on linear axes. In the range of standards used the dose response curve was usually linear, (Fig. B1.5) although occasional deviations from linearity towards S and log type curves were seen (Figs. B1.7 and B1.6).

B1.3.7 Measurement of cholecystokinin in human serum

Serum samples obtained and stored previously (1.3.5) were

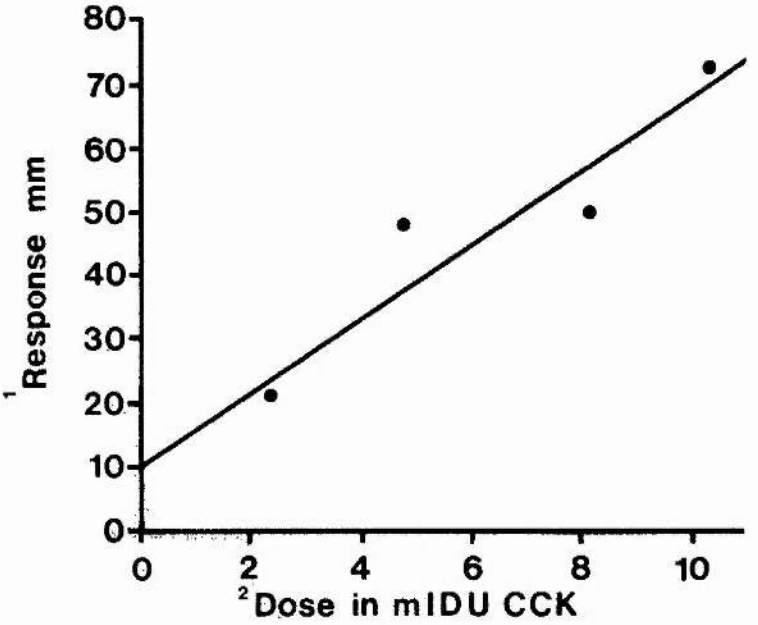

Fig B1.5 Dose-response curve (linear) to standard solutions of cholecystokinin

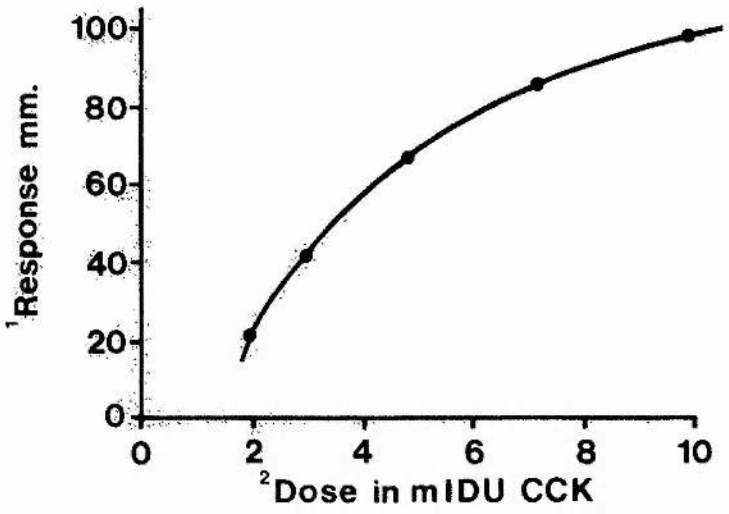

Fig B1.6 Dose-response curve (logarithmic) to standard solutions of cholecystokinin

1,2 See notes under Fig B1.7

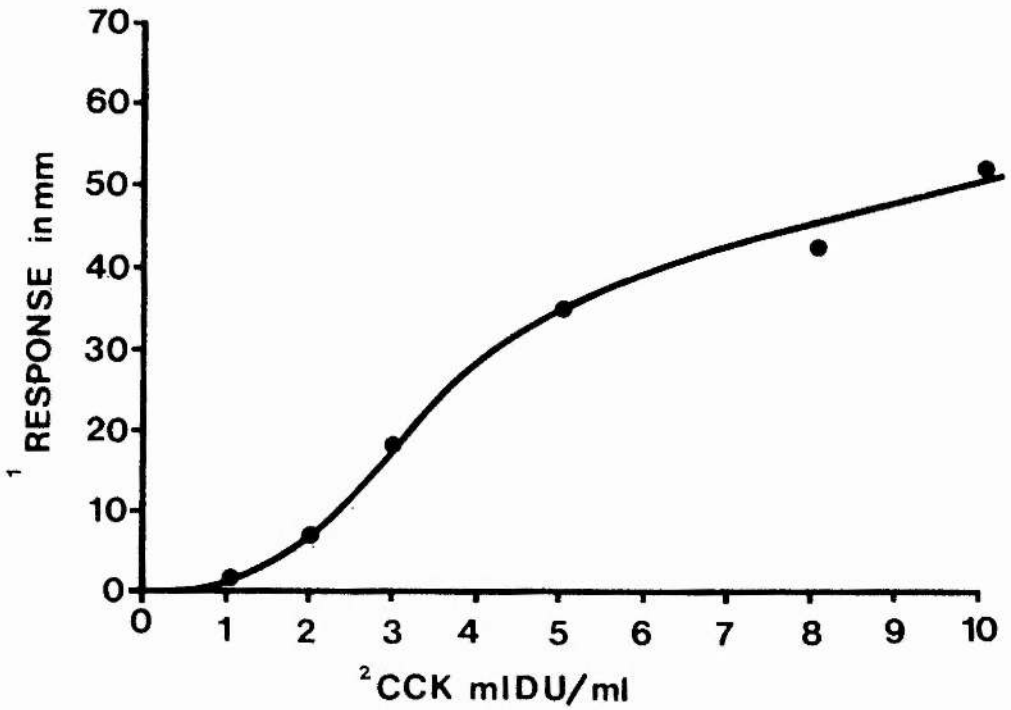

Fig B1.7 Dose-response curve (S-type) to standard solutions of cholecystokinin.

1 Response throughout this thesis always refers to the height of the peak obtained on the chart recorder.

2 Dose throughout this thesis always means the concentration of CCK in mIDU/ml superfusing solution.

defrosted by holding the tubes in a stream of warm running water. 1ml of each sample was diluted to 25ml total volume with Krebs solution. This test solution was placed in the 37°C water bath with the 95% oxygen/5% carbon dioxide mixture bubbling through it for 4 mins before the assay supply tube was introduced. When the system was full of the test solution the return tube was also introduced to the measuring cylinder and the test solution recirculated until a plateau or maximum peak height was reached.

The supply tube was returned to the Krebs reservoir and the solution allowed to run to waste for 4 mins; Krebs solution was then recirculated until a steady base line was again obtained - usually a further 4 mins - and the next serum sample was then introduced.

The height of the peak produced on the chart was measured. This hheight was converted to mIDU CCK/ml test solution from the calibration line already obtained. The amount of cholecystokinin in the serum was then determined from the formula:

$$\text{Serum CCK(mIDU/ml)} = \frac{25 \times \text{mIDU/ml test sample}}{\text{ml serum diluted}}$$

For serum samples containing little or large amounts of cholecystokinin more (2-3ml) or less 0.25 - 0.75ml) serum was used for the test solution which was still made to a final volume of 25ml with Krebs solution.

Chapter B2 The First Automation System

B2.1 Introduction

The majority of automated assays in regular use rely upon a change in some measurable physical property occurring in a solution. However this change is produced, whether by chemical or other means, the final measurement is often made with a colorimentric technique by noting a change in the intensity of absorbance or fluorescence, or a movement in the wavelength of maximum absorbance. Other physical properties, such as a refractive index, may also be utilised. The outputs from these instruments are recorded permanently on charts or digital print-outs from which the results are calculated.

These methods are frequently adopted where they are needed to process large numbers of small samples quickly and accurately or to monitor continuously the effluent from a column. Often they are more efficient than a manual process - as they can operate continuously overnight - and they rarely require more than a few (10) millilitres of sample.

This assay differs from the above in that: (1) the physical effect which is measured is a property of a biological tissue not of a solution; (2) for the best results the maximum rate of process of samples is four per hour, but three per hour is preferable; and (3) a sample volume size of 25ml - 75ml is required depending upon theapparatus.

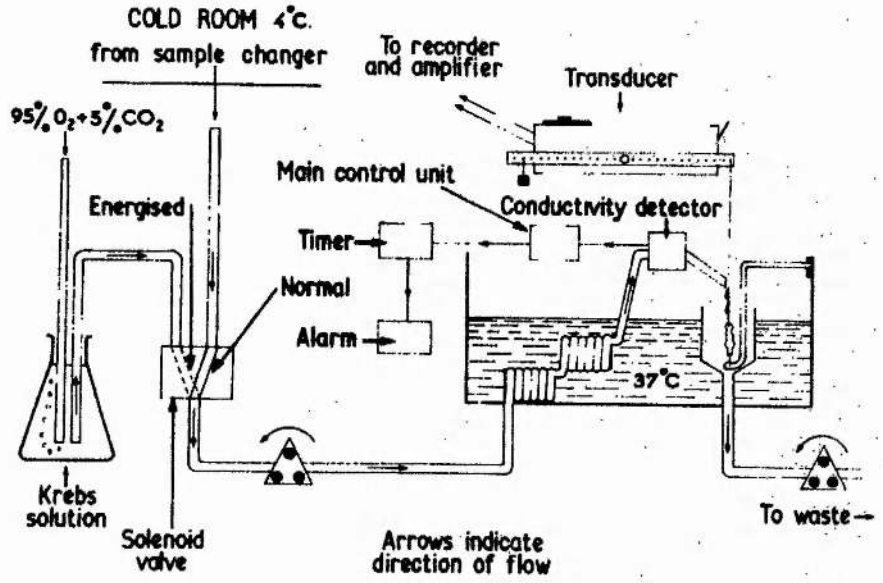

Fig B2.1 Open system of apparatus for first automatic assay

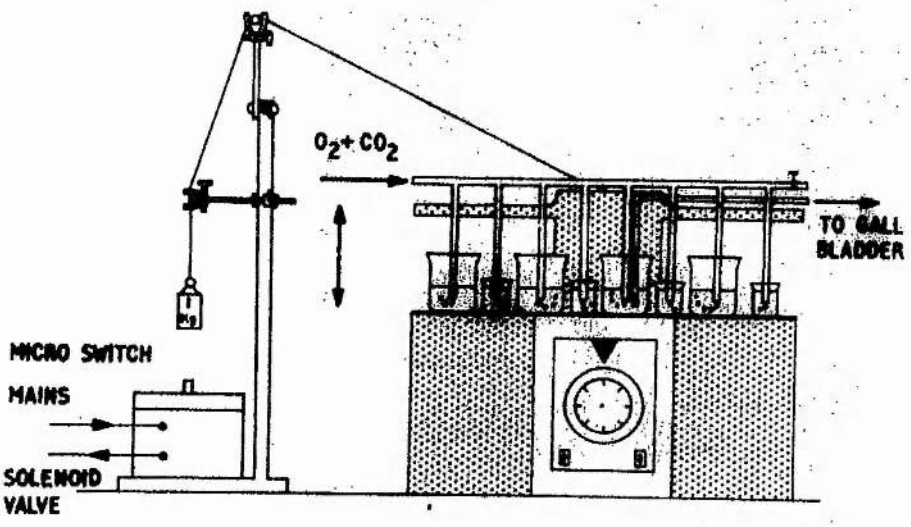

Fig B2.2 Modified slide staining machine as sample changer for first automatic assay

It is nevertheless an ideal system for automation requiring only a change from sample to wash in a regular manner similar to that provided by an Auto Analyser sample changer. The major problems to be overcome are the volume of solution required for each sample and intermediate wash, and the much longer time scale involved.

B2.2 The new system

The principle of the original assay was altered only in the substitution of an open system for the original recirculating system (Fig. B2.1).

The strip of rabbit gall-bladder was prepared and suspended from the transducer as already described (1.3.2). It was then equilibrated by superfusion with recirculating Krebs solution for a minimum of two hours.

A single standard solution containing 5mIDU CCK/ml was first passed over the gall-bladder (original system, Fig. B1.1) to determine the gall-bladder's sensitivity and the counter balance (the normal counter balance on the transducer was 1 paper clip 800mg placed at the point of maximum leverage, for exceptionally strong (very sensitive) or weak (relatively insensitive) gall-bladders, the counter balance was increased to between 1½ and 3 paper clips or decreased to ½ a paper clip respectively) required on the transducer. The amplification and recording systems were unaltered.

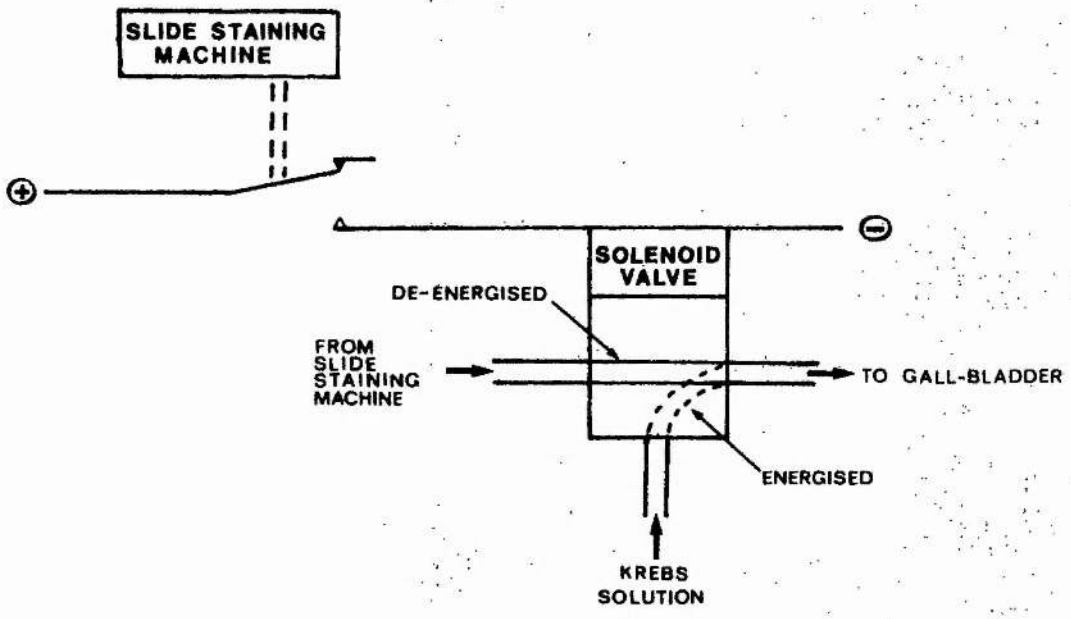

Fig B2.3 Circuit diagram for first automatic system

All further standards and samples were applied automatically from a modified 24-station slide staining machine (British American Optical Co. Ltd. now Reichert-Jung U.K.) (Figs. B2.1, B2.2 and B2.3) situated in an adjoining cold room maintained at 4°C.

Thirteen of the twenty four stations were supplied with the 95% oxygen/5% carbondioxide mixture through tubes attached to the machine by splints. The sample was collected through a second tube attached to the splint at the sampling point, this tube being adjusted so that it just reached the bottom of the sample containers.

The stations on the machine were occupied alternately by vessels containing 75ml sample or standard and 500ml Krebs solution, respectively. The machine was programmed so that negative pressure from the self-priming peristaltic pump drew sample for five minutes followed by Krebs solution for 15 minutes.

The slide staining machine required approximately 30 seconds to change from one station to the next. During this time the probe was out of the solutions for about 16 seconds. A gap of this length of time had been previously seen to upset the response of the gall-bladder to following samples.

The airgap was therefore eliminated by the insertion of a three-way solenoid valve (Dewrance Controls Ltd., Skelmersdale, Lancs., (now Dewraswitch Ltd.) Type 831443) operated by the slide staining machine (Fig. B2.3). As the top of the machine lifted prior to moving to the next station, it lowered a small weight on to a micro-switch (Fig. B2.2) energising the solenoid and allowing Krebs solution

from a separate reservoir into the system for the greater part of the time the probe was not immersed.

To guard against machine or pump failure an alarm system dependent on electrical capacity (Dielectric constant) was incorporated (FI-Monitor, Fisons Ltd.,). The timer delays the sounding of the alarm for 8 seconds, thus small air bubbles are ignored.

All equipment operated by mains electricity was switched off, shortly after the last sample had been taken, by an 'off only' time switch.

B2.3 Operation of the automatic assay

Following determination of the sensitivity of the gall-bladder strip (B2.2) and completion of adjustments to the counter balance on the transducer (range 250 - 1700 mg) and the position of the attenuator on the amplifier, the equipment was ready to begin automatic processing.

The slide staining machine was programmed to run at three or four samples per hour - i.e. to draw sample for five minutes then wash for ten or fifteen minutes according to the speed with which the gall-bladder relaxed following stimulation.

The gall-bladder was first calibrated in a similar manner to the manual technique, sample solutions containing 0.5, 1, 2, 3, 4 and 5 mIDU/ml CCK being applied consecutively to the gall-bladder. Serum samples, generally 2ml serum diluted to 15ml followed.

Twelve standards and samples could be measured during the normal working day. The machine could then be reset, the wash reservoirs refilled, and a further 12 solutions measured during the evening, the

trace being examined the next day. An 'off only' time switch turned off all mains electricity operated equipment shortly after the last sample had been measured.

The last four samples were usually standards ensuring that sensitivity variations were taken into account in the calibration of results (formula see 1.3.7). The slight carry over of one sample to the next was not found to be sufficient, even when moving from a solution of high cholecystokinin concentration to one of low cholecystok inin concentration, to affect the response of the gall-bladder to the following solution. It was however desirable that this should, if possible, be eliminated.

Chapter B3 Development of the Automatic System

B3.1 Stimulus for development

The automatic system described in Chapter B2 was a major advance over the old manual technique and two advantages were immediately obvious: (i) the cholecystokinin content of twice as many samples as before could now be estimated in a single day, that is 24 instead of a maximum of 12 in a normal working day; (ii) the person operating the equipment was released, once all the solutions had been prepared and positioned on the machine, for further useful work elsewhere, needing only to reset the machine with a further twelve samples at the end of the day. Results were worked out the following day.

With improved efficiency came an increased demand for cholecystokinin measurement, until a backlog of samples once again began to accumulate and an increased capacity was once more an obvious need, but how to achieve it?

Use of all 24 stations on the slide staining machine as sample positions, with Krebs wash solution coming from a separate reservoir was the most logical development and ways to do this were immediately explored.

B3.2 The second automatic system

The first design of apparatus to use all twenty four stations on the slide staining machine as sample positions is shown in Fig. B3.1.

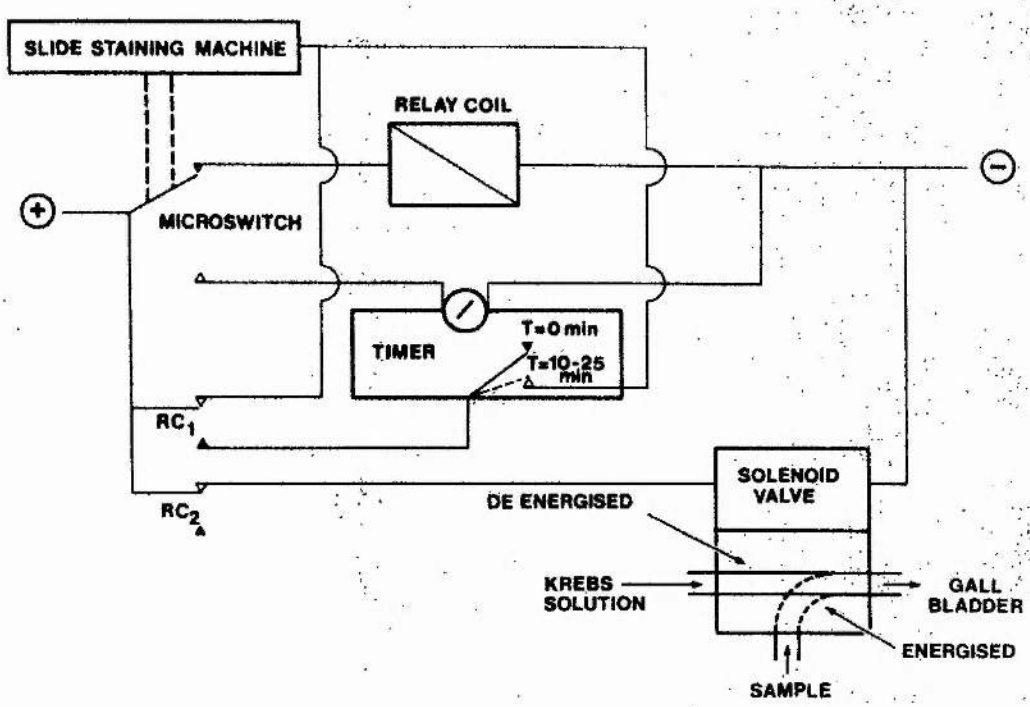

Fig B3.1 Circuit diagram for second automatic system

The slide changing machine was programmed to change station every five minutes. When the slide staining machine came to a station the relay coil was activated through operation of the microswitch, the relay contacts RC_1 and RC_2 then supplied power to the slide staining machine and the solenoid valve. In its energised state the three-way valve allowed sample to go to the gall-bladder; when de-energised Krebs solution from a separate reservoir was fed through to the gall-bladder.

After sample had been drawn for five minutes the slide staining machine began its changeover process. As the top of the machine lifted it operated the microswitch and cut off power to the relay coil and hence to itself and the solenoid valve. It remained suspended between stations while the interval timer (type STPYMP 30 min.), activated by operation of the microswitch, ran for a predetermined length of time; 10, 15 or 25 minutes for 4, 3 or 2 samples an hour. At the end of this wash period the timer contacts closed restoring power to the slide staining machine through contacts RC_1. The slide staining machine then completed its station changing performance, operating the microswitch again as it came to rest in its new position. This operation of the microswitch cut the power to the timer, thereby causing it to reset, and activated the relay coil thus giving power to the solenoid valve and itself through the relay contacts. The cycle was then repeated until all samples had been processed.

While the above system was theoretically correct electrically, and indeed was tried and proved on the laboratory bench, it was found

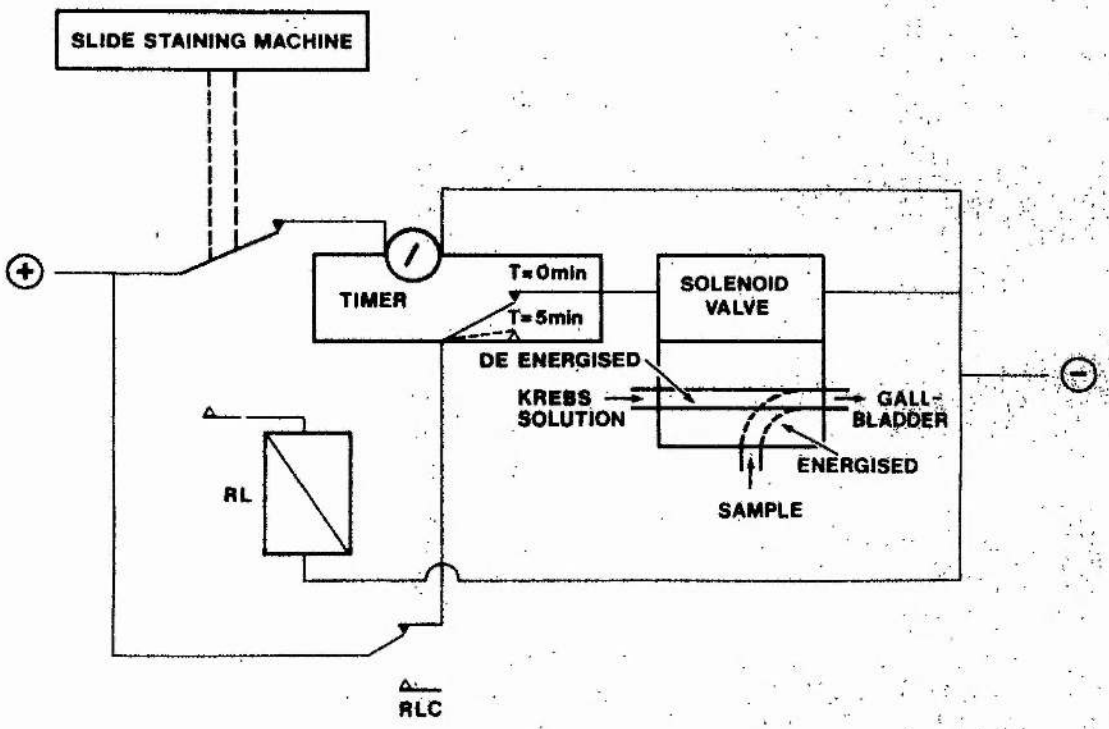

Fig B3.2 Circuit diagram for third automatic system

to be of no practical use. The single reason for its failure was the refusal, when positioned in the cold room, of the slide staining machine's clock to restart at the beginning of each cycle. For this reason alone another system had to be designed.

B3.3 The third automatic system

In the light of the above experience it was clear that any design for a system to use all twenty four stations on the slide staining machine would have to ensure that the machine itself was running continuously. With this in mind a third automatic system was designed (Fig. B3.2).

Electrically simpler than system 2 it still used the same electrical components. This time, however, the number of samples per hour was programmed on to the slide staining machine rather than through the timer.

The operation of this system is best described by observing the events of a station to station move: as the top of the slide staining machine lifts it operates the microswitch, this disconnects the timer and causes it to reset, the circuit to the solenoid valve would therefore be restored if the simultaneous operation of the relay had not broken the circuit at a second point (RLC). Reaching the new station the microswitch is returned to its original position activating the timer, deactivating the relay coil and thereby energising the solenoid valve and allowing sample to flow to the gall-bladder. Five

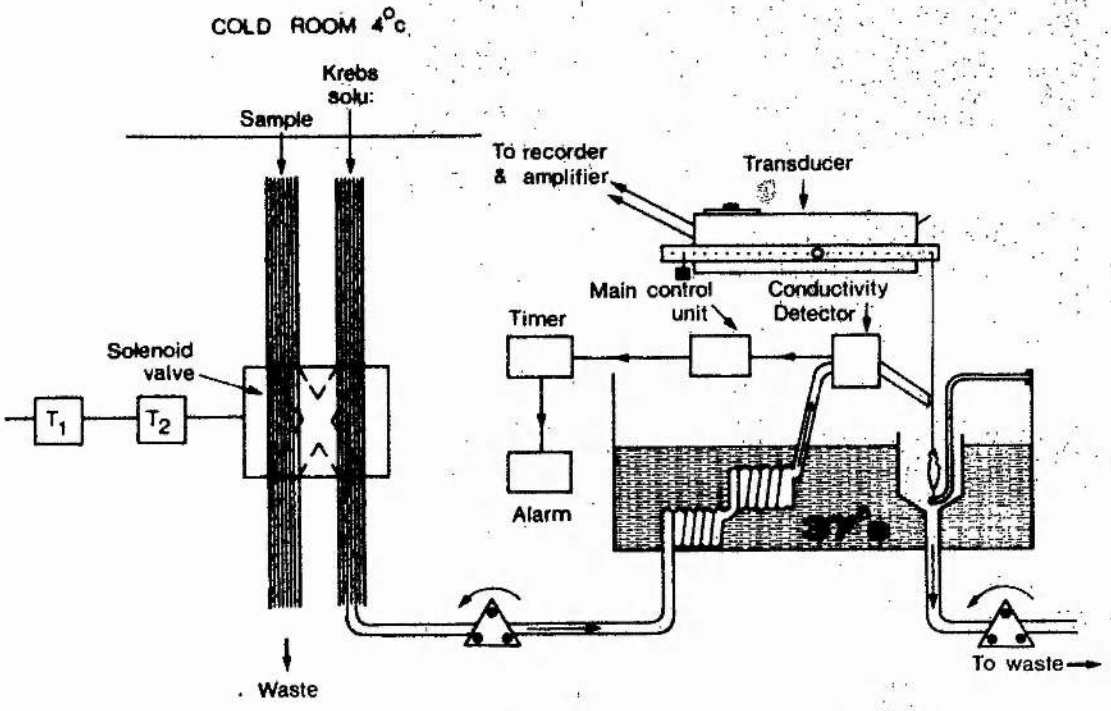

Fig B3.4 Final arrangement of apparatus for the automatic bioassay of cholecystokinin.

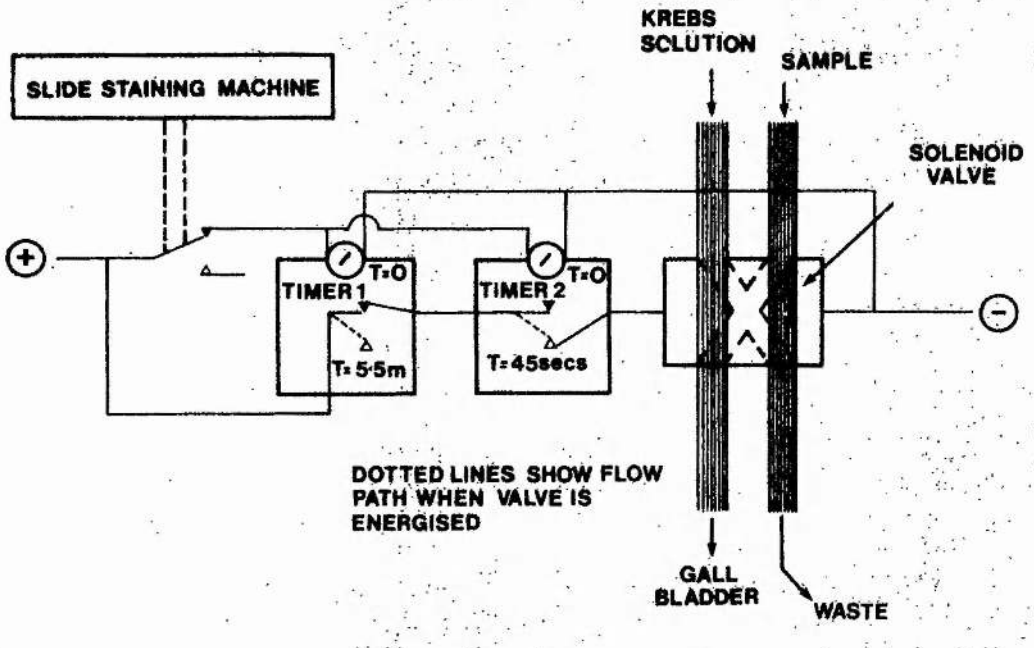

Fig B3.3 Circuit diagram for the final automatic system.

minutes later the timer switches off the solenoid valve and Krebs solution is allowed to wash over the gall-bladder for 10, 15 or 25 minutes according to a sampling rate of 4, 3 or 2 samples per hour respectively. At the end of the wash period the slide staining machine moves to its next station and the whole cycle of events is repeated until just after the last sample has been processed when an 'off only' time switch cuts the power to all equipment operated by mains electricity.

Both this system, and system 2, allowed a small amount of carry over of one sample to the next. This although undesirable, did not appear to affect the results adversely.

B 3.4 Final developments

Although, as has been stated above, the 'carry over' from one sample to the next was small and apparently did not affect the results, it nevertheless remained a possible source of error which should, if practicable, be eliminated.

The discovery and purchase of a four way solenoid valve (Dewraswitch Ltd., Skelmersdale, Lancs. type 8342A1) together with an additional timer (type STPYMP 72s, I.M.O. Controls, London.) made this possible. The circuit diagram is shown in Fig. B3.3 and the final arrangement of the apparatus in Fig. B3.4.

Again it was the slide staining machine that was programmed to determine the number of samples per hour, and its movement from one station to the next that controlled the electronics. The changing of the slide staining machine from one station to the next reset and then

restarted the two electric timers. Timer 2 delayed energising the solenoid valve until the sample had reached and filled the valve (Approximately 45s), this avoided a long air gap between the Krebs wash solution and the sample solution. Timer 1 switched off the solenoid valve after it had been energised (allowing sample to pass over the gall-bladder) for five minutes. In its de-energised s ate the valve allowed Krebs solution to go to the gall-bladder, any remaining sample being pumped to waste. In this way any carry over of one sample to the next was prevented.

As a further refinement the supply line to the gall-bladder was split between the valve and the pump and both sample and wash solutions were fed through a parallel line to another set of apparatus. Thus the effect of a single sample solution could be observed simultaneously on two gall-bladder strips in parallel apparatus. The two gall bladder strips often responded very differently to the same dose of cholecystokinin, but each gall-bladder strip had its own dose-response curve from which the cholecystokinin content of serum samples could be calculated.

With every station on the slide staining machine now used as a sample position, it was possible, having already processed up to twenty samples, for the machine to be reset with a full (24 samples) load to run overnight. It thus became practicable to estimate at least 40 cholecystokinin-containing solutions in duplicate everyday. All equipment operated by mains electricity was switched off by the 'off only' time switch when the last sample had been processed some

8 - 9 hours later. This capacity for a theoretical maximum of 48 samples, with 96 individual results per day turned a technique that was purely a research tool into something more practical and nearer the needs of a routine laboratory.

B3.4.1 Sample preparation

Using two sets of apparatus in parallel it was found that 75ml of sample solution was insufficient. The sample size was therefore increased to 100ml; 0.2, 0.4, 0.8, 1.6, 2.4, 3.2 and 4.0ml of the standard stock solutions (1.3.3) giving standard solutions of 0.25, 0.5, 1, 2, 3, 4 and 5mIDU/ml CCK respectively when diluted to 100ml. Test samples of serum were prepared by diluting 2ml of serum to 100ml; this only rarely resulted in a sample either too weak or too strong in cholecystokinin to obtain a result from the calibration line.

B3.4.2 Published material

Details of two of the automatic systems have been published (Marshall, 1976; Marshall, Egberts and Johnson, 1978).

Chapter B4 Standards: Investigation of Potency

B4.1 Introduction

Preliminary reports by Oliver and Harvey (1977) and Oliver, Rey and Harvey (1978) have shown that commercial preparations of cholecystokinin from the Boots Company are up to eight times as activeaas preparations produced by the G.I.H. Research Institute, Karolinska, when both preparations are diluted to give the same theoretical concentration of cholecystokinin in Ivy Dog Units. Their experiments showed conclusively that Boots pancreozymin consisted primarily of small active molecules and the Karolinska cholecystokinin to be mainly composed of the full 33 or 39 amino acid molecules. However, no evidence was offered to show which of the commercial products was correctly calibrated.

We had experienced difficulty in obtaining dose-response curves using the Karolinska cholecystokinin as standard, and routinely used either Boots pancreozymin or the synthetic C-terminal octapeptide whose activities were consistently comparable.

This study, inspired by questioning the relative activity of two different batches of Karolinska cholecystokinin, and completed on the 12th February 1978, tried to establish which preparation had been correctly calibrated.

B4.2. Materials and methods

The stock solutions of each standard were prepared as previously described (section B1.3.3) and the experiment performed with the final automatic apparatus (section B3.4).

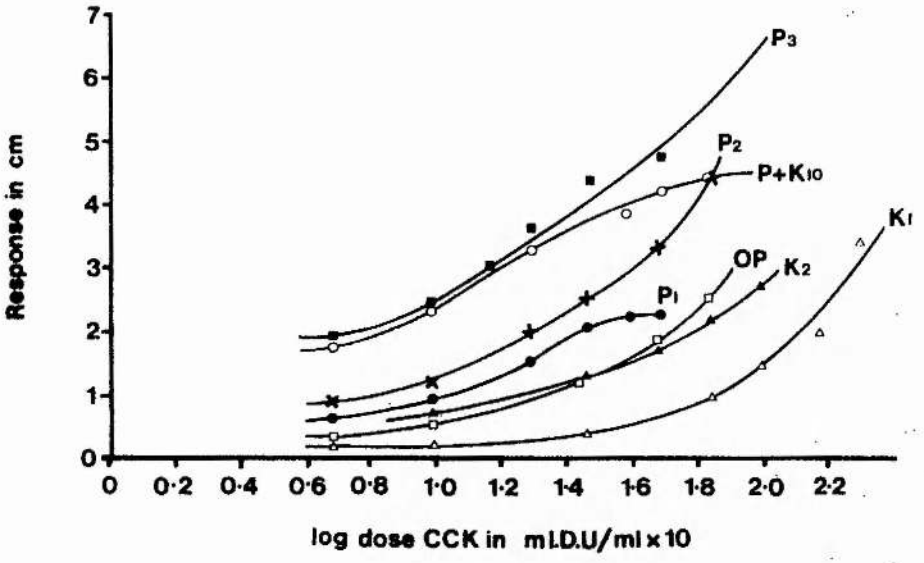

Fig B4.1 Dose-response curves on linear axes for different preparations of cholecystokinin

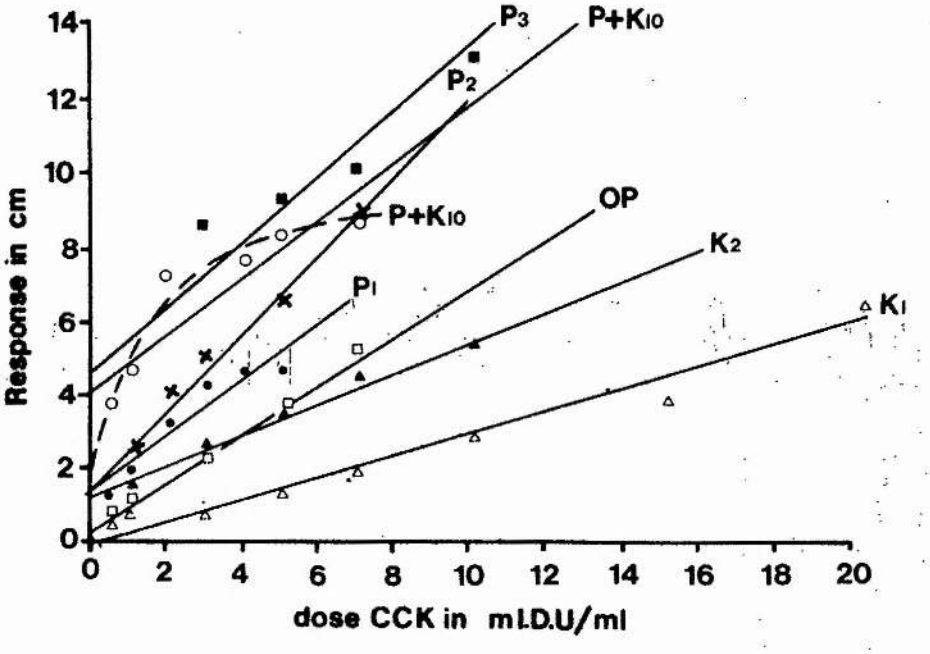

Fig B4.2 Dose-response curves on log-linear axes for different preparations of cholecystokinin

Each strip of rabbit gall-bladder was first primed (see section B6.9) by the passage of two serum samples (Marshall, Egberts and Johnson, 1978), the three types of cholecystokinin (Boots Pancreozymin, Karolinska Cholecystokinin, and the synthetic C-terminal octapeptide of cholecystokinin) were then presented to the gall-bladders in graded doses, 0.5 - 20 mIDU/ml, prepared by dilution of the standard stock solutions described above, at a rate of 3 or 4 samples per hour. In one experiment the two batches of Karolinska Cholecystokinin were tested for comparable activity.

The response of the gall-bladders to each sample was recorded as the height of the peak (in cm.) on the recorder attached to the isotonic transducers. Results were displayed as dose-response curves.

B4.3 Results

The actual peak heights recorded are depicted in Tables B4.1 - B4.3 and Figs. B4.1 and B4.2. (See also Figs. AP1 - AP6 in Appendix D). The responses obtained from the three types of cholecystokinin showed clearly that the Boots Pancreozymin preparation had between two and seven (mean 4.23) times the activity of the Karolinska Cholecystokinin as determined by the difference in the slopes of the dose-response curves for the two preparations. The activity of the octapeptide fell between that of Boots Pancreozymin and Karolinska Cholecystokinin, while no real difference was detected between the two batches of Karolinska Cholecystokinin.

B4.4 Discussion

While preparing the standard solution of the octapeptide of cholecystokinin it was noted that the machine used for dispensing 1 ml

TABLE B4.1

Test for equivalence of activity in two batches of Karolinska Cholecystokinin

Batch No	dose applied mIDU/ml	Response of gall-bladder in cm gall-bladder A	gall-bladder B	Boots Pancreozymin equivalent in MIDU/ml A	B	Mean
27742	0.25	0.9	1.75	0.3	1.6	0.95
	0.5	1.1	1.9	0.5	1.75	1.125
	1.0	1.2	1.7	0.575	1.55	1.063
	2.0	1.6	1.8	0.9	1.65	1.275
27725	0.25	1.2	1.5	0.575	1.375	0.975
	0.5	1.2	0.9	0.575	0.8	0.69
	1.0	1.4	1.1	0.8	1.0	0.9
	2.0	1.5	1.6	0.875	1.475	1.175

TABLE B4.2

Testing the relationship between two commercial preparations of CCK

Test Solution in mIDU/ml	Gall-bladders response in cm		Test Solution	Gall-bladders response in cm	
	A	B		A	B
P 0.5	0.2	0.1	P 5.0	3.3	0.9
P 1.0	0.2	0.2	P 7.0	3.6	1.4
P 2.0	0.6	0.2	P 10.0	4.6	1.35
P 3.0	1.3	0.4	P 10.0 + K 10.0	3.9	0.7
P 4.0	1.7	0.4	P 7.0 + K 10.0	3.7	0.6
P 5.0	2.2	0.5	P 5.0 + K 10.0	3.0	0.6
K 15.0	2.3	0.3	P 3.0 + K 10.0	2.5	0.4
K 10.0	1.6	0.2	P 1.0 + K 10.0	1.4	0.3
K 5.0	0.7	0.1			
K 3.0	0.5	0.15			
K 1.0	0.2	0.1			
K 0.5	0.15	0.1			
P 1.0	0.75	0.2			
P 3.0	2.6	0.7			

Pancreozymin = P

Karolinska = K (batch 27725)

*

TABLE B4.3

Testing the relationship between three preparations of CCK

Test Solution in mIDU/ml	Gall-bladders response in cm		Test Solution	Gall-bladders response in cm	
	A	B		A	B
OP 0.5	0.6	0.5	P 0.5	1.7	0.7
OP 1	0.9	0.7	P 0.5 + K110	3.6	1.5
OP 3	2.15	1.0	P 1 + K 10	4.6	2.5
OP 5	3.5	1.4	P 2 + K 10	6.4	3.6
OP 7	4.9	2.4	P 4 + K 10	7.6	5.1
K 0.5	0.3	0.2	P 5 + K 10	8.3	6.2
K 1	0.3	0.4	P 7 + K 10	8.7	7.0
K 3	0.6	0.7	K 1	1.4	0.4
K 5	1.2	1.0	K 3	2.5	0.4
K 10	2.8	1.85	K 5	3.4	0.6
K 15	3.9	2.9	K 7	4.4	1.1
K 20	6.5	4.2	K 10	5.3	1.2
P 7	8.7	6.0	P 10	13.0[E]	8.7
P 5	6.5	4.2	P 7	10.1	7.2
P 3	5.0	2.1	P 5	9.3	5.0
P 2	4.0	1.6	P 3	8.6	3.5
P 1	2.3	0.8	P 2	7.2	2.0
			P 1	4.8	
			P 0.5	4.0	

Pancreozymin = P

Karolinska = K (batch 27742)

Octapeptide = OP

E = Estimate, top of peak off chart

portions at each stage of the serial dilution (Compupet, General Diagnostics Ltd.) was in fact delivering very slightly less than 1ml. With the very large dilution factor involved - $1:10^7$ at final stage - it would not be surprising to find that small dispensing and dilution errors have crept in and been magnified so that the actual activity in the final solution is lower than calculated; the above observation makes it most unlikely that these errors would result in an activity higher than expected.

It is therefore logical in view of the evidence of Figs. B4.1, B4.2 and AP1 - AP6 to conclude that the pancreozymin activity reported by the Boots Company on their vials is correct. The fact that the logarithmic curves do not really appear (though this has not been tested statistically) to differ in parallelism is an indication that the same substance is being measured each time at considerably different concentrations . With the number of points on each curve limited to six it is unlikely that the apparent deviation from parallelism (Figs. B4.1 and B4.2), when Boots and Karolinska preparations were combined is due to some form of competitive inhibition. Indeed in the face of Oliver and Harvey's (1977) results, Boots retested their original standard, now 10 years old, in their cat system; they found no change in its activity (Harvey, private communication).

The discrepancy can only be explained by three assumptions: (i) the assertion that 4 CHR units = 1 ID unit is wrong, (ii) the

Karolinska assay system is producing high results causing incorrect labelling of the activity of their produce, or (iii) the shelf life of the Karolinska material is much shorter than claimed.

It does not seem likely that any inhibitors are involved, but this experiment does bring sharply into focus the question of which standard should be used for a bioassay of cholecystokinin, and in what units the results should be expressed. 5mIDU is equivalent to approximately 250pg (28pmol) of the octapeptide (22,000 IDU/mg, Ondetti et al. 1970) but would be 1.7ng (43pmol) of the full molecule (3,000 IDU/mg, Jorpes and Mutt, 1968). Centres using different standards will therefore get different results for the same unknown and the discrepancy could be exaggerated further by the use of different units, with a different base, to express the result.

However, since it is still impossible to be certain which molecular form of cholecystokinin is responsible for the greater part of the activity in serum it is probably pointless to try and express results in any way other than biological units.

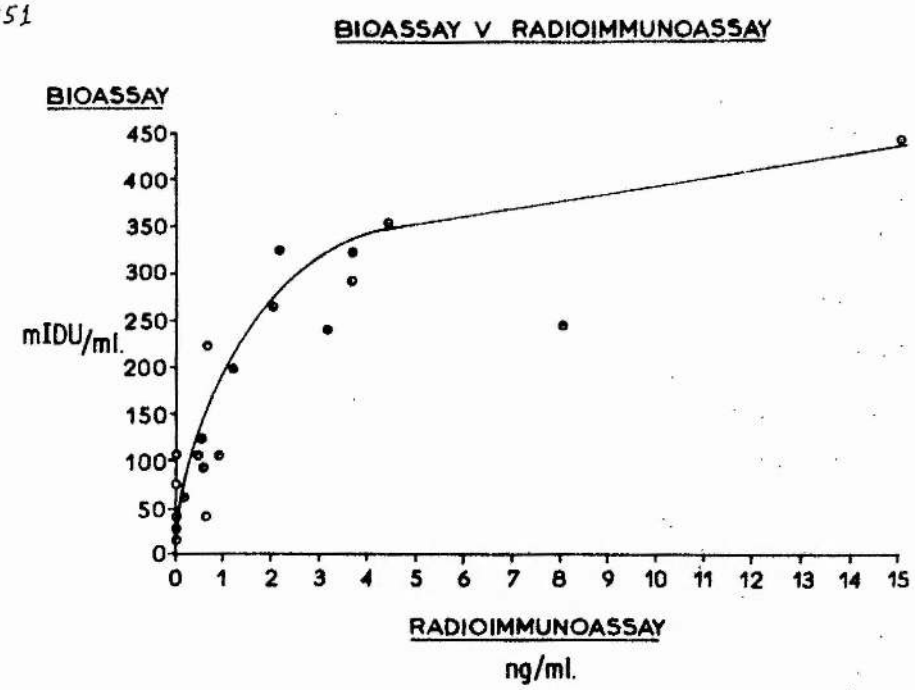

Fig B5.1 Comparison of results obtained by radioimmuno and bioassays for the amount of cholecystokinin in human serum samples, using linear scales

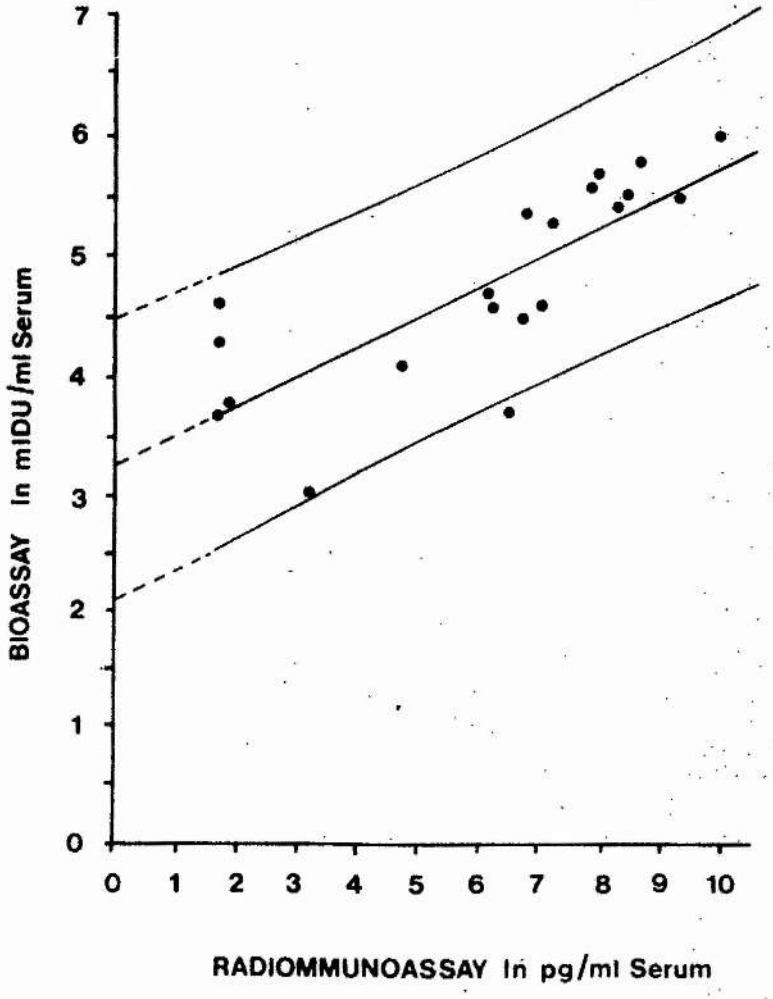

Fig B5.2 Comparison of results obtained for cholecystokinin content of human serum samples, by radioimmuno and bioassays, using natural logarithmic scales

Chapter B5 Comparison of Radioimmuno and Bioassays for Cholecystokinin

B5.1 Introduction

While the developments described in the preceding chapters were taking place I was in contact with a group of workers in Bristol who were also interested in cholecystokinin, but who were at that time concentrating on the development of a radioimmunoassay (Harvey et al, 1974). It was of interest to both groups of workers to see if a correlation between the two very different types of assay could be established.

B5.2 Method

Blood samples were taken from twenty volunteers, some fasting, some stimulated with milk or magnesium chloride. These were divided evenly, the serum collected and stored frozen at $-20^{\circ}C$ until required for assay. Radioimmunoassay was performed by the Bristol group according to their established technique (Harvey et al. 1974), cholecystokinin bioactivity was estimated using the first automatic technique (chapter B2). Each centre performed the assay 'blind' without knowledge of the type of sample or the other centre's results.

B5.3 Results

Results from this experiment are shown in Figures B5.1 and B5.2; when the results from the two centres were plotted on linear axes (Fig. B5.1) a non-linear relationship was seen, a small rise in radioimmunoactivity initially corresponding to a large increase in bioactivity, then at high bioactivity levels a small rise in bioactivity corresponding to a large rise in radioimmunoactivity. This is typical of a logarithmic relationship.

On performing a log - log transform a linear relationship between the two assays was established (Fig. B5.2); the regression line had a high coefficient of determination (r^2) of 0.625 and this is shown together with the 95% confidence limits.

B5.4 Discussion

That this experiment achieved its objective in showing a direct relationship between the results from the two laboratories was quite clear from Fig. B5.2. However, the intercept on the bioassay axis strongly suggested that the bioassay was measuring something which the radioimmunoassay was not. From the intercept the average value of this unknown substance was calculated to be approximately 28mIDU/ml for each sample.

To test this theory a 4ml sample of a large pool of serum was extracted with dextran-coated charcoal to remove all the small peptides including cholecystokinin (Morris and Morris, 1964). The cholecystokinin content of the extracted serum was estimated to be 31mIDU/ml, very close to the predicted 28mIDU/ml.

The finding of this residual activity, which was termed the 'serum blank', was a bonus from this experiment.

Thus this experiment succeeded in showing that results, from two widely separated laboratories, using totally different procedures to measure the same substance can bear a linear relationship to one another. That the two assays utilised different units which were not readily interchangeable - did not matter, since the form of cholecystokinin in human serum, and hence its molecular weight were unknown. Also unknown was the complete

specificity of the radioimmunoassay for the different fragments of cholecystokinin to which it was sensitive. Each assay had its own dose-response curves from which the results were calculated, and these in their final form were what mattered.

The findings from this experiment led directly to the experiments described in the following chapter.

Chapter B6 Validation of the Technique

B6.1 Introduction

Early experiments (Johnson and McDermott, 1973) gave unexpectedly high values for serum concentrations of cholecystokinin when compared with values for other gastrointestinal hormones. Possible reasons for this discrepancy have now been investigated and the present experiments were designed to answer four main questions:

(i) is the response of an isolated rabbit gall-bladder to a stimulus constant for the duration of the experiment?

(ii) does serum itself affect the response of the isolated gall-bladder?

(iii) can cholecystokinin-mimicking substances be isolated and removed?

(iv) can the apparent cholecystokinin content of a serum sample alter during processing and storage?

The final question has been asked because it has been shown (Ondetti, Rubin, Engel, Pluscec and Sheehan, 1970) that some of the smaller carboxyterminal fragments of cholecystokinin may be up to seven times more active on a molar basis than the full molecule (e.g. C-terminal octapeptide 2.5 times as active, C-terminal dodecapeptide 7 times as active). Hormonal activity normally drops during processing and storage of a sample but if enzymic breakdown of the full cholecystokinin m lecule occurred, a considerable increase in cholecystokinin bioactivity could be generated before the activity of the sample was measured.

B6.2 Procedures

These experiments were performed as a progression from each other over a period of time as new thoughts and questions came to mind. The actual technique used for a particular experiment is indicated at the beginning of the relevant paragraph.

It is not known which form of cholecystokinin is the active constituent in serum, i.e. the full molecule or small fragments or a combination of both. It is therefore more meaningful to express results in terms of biological activity (mIDU/ml), although for comparison the molar equivalentoof the full molecule in pmol/ml has been placed in brackets after quoted biological units using conversion factors based on 3000 IDU/mg (Jorpes, Mutt and Toczko, 1968) and accepting the molecular weight of cholecystokinin as 3940.

B6.3 The effect of time

B6.3.1 Method

Using the original manual technique (chapter B1) to study the effect of repeated stimulation over a period of 12 - 14 hours, the responses of the isolated strips of rabbit gall-bladder to standard solutions of cholecystokinin containing 0.5, 1, 2, 4 and 8 mIDU/ml (0.04, 0.09, 0.17, 0.34 and 0.68 pmol/ml) cholecystokinin as pancreozymin (Boots) were measured. The solutions were repeatedly passed over the strips of gall-bladder in the above order. No serum samples at all were passed during these experiments.

B6.3.2 Results

Figure B6.1 shows the result of one experiment where standard

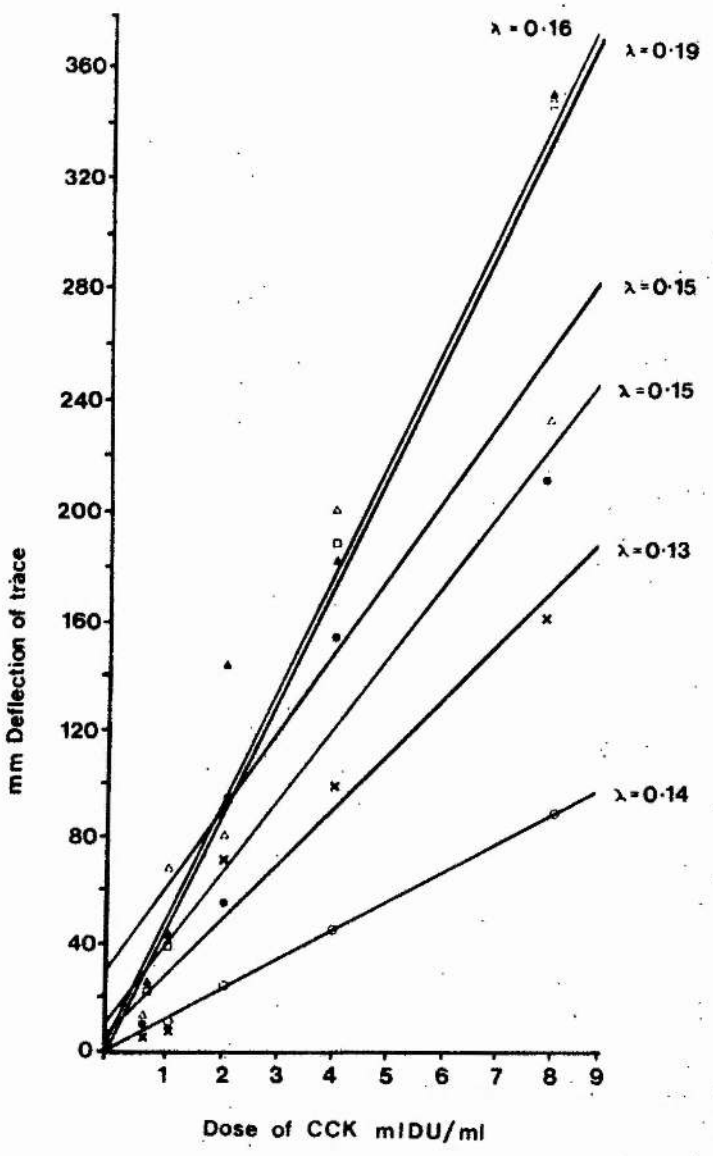

Fig B6.1 Dose-response curves for cholecystokinin, given as milli Ivy Dog Units/ml, showing progressive increases in the sensitivity of strips of rabbit gall-bladder with time.
○, 3 - 4h; ×, 4 - 5.5h; ●, 6.8 - 8h; △, 8 - 10h; □, 10 - 12h; ▲, 12 -14h.

concentrations of cholecystokinin were applied repeatedly for 12 - 14 hours. Each line is a dose-response curve to the five standard solutions introduced consecutively. Similar results were obtained with other strips of gall-bladder. The slopes of the dose-response curves increased steadily for the first 7 hours before stabilizing. The slope of the final dose-response curve in three experiments was found to be greater then the slope of the initial line by a factor of 2.29 $\pm$ 1.295 (S.D.) ($P<0.001$ from t-test for comparison of slopes) but the slopes of the last two lines were very similar ($P>0.2$) confirming stabilization.

B6.4 The effect of serum

B6.4.1 Method

Using the first automatic system (chapter B2) to observe the effect of normal serum on the responses of the isolated rabbit gall-bladder to standard solutions of cholecystokinin, samples were superfused over the gall-bladder strips at a continuous rate of three samples per hour. A five point dose-response curve was obtained. Two consecutive samples containing 2ml human serum (stored frozen at -20°C until required), diluted to 75ml with Krebs solution, were then passed over the strips of rabbit gall-bladder. The cholecystokinin doses were then repeated, further serum samples passed and the cholecystokinin doses repeated again. In two similar experiments serum from blood treated with Trasylol (aprotinin, see section B6.6) was used.

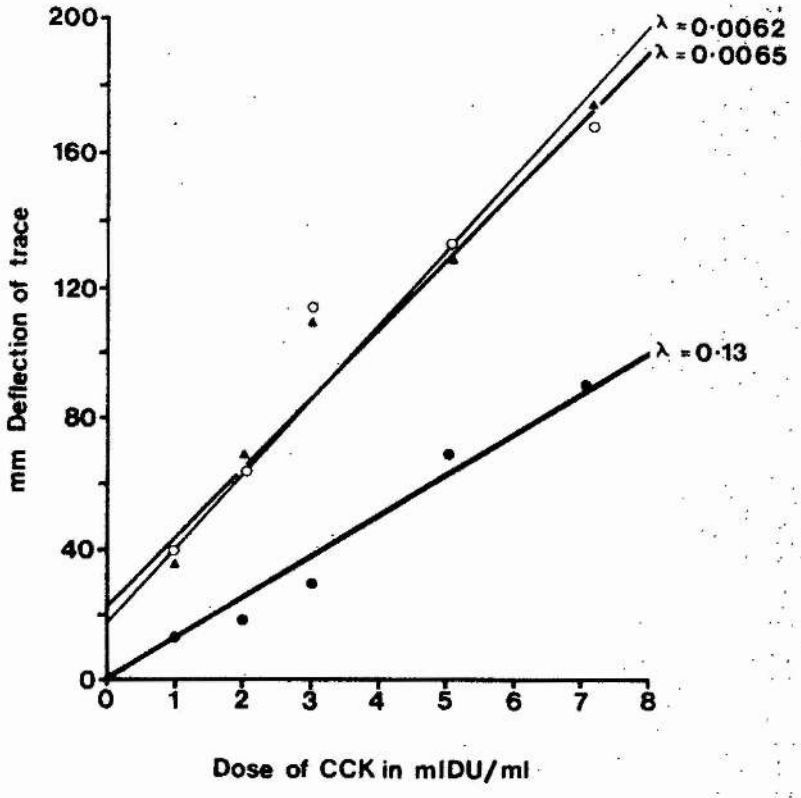

Fig B6.2 Dose-response curves for cholecystokinin, given as milli Ivy Dog Units/ml, showing the alteration in slope from the initial curve (●) to that after exposure of the rabbit gall-bladder to a single serum sample (▲) which had not altered after 8h (○).

B6.4.2 Results

The effect of serum on the dose-response curve to cholecystokinin in one out of five similar experiments is shown in Fig. B6.2; immediately after the passage of the first serum sample the slope of the cholecystokinin dose-response curve increased by a factor of 2.75 ± 1.058 ($P < 0.001$ from t-test for comparison of slopes) (cf. 2.29 see Fig. B6.1) over the slope of the initial dose-response curve. Further serum samples did not alter the slope of the dose response curve again; this remained stable for the duration of the experiment (12hr; $P > 0.2$; five experiments). Trasylol made no difference to this serum shift effect; the ratios of the slopes of the lines were 2.5 and 2.6 in the two experiments.

B6.5 Cholecystokinin - mimicking substances

B6.5.1 Method

Using the final automatic system (chapter B3.4) the possibility that non-peptide molecules in the serum were contributing to the cholecystokinin-like activity was tested using charcoal to extract the peptides from the serum by adsorption (Morris and Morris, 1964), leaving other active substances behind. An insolubilised specific antibody for cholecystokinin would have been preferable and left no doubt that only cholecystokinin was extracted, this however, was not available. Blood samples (20ml) were taken from fasting volunteers. The samples were dispensed into tubes containing 10,000 kallikrein inactivator units (KIU) of Trasylol per 10ml blood. When the blood had clotted the serum was collected.

The cholecystokinin-like activity was then estimated;

(i) For total cholecystokinin activity, 2ml portions of each serum sample were diluted to 100ml with Krebs solution.

(ii) For cholecystokinin-mimicking substances, 4ml portions of each serum sample were shaken with approximately 80mg dextran-coated charcoal (10g activated charcoal, HCl washed, Sigma; 1g Dextran T70, Pharmacia Fine Chemicals). The charcoal was removed by centrifugation at 1700 x g for 10 minutes and the extracted serum diluted to 100ml with Krebs solution.

To confirm that the charcoal was extracting cholecystokinin and peptides of a similar molecular weight, 25ml blood samples were taken from four non-fasting volunteers. The samples were evenly distributed among ten plain glass tubes. Once the blood had clotted the tubes were centrifuged (1000 x g for 10min.) and the serum removed and pooled. Portions of this pool of serum were then treated as follows:

(i) 2ml remained untreated (for total CCK activity),

(ii) 2ml + 125mIDU (10.5 pmol) CCK (pancreozymin),

(iii) 4ml extracted with dextran-coated charcoal,

(iv) 4ml + 125mIDU (10.5 pmol) CCK extracted with dextran-coated charcoal

(v) 4ml extracted with dextran coated charcoal then digested with trypsin

(vi) 4ml extracted with dextran-coated charcoal, digested with trypsin then re-extracted with dextran coated charcoal,

(vii) 4ml +125mIDU (10.5 pmol) CCK extracted with dextran-coated charcoal then digested with trypsin,

(viii) 4ml + 125mIDU (10.5 pmol) CCK extracted with dextran-coated charcoal, digested with trypsin the re-extracted with dextran-coated charcoal.

Trypsin (treated with dextran-coated charcoal, Sigma) was prepared at a concentration of 2mg/ml in 1% aqueous ammonium bicarbonate and stored at -20°C until required. 2ml of this solution was added to each serum portion to be digested and the reaction was allowed to proceed for 30 min. at 37°C when it was stopped by the addition of 10,000 KIU Trasylol. The treated solutions were stored at -20°C for 24 hours before measurement of cholecystokinin bioactivity.

B6.5.2 Results

Table B6.1 shows the quantitative removal by dextran-coated charcoal of both endogenous and exogenous cholecystokinin from serum. It also illustrates the good recovery of exogenous cholecystokinin and shows that digestion of extracted serum with trypsin does not release small fragments of cholecystokinin with additional activity.

It was found that, whereas the level of cholecystokinin in the serum of subjects varied after a meal, the amounts of cholecystokinin-mimicking substances remained small and constant (range 5-45 mIDU/ml serum; 0.4-3.8 pmol/ml), but the proportion

Table B6.1

Effect of extracting normal serum and normal serum with added Cholecystokinin (CCK) with dextran-coated Charcoal (80 mg), and digesting the extracted serum with trypsin and re-extracting the digested serum.

Serum sample	milli Ivy Dog Units/ml serum (pmol/ml)
A	141 (11.9)
B	246 (20.8)
A Extracted	8 (0.7)
B Extracted	11 (0.9)
A Extracted,digested	7.5(0.6)
B Extracted,digested	8 (0.7)
A Extracted,digested,re-extracted	7 (0.6)
B Extracted,digested,re-extracted	9 (0.8)

A, Normal serum; B, normal serum + 125 milli Ivy Dog Units (10.6 pmol)

of these substances in the total cholecystokinin activity of the serum of any individual could not be predicted.

B6.6 Possible degradation during processing and storage

B6.6.1 Method

Using the final automatic system (chapter B3.4) the possible effects of the experimental procedures on the final estimates of cholecystokinin activity were tested in several ways. First, 20ml blood samples were taken from five fasting volunteers; 10ml blood were transfered to a normal plain glass tube as a control, and the other 10ml were transfered to another plain glass tube containing 10,000 KIU Trasylol. Both samples were treated in the same manner to produce serum and were then frozen and stored at -20°C for 4-5 days until examined for cholecystokinin bioactivity. Further experiments were conducted to observe the effect of Trasylol on the cholecystokinin-mimicking substances. Samples of blood were divided into two; one half was treated with Trasylol as before, the other retained as control. These two samples were then further subdivided giving four measurements for each volunteer:

(i) control,

(ii) cholecystokinin-mimicking substances,

(iii) Trasylol treated,

(iv) Trasylol treated cholecystokinin-mimicking substances.

To test the recovery of cholecystokinin added to serum, a 50ml sample of blood was taken from a non-fasting volunteer. This was evenly distributed among five plain glass tubes each containing 10,000 KIU Trasylol. Once the blood had clotted, the tubes were centrifuged and the serum was pooled.

This pool of serum was divided into five 5ml portions; the first was to act as control, to the others were added 0.1, 0.2, 0.3, or 0.4ml respectively of a solution containing 1.25 IDU/ml (10.5 pmol/ml) of cholecystokinin. Each portion of serum then had an additional 125, 250, 375 or 500 mIDU (10.5, 21, 31.5 or 42 pmol) cholecystokinin respectively. 2ml of serum from each tube were diluted to 100ml with Krebs solution and the cholecystokinin bioactivity was measured on two strips of rabbit gall-bladder set in parallel.

Finally, to assess the effect of all factors, the levels of cholecystokinin in the serum of ten normal fasting volunteers were measured with all four factors described above taken into account (i.e. Trasylol added to blood samples, estimation of CCK-mimicking substances made, gall-bladders primed with serum before calibration) were measured, and the release of cholecystokinin in five patients following a liquid meal of 'Build Up' (Carnation) was followed for one hour.

B6.6.2 Results

The differences in cholecystokinin-like bioactivity between serum control samples from five fasting volunteers and the same samples with added Trasylol are shown in table B6.2. There was a very marked reduction in apparent cholecystokinin activity when Trasylol was used. Table B6.3 records the average of two estimations of cholecystokinin in blood samples from non-fasting subjects. This illustrates that while Trasylol appreciably affected the total measureable cholecystokinin, it did not significantly alter the 'serum blank' (cholecystokinin-mimicking substances, $P>0.2$, paired t-test). The reduced effect of Trasylol on these samples (cf. table B6.2 was probably due to the non-fasting state of the volunteers (see Discussion). Trasylol did not alter the response of the gall-bladder to standard cholecystokinin (neither Boots preparation nor the octapeptide) in Krebs solution.

Table B6.4 shows that both satisfactory recovery of added cholecystokinin and a good correlation can be obtained from two strips of gall-bladder set in parallel.

After modification of the method (blood taken into Trasylol, measurement of serum blank, and priming of the gall-bladders), the mean fasting level of cholecystokinin in the serum of ten young normal healthy volunteers was found to be 28 ± 10 mIDU/ml serum (2.4 ± 0.8 pmol/ml); this was about one

Table B6.2

Effect of adding aprotinin (Trasylol, 10 000 kallikrein inactivator units/10ml) to individual samples of human blood from five fasting volunteers on the concentration of cholecystokinin (CCK) in the serum.

Serum sample	milli Ivy Dog Units CCK/ml serum (pmol/ml)	CCK after Trasylol (% of control)
A Without Trasylol	190 (1.60)	
A With Trasylol	13 (1.1)	6.6
B Without Trasylol	73 (6.1)	
B With Trasylol	5 (0.4)	6.9
C Without Trasylol	150 (12.7)	
C With Trasylol	11 (0.9)	7.5
D Without Trasylol	128 (10.8)	
D With Trasylol	28 (2.3)	21.5
E Without Trasylol	78 (6.6)	
E With Trasylol	20 (1.7)	25.8

Table B6.3

Effect of adding aprotinin (Trasylol, 10 000 kallikrein units/10ml) to individual samples of blood from five non-fasting volunteers on the level of cholecystokinin (CCK)-like activity in the serum after extraction with dextran-coated charcoal (serum blank) (values show the mean of two estimations on each sample).

CCK in milli Ivy Dog units/ml serum (pmol/ml)

Serum sample	Control	Control blank	True control blank	With Trasylol	Blank with Trasylol	True Value
F	139(11.8)	21(1.8)	118(10.0)	104 (8.8)	26(2.2)	78(6.6)
G	112 (9.5)	17(1.4)	95 (8.0)	108 (9.1)	32(2.7)	76(6.4)
H	154(13.0)	15(1.3)	139(11.8)	82 (6.9)	27(2.3)	55(4.7)
I	191(16.2)	38(3.2)	159(13.5)	99 (8.4)	42(3.6)	57(4.8)
J	365(30.9)	43(3.6)	322(27.2)	118(10.0)	34(2.9)	74(6.3)

FP63.3

Table B6.4

Efficacy of recovery of cholecystokinin (CCK; in milli Ivy Dog units (mIDU)) added in graded doses to 5 ml normal human serum.

Sample	mIDU/ml (pmol/ml)	mIDU (pmol) in total volume	Expt - control	% recovered
1st gall-bladder strip				
Control	90.0 (7.6)	450(38.1)	---	---
+ 125 mIDU	112.5 (9.5)	573(48.5)	124(10.4)	99.2
+ 250 mIDU	125.0(10.6)	650(55.0)	200(16.9)	80.0
+ 375 mIDU	160.0(13.5)	848(71.7)	398(33.6)	106.1
+ 500 mIDU	195.0(16.5)	1053(89.1)	603(51.0)	120.6
2nd gall-bladder strip				
Control	105.0 (8.9)	525(44.4)	---	---
+ 125 mIDU	125.0(10.6)	637(53.9)	113 (9.5)	90.4
+ 250 mIDU	138.0(11.7)	715(60.5)	190(16.1)	77.0
+ 375 mIDU	178.0(15.1)	940(79.5)	416(35.1)	110.9
+ 500 mIDU	210.0(17.8)	1134(95.9)	609(51.5)	121.8

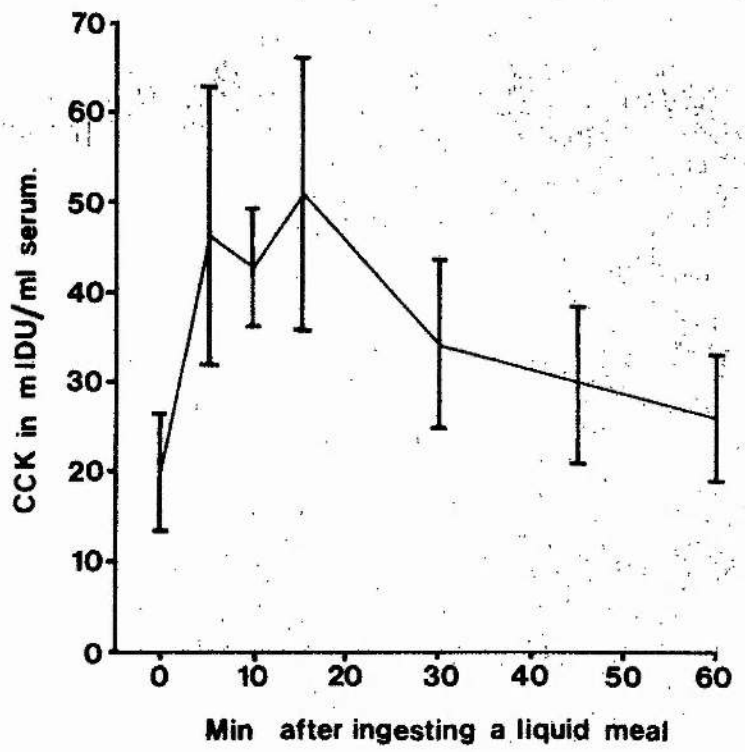

Fig B6.3 Time course of release of cholecystokinin over 1h after ingestion of a liquid meal. Values are means + S.E.M. for five patients. The CCK release was measured in milli Ivy Dog Units/ml serum.

third of the level found before modification (Johnson and McDermott, 1973).

Figure B6.3 shows the time course for the release of cholecystokinin following a liquid meal. It should be realised however that the size and timing of the peak response varied considerably from patient to patient occurring as early as five minutes or as late as 60 minutes after ingestion of the meal; this has the effect of depressing the average response indicated in Fig. B6.3 and flattening the curve.

B6.7 Determination of the optimum amount of Trasylol to add to each blood sample

B6.7.1 Method.

30ml of blood was taken from each of three non-fasting volunteers. Each sample was divided evenly between three plain glass tubes containing 5000, 10,000 and 20,000 KIU of Trasylol respectively (0.25, 0.5 and 1ml of the commercial preparation of Trasylol. The blood was allowed to clot, the tubes centrifuged and the serum separated and stored frozen at - 20°C until required. Each sample was then assayed for cholecystokinin bioactivity using the final automatic system (chapter B3.4) and two strips of rabbit gall-bladder set in parallel.

B6.7.2 Results

Table B6.5 shows the result of this experiment with the individual results obtained on the two gall-bladder strips A and B. Each portion of blood was assumed to yield 5ml of serum. The total volume of the three test solutions from each blood sample was then 5.25, 5.5 and 6.0ml corresponding to 10ml blood treated with 5,000, 10,000 and 20,000 KIU Trasylol respectively.

The results from the three volunteers differed widely, from a reduction by a factor of approximately 3 in the amount of cholecystokinin bioactivity by 20,000 KIU compared with 5,000 in two samples, to virtually no difference in the effect of the two amounts of Trasylol in the third.

10,000 KIU Trasylol effected some reduction in activity in all cases and was adopted as the standard amount to be added to 10ml of blood. This amount (0.5ml) was felt not large enough to have significant dilution effects, but sufficient to inhibit at least some of the enzymic degradation during processing and storage.

B6.8 Discussion

The isolated rabbit gall-bladder increases in sensitivity to repeated standard solutions (section B6.3) of cholecystokinin

Table B6.5

mIDU sample with amount of Trasylol

Serum	5,000 KIU gall-bladder		10,000 KIU gall-bladder		20,000 KIU gall-bladder	
	A	B	A	B	A	B
K	312.5	277.5	305.0	267.0	87.5	75.0
L	280.0	337.5	167.5	160.0	90.0	110.0
M	195.0	185.0	140.0	145.0	155.0	170.0

FP65

over a period of about 6-8 hours after which it remains stable. A single serum sample increases the sensitivity of the gall-bladder to its maximum immediately (i.e. equivalent to 6-8 hour stimulation with standards, section B6.4). Both Figs. B6.1 and B6.2 have a true zero and therefore direct comparison is possible. Thus a strip of gall-bladder can be 'primed' with an initial serum sample before determining the cholecystokinin dose-response curve; its sensitivity will then remain unchanged for the duration of the experiment and this can be confirmed by passing further standards during and at the end of the experiment.

Results in table B6.1 (section B6.5.2) make it unlikely that residual cholecystokinin like activity after extraction with dextran-coated charcoal is due to a high molecular weight cholecystokinin. We have termed this residual activity whose nature is unknown the 'serum blank'. Berry and Flower (1971) and Johnson and McDermott (1973) tested a number of substances including secretin, gastrin and 5-hydroxytryptamine; in addition we have also tested glucagon (5 and 10ug/ml superfusing solution) and these substances alone and in conjunction with cholecystokinin had no effect on the response of the gall- bladder. The 'serum blank' is not a breakdown product of cholecystokinin as it is

still present when Trasylol is used. At present the only way to eliminate the 'serum blank' is to measure it and subtract it from the total cholecystokinin activity found in the untreated serum.

Trasylol reduced the cholecystokinin-like activity of the serum sample but not the action of cholecystokinin or cholecystokinin octapeptide on the strip of gall-bladder (section B6.6). Thus since Trasylol inhibits proteinases which are activated in tissues damaged by ischaemia and hypoxia (for instance in shock or through tourniquet application, Glen and Lefer, 1972), it is likely to be preventing the breakdown during processing of the full cholecystokinin molecule to the much more active dodeca-/and octapeptides.

The lower cholecystokinin-like activity in serum treated with Trasylol strongly suggests that in its absence activity is generated in vitro. Since Johnson and McDermott (1973) found a slight loss of activity when repeated measurements were made on five consecutive days after deep freezing, it is likely that the increase in activity occurs during processing.

The effects of Trasylol are much greater on samples from fasting subjects than on those samples taken at the peak period after ingestion of food.

In the case of the related hormone gastrin, the high molecular weight G34 form predominates in the serum of fasting subjects, whereas the smaller G17 is released after a stimulus (Rehfeld, Stadil and Vikelsoe, 1974). It is possible that similarly significant amounts of the dodeca and/or octapeptides of cholecystokinin (which cannot be broken down further to more active fragments) are released after a meal, but are not present in the serum of fasting subjects.

Four factors then appear to have contributed towards the surprisingly high estimates of serum cholecystokinin measured biologically in vitro.

(i) The sensitivity of the gall-bladder increases with the passage of time, early standards will therefore give artificially low values for the response of the gall-bladder to that dose of cholecystokinin.

(ii) The passage of serum over the isolated gall-bladder immediately increases its sensitivity to the maximum; a gall-bladder can therefore be 'primed' before it is calibrated and the errors due to (i) eliminated.

(iii) Substances mimicking cholecystokinin activity are present in all sera and must be measured and subtracted from the total cholecystokinin activity.

(iv) Unless an enzyme inhibitor such as Trasylol is added to the blood sample a considerable *in vitro* generation of cholecystokinin activity may occur.

However these factors apply to all samples and do not necessarily invalidate the relative changes in the same patient following a stimulus such as a meal.

B6.9 Conclusions

From these experiments it was concluded that, in the routine use of this method, the first three solutions of cholecystokinin presented to the gall-bladders should be:

(i) a medium dose of 5mIDU/ml (0.4 pmol/ml) of standard cholecystokinin in Krebs solution.

(ii) Two portions of 2-3ml pooled serum diluted to 100ml with Krebs solution.

The first solution gives an approximate idea of the sensitivity of the gall-bladder and will indicate if any adjustment of the transducer's counter-balance is required, and the second two ensure the complete resetting of the dose-response curve, that is priming of the gall-bladder before calibration.

A further dose-response curve at the end of the run and a series of standard cholecystokinin solutions at intervals during the run should be used as an extra precaution. In addition, all blood samples should be taken into Trasylol (10,000 KIU/10ml blood) and one estimation of the serum blank made for each patient.

Chapter B7 The effect of α_2 - macroglobulin bound trypsin on cholecystokinin, and modification of this action by Trasylol, leupeptin and antipain.

B7.1 Introduction

With the observation (section B6.6) that Trasylol can, in some circumstances, have a dramatic effect on the result obtained for the amount of cholecystokinin bioactivity in a given sample, it was of interest to discover what effect, if any, trypsin bound to α_2 - macroglobulin had on cholecystokinin activity, and also to note the effect of inhibitors on this effect.

Since trypsin splits the CO-NH peptide bond on the acid side of basic amino acids any action of trypsin on cholecystokinin would show as an increase in bioactivity, due to the release of the dodeca and octa peptides at the active C-terminal end of the molecule (for structure see section A3.1). In fact, since the arginine bond that would release the octapeptide is surrounded by aspartic acid residues, it would be more likely that the active fragment released by the enzymic action of α_2- macroglobulin bound trypsin would be the dodeca-peptide, which is 10-15 times as active as the full molecule on a weight basis (Ondetti et al., 1970). Cleavage at the third arginine residue would have no effect in subsequent bioactivity.

B7.2 Method

Trypsin bound to α_2 - macroglobulin was a gift from the American Red Cross Blood Research Laboratory, Bethesda, Maryland, U.S.A. A small amount (365ng) of cholecystokinin-variant (39 amino acids, section A3.1 a gift from Professor V. Mutt) was incubated at room temperature (~20°C) with 5 ul α_2 - macroglobulin bound trypsin (12 mg/ml α_2 Macroglobulin with 300 ug active Trypsin/ml in 50 mM Tris-hydrochloride buffer pH 7.3 containing 10 mM $CaCl_2$) for 15 minutes, At the end of this time the reaction was stopped by dilution with ice cold Krebs solution.

To investigate the effects of the trypsin inhibitors Trasylol, leupeptin and antipain, series of reaction vessels were prepared each containing the same amount of cholecystokinin variant (365ng) plus graded amounts of the inhibitors reduced by serial dilution from concentrations of 2.8mg/ml for Trasylol, 200uM for leupeptin and 150 uM for antipain to minimum concentrations of 22 ug/ml, 1.57 uM and 1.17 uM respectively. A constant (5 ul) amount of α_2 - macroglobulin bound trypsin was added to each tube, and the tubes incubated at room temperature (~20°C) for 15 minutes. At the end of this time the reaction was stopped as before by dilution with ice cold Krebs solution.

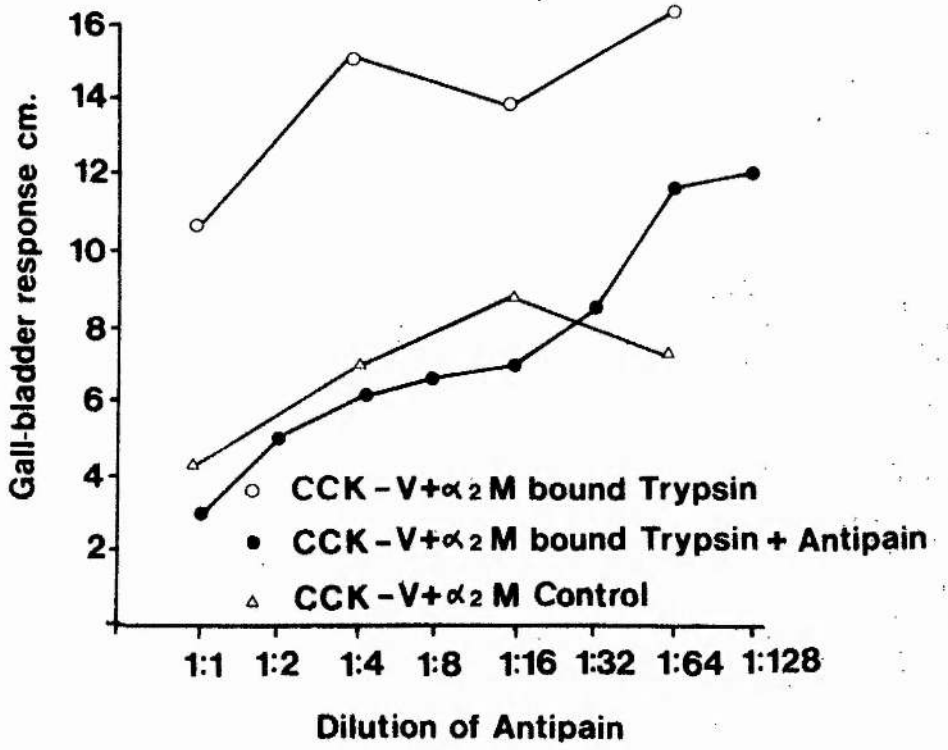

Fig B7.2 Effect of antipain on the degradation of cholecystokinin by α_2 - bound trypsin

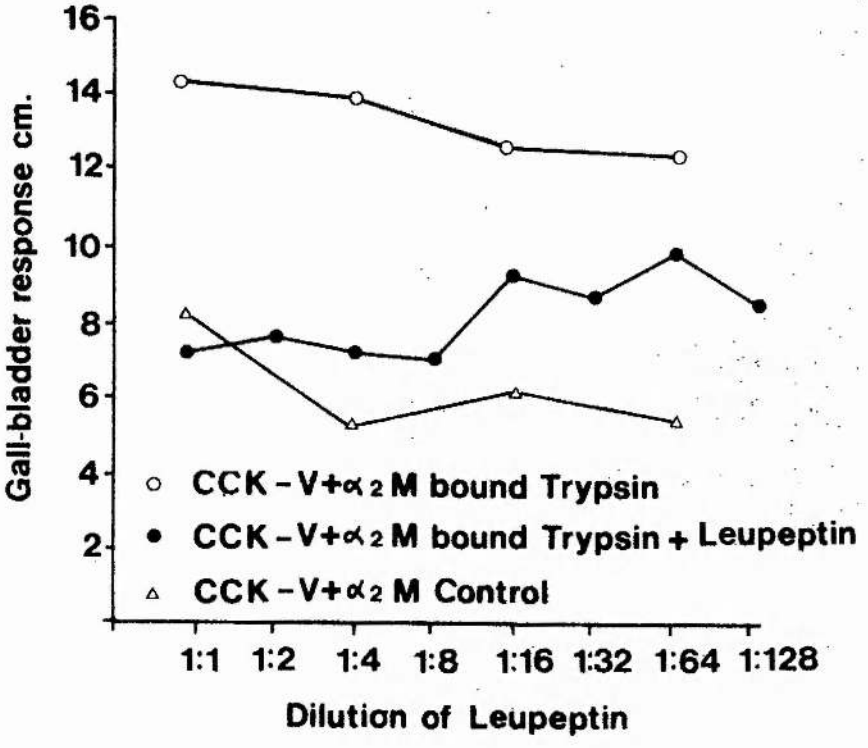

Fig B7.3 Effect of leupeptin on the degradation of cholecystokinin by α_2 - bound trypsin

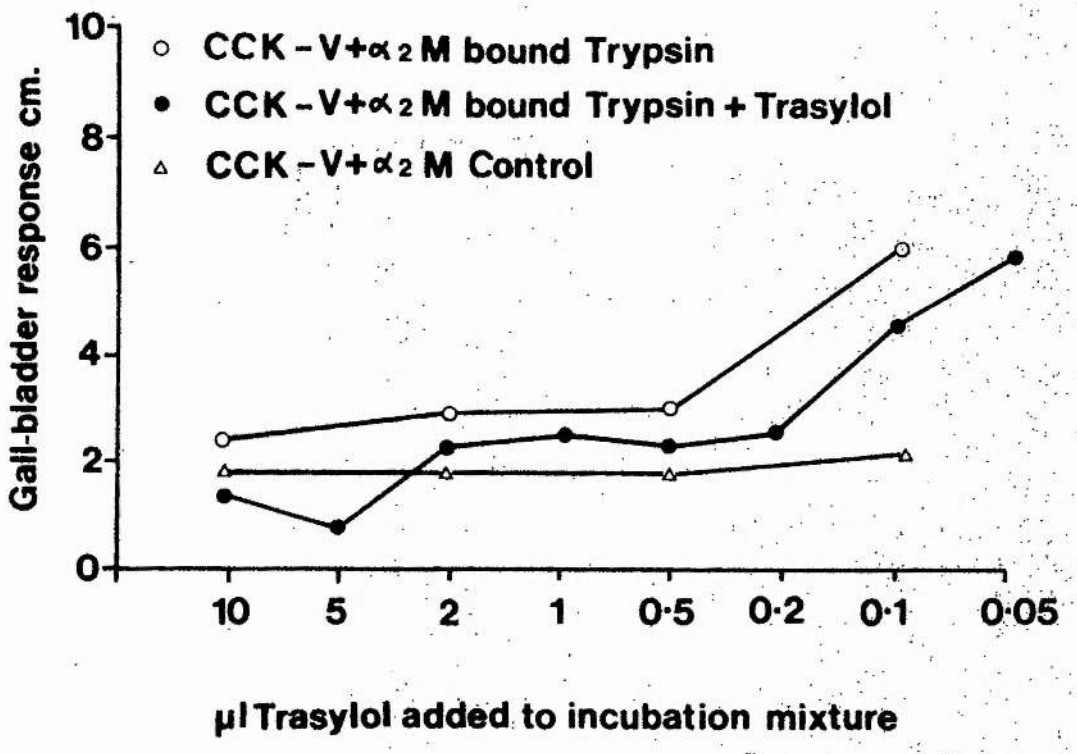

Fig B7.1 Effect of aprotinin (Trasylol) on the degradation of cholecystokinin by α_2 - bound trypsin.

In addition each series was controlled with tubes containing only cholecystokinin-variant and α_2 - macroglobulin (these gave the amount of CCK bioactivity added to each tube), and by tubes containing cholecystokinin-variant and α_2 - macroglobulin bound trypsin added as above (these gave the maximum amount of CCK bioactivity which could be expected after enzyme treatment).

All samples were then diluted to 100ml with Krebs solution and their cholecystokinin bioactivity estimated with the final automatic system (section B3.4).

B7.3 Results.

The cholecystokinin-variant treated with α_2 - macroglobulin bound trypsin showed a 4.5 fold increase in bioactivity over the control sample which did not contain the enzyme. The effects of the three inhibitors Trasylol, leupeptin and antipain are shown in Figs. B7.1, B7.2, B7.3 and AP7. From these it can be clearly seen that while leupeptin and antipain considerably reduced the enzyme effect of α_2 - macroglobulin bound trypsin upon the cholecystokinin-variant, Trasylol had little or no effect.

B7.4 Discussion

These results demonstrate conclusively that cholecystokinin bioactivity can be generated *in vitro* by enzymic splitting of the whole molecule to fragments that are more active. If it were certain that the main peptidase in serum was trypsin it might be more relevant to take blood samples into leupeptin or antipain, considering the relative ineffectiveness of Trasylol.

However, since in practice a number of peptidases will be present in serum, Trasylol as a more general inhibitor, is still probably the material of choice, despite the fact that these experiments, which were performed since those described in chapter B6, suggest that Trasylol may not be doing its job as efficiently as it might at the concentration used.

While use of a higher concentration of Trasylol (section B6.7) has shown Trasylol to be an effective inhibitor of proteinase activity, routine use of these higher concentrations would introduce unacceptable dilution problems, correction for which, due to the varying amount of serum produced by each individual, would be virtually impossible.

Chapter B.8 The effect of drugs, and other peptide hormones, on the response of the isolated rabbit gall-bladder to cholecystokinin - containing solutions.

B8.1 Introduction

Any analytical system must necessarily be sufficiently specific for the substance being analysed, to permit quantitative estimates of the amount of the substance in test solutions to be made with confidence. It was therefore important to eliminate the possibility of interference with the system by other substances. This would be a fairly easy procedure for assay systems relying on chemical or physical changes in the solutions. The more complex bioassay system must be tested in a similar manner but much more care is needed in the interpretation of the results.

The following experiments set out to ensure; (1) that cholecystokinin acts directly on the gall-bladder and that its action was not mediated nor modified by associated nervous pathways. (2) That other peptide hormones, in particular secretin, motilin, somatostatin, pancreatic polypeptide, glucagon or pentagastrin, did not influence the effect of cholecystokinin on the gall-bladder when they were present in normal physiological amounts.

B8.2 The effect of neurotransmitter blocking drugs

The effects of the anticholinergic drug atropine, the β-blocker propranolol (Inderal), the α-blocker phentolamine mesylate (Rogitine) and the anaesthetic Lignocaine, on the response of the isolated rabbit gall-bladder to standard solutions of cholecystokinin were studied using the final automatic equipment (section 3.4).

The gall-bladder strips were calibrated with solutions containing 0.5, 1, 2, 3, 4 and 5mIDU/ml of cholecystokinin (as Boots Pancreozymin). This calibration was then repeated but with all the solutions containing 5ug/ml of one of the drugs. Calibrations continued alternating between the cholecystokinin control and the same solutions containing 5ug/ml of one of the drugs. The height of the peaks obtained in response to these solutions was measured and the dose-response curves constructed.

B8.3 The effect of other peptide hormones

These experiments were performed in an identical manner to those described in section B8.2 for the blocking drugs. After priming the gall-bladder strips with two diluted serum samples dose-response curves were constructed with standard solutions of cholecystokinin (as Boots Pancreozymin) as before. The calibration was then repeated with each

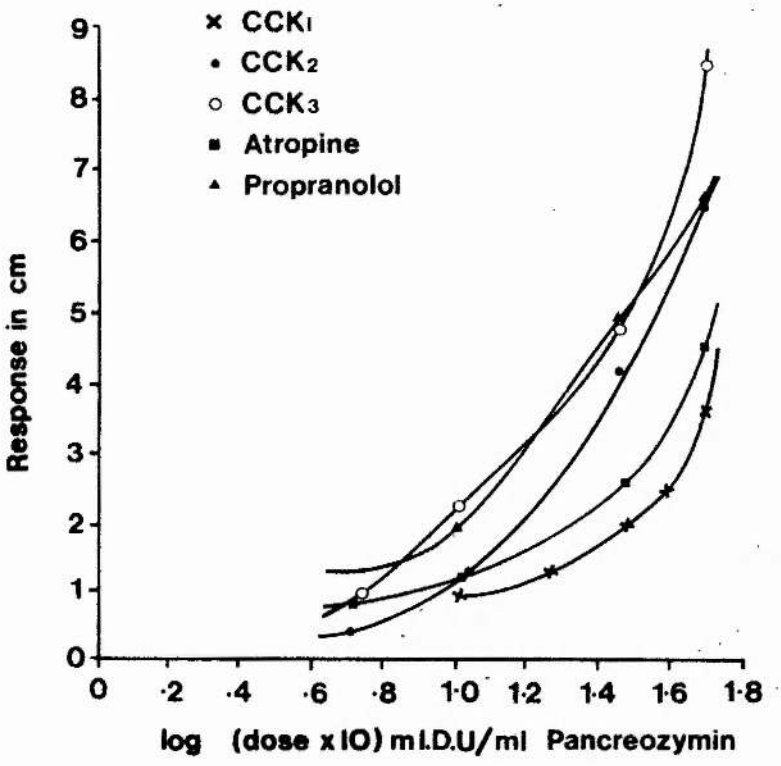

Fig B8.1 Dose-response curves for rabbit gall-bladder to graded doses of cholecystokinin alone and in the presence of the drugs atropine or propranolol.

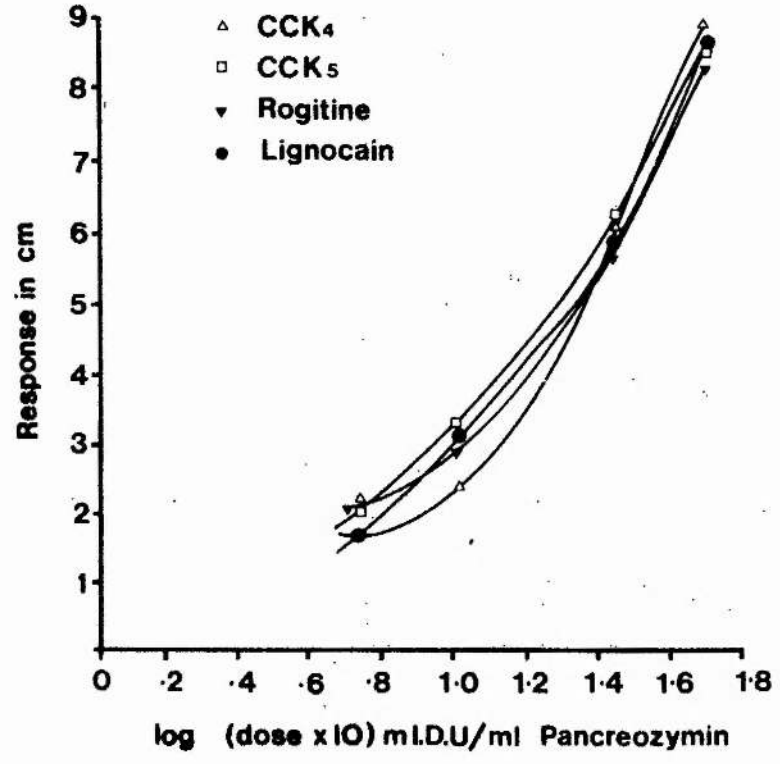

Fig B8.1A Dose-response curves for rabbit gall-bladder to graded doses of cholecystokinin alone and in the presence of the drugs phentolamine mesylate or lignocaine.

solution containing a constant amount of the peptide hormone under test; secretin 100mU/ml (as either Boots or Karolinska preparation), motilin 20pg/ml, pentagastrin 100pg/ml, pancreatic polypeptide 80pg/ml, somatostatin 4ng/ml and glucagon 200ng/ml. The amount of hormone used was dictated in part by the amount of each hormone available (e.g. total motilin available was 20ng) but amounts were also chosen to correlate with known physiological levels. In addition to the calibration solutions one sample containing only the test hormone at the concentration selected was also presented to the gall-bladders.

In a similar manner the desulphated C-terminal octapeptide of cholecystokinin was applied to the gall-bladder in graded amounts up to 1200pg/ml and also at a constant concentration of 800pg/ml in conjunction with graded doses of cholecystokinin.

B8.4 Results

There was no significant difference between the response of the gall-bladders to cholecystokinin alone and the response to the same dose in the presence of atropine, propranolol, lignocaine or phentolamine mesylate (Fig.B8.1). This confirms the accepted belief that cholecystokinin acts directly on the gall-bladder tissue and not through any

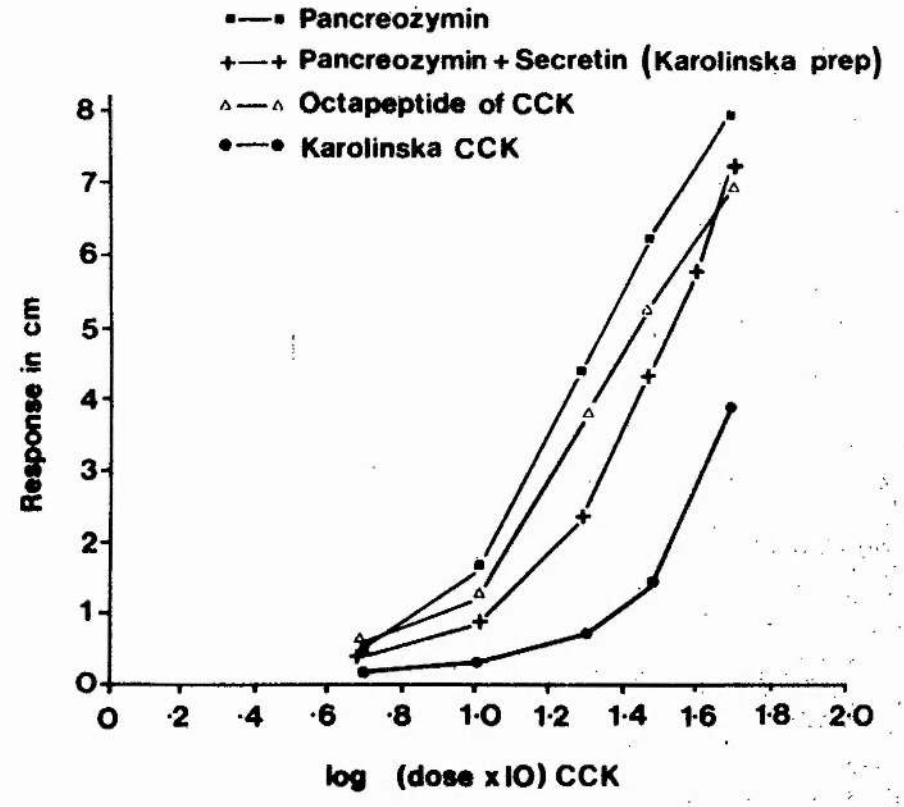

Fig B8.4 Dose-response curves for rabbit gall-bladder to graded doses of cholecystokinin alone and in the presence of secretin (Karolinska).

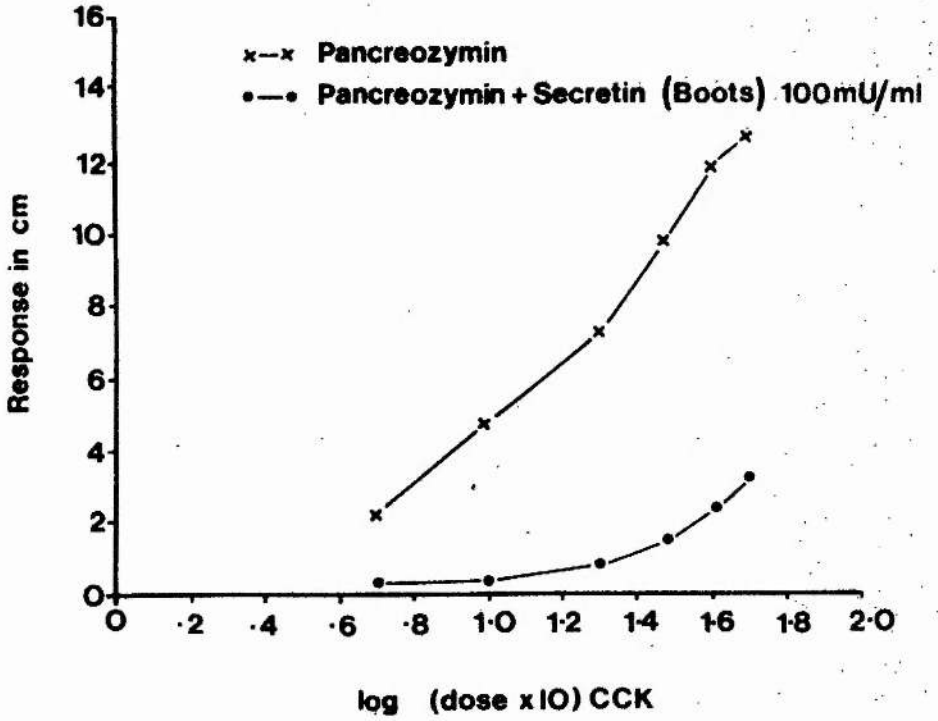

Fig B8.5 Dose-response curves for rabbit gall-bladder to graded doses of cholecystokinin alone and in the presence of secretin (Boots).

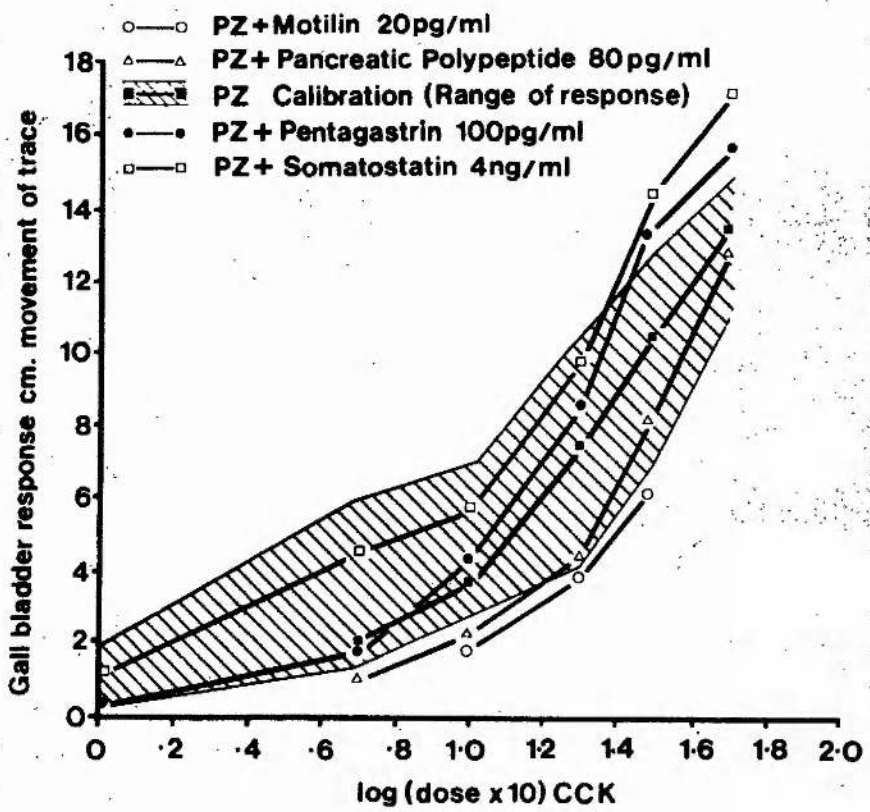

Fig B8.2 Dose-response curves for rabbit gall-bladder to graded doses of cholecystokinin alone and in the presence of motilin, pancreatic polypeptide, pentagastrin or somatostatin.

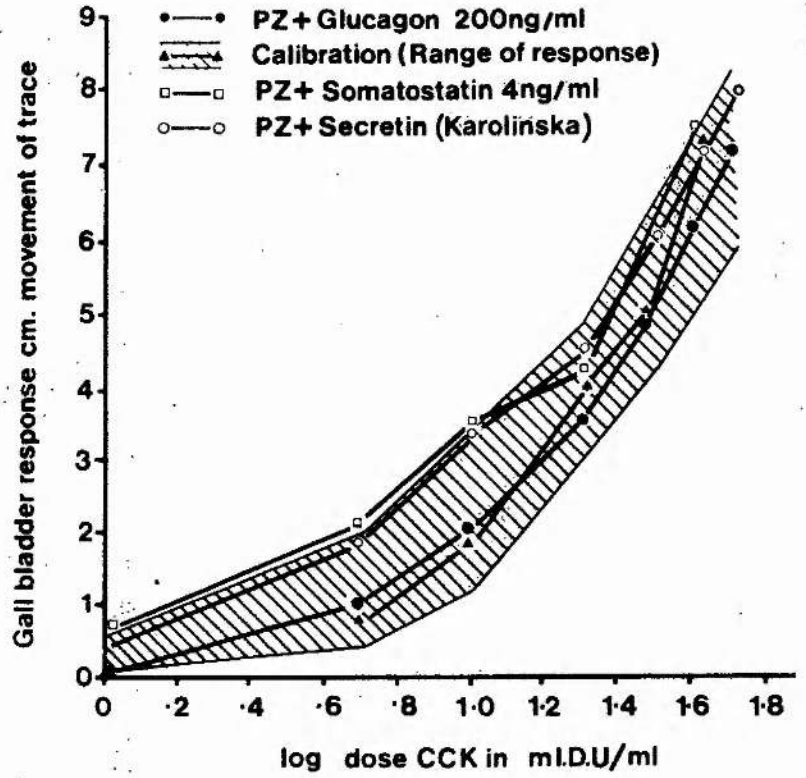

Fig B8.3 Dose-response curves for rabbit gall-bladder to graded doses of cholecystokinin alone and in the presence of glucagon, somatostatin or secretin (Karolinska).

intermediate pathway.

Interaction with other peptide hormones however manifested itself as either; no effect, slight inhibition (except for the Boots preparation of secretin Fig. B8.5) or slight enhancement (Figs. B8.2, B8.3, B8.4). These effects are summarised in Table B8.1.

The desulphated C-terminal octapeptide of cholecystokinin had no significant activity on its own, even at a concentration of 1200pg/ml, while when applied to the gall-bladder in conjunction with cholecystokinin it exerted a slight inhibitory effect (see Table B8.2).

B8.5 Discussion

These results confirm that the action of cholecystokinin is directly on the gall-bladder tissue and is not mediated through any nervous pathway. The interactions with the other peptide hormones are interesting but it appears that the inhibitory effects just about match the enhancing effects creating an overall balance of no effect. Thus these interactions, would not therefore appear to alter significantly the gall-bladder response to a dose of cholecystokinin when all the hormones are present together.

Table B8.1

Effect of other peptide hormones on the response of the isolated rabbit gall-bladder to cholecy stokinin.

Hormone	Concentration	Effect	Effect alone
Glucagon	200ng/ml	none	none
Somatostatin	4ng/ml	Slight enhancement	none
Motilin	20pg/ml	Slight inhibition	none
Pentagastrin	100pg/ml	Slight enhancement	none
Pancreatic polypeptide	80pg/ml	Slight inhibition	none
Secretin (Boots)	100mU/ml	Considerable inhibition	= 1 mIDU/ml
Secretin (Karolinska)	100mU/ml	Slight inhibition	none

Table B8.2

Response of the isolated rabbit gall-bladder to the desulphated C-terminal octapeptide of cholecystokinin and to graded doses of cholecystokinin in the presence or absence of the desulphated octapeptide.

dose of cholecystokinin in mIDU/ml	dose of desulphated octapeptide in pg/ml	gall-bladder response height of peak in cm.
	400	0.4
	800	0.6
	1200	0.5
5	-	13.0
3	-	9.8
2	-	6.1
1	-	3.7
0.5	-	2.1
5	800	12.0
3	800	8.7
2	800	5.7
1	800	3.1
0.5	800	1.4

The experiments with the desulphated C-terminal octapeptide of cholecystokinin demonstrate the total loss of activity that occurs with the removal of the tyrosine sulphate grouping. It was not surprising therefore to find that this desulphated molecule had some inhibitory effect on the normal molecule. Although the sulphate grouping is essential for biological activity it is unlikely that this grouping alone determines binding at the receptor sites. The desulphated molecule will almost certainly bind to the receptor sites and block them, since this binding must be reversable, inhibition of the natural molecule by the desulphated molecule is most likely to be of a competitive nature.

Chapter B9 Identification of different molecular forms of cholecystokinin in human serum

B9.1 Introduction

This work began from the observation that very few radio-immunoassays for cholecystokinin had been established through the world , and those that were established were making few appearances in the literature. The difficulties were not confined to problems encountered in labelling the pure cholecystokinin (see Section C); severe difficulties also seemed to be encountered in obtaining antibodies to cholecystokinin, and it could take up to 18 months of immunisation before antibodies could be detected in the serum of even one of a batch of test animals (Young, Lazarus and Chisholm, 1969). Even then there was no guarantee that these antibodies raised to porcine cholecystokinin would react with human cholecystokinin, and suggestions were made that a major structural difference could exist between the porcine and human cholecystokinin (Go, Ryan and Summershill, 1971).

To investigate this possibility it was decided to try to isolate sufficient cholecystokinin from human serum to enable an amino acid analysis to be performed. Early experiments in this direction were not encouraging; it was

impossible to locate cholecystokinin acitivity in fractions of column effluent, and it also became clear that many litres of serum would be required to obtain sufficient cholecystokinin for an amino acid analysis to take place. Therefore after the early experiments the research was directed at identifying cholecystokinin activity in column effluents and the idea of obtaining the amino acid sequence was temporarily shelved.

B9.2 First Experiment

A 1.5 x 100 cm column (Pharmacia Fine Chemicals Ltd. K26 column) was packed with Sephadex G50 (fine) and equilibrated with Krebs solution/saturated with Benzene.

50ml fresh serum was applied to the column which was then eluted with Krebs solution saturated with benzene. The column effluent was collected in 15ml fractions.

Each fraction was diluted to 100ml and examined for cholecystokinin activity with the final automatic system.

No detectable cholecystokinin was found in any fraction.

The experiment was repeated, but this time the fractions were pooled in sixes (1-6, 7-12 etc) forming samples of 100ml for testing for cholecystokinin activity as before. Again no cholecystokinin activity was found in any of the samples from pooled fraction.

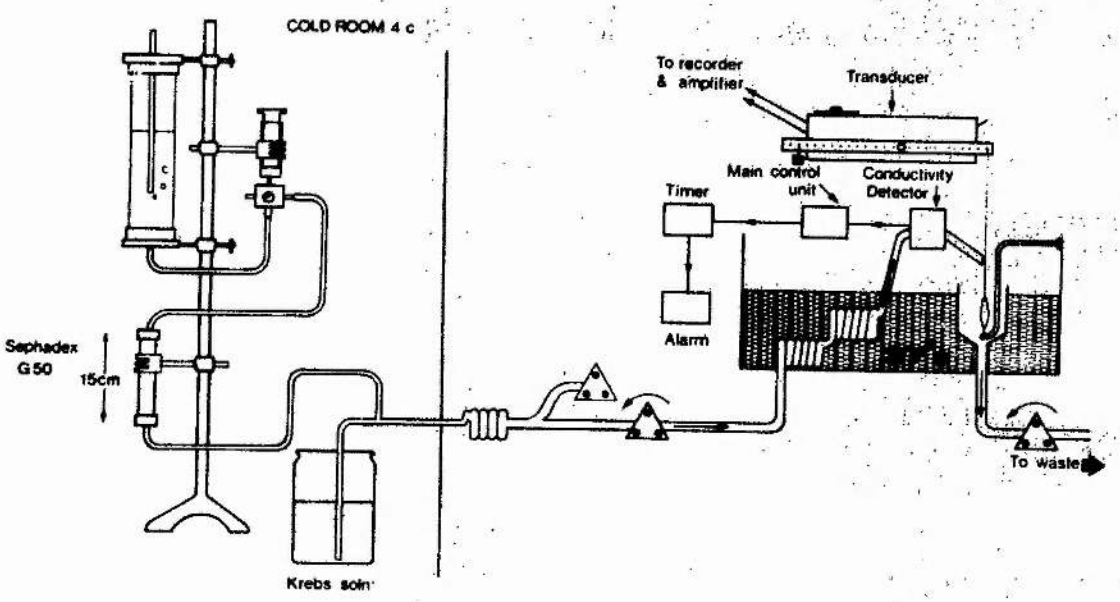

Fig B9.1 Modified apparatus for monitoring column effluent for cholecystokinin bioactivity.

B9.3 Second Experiment

The elution for the first experiment had been carried out at room temperature and the fractions stored at room temperature until tested. It was felt that this was at least part of the reason for failure to detect cholecystokinin activity in the column effluent. It would have been preferable to feed the column effluent directly onto the assay system but a previous experiment using a column of this size (1.5 x 100cm) had failed. The scale of the experiment was therefore reduced.

B9.3.1 Apparatus and Column Calibration

For this experiment the automatic equipment was disconnected from the bioassay apparatus and the effluent from a 1 x 15cm column of Sephadex G50 was fed directly into the main stream of Krebs solution (Fig. 9.1). A short coil was added to ensure adequate mixing before the stream was split to supply the two gall-bladders. The column was eluted with Krebs solution, and the final dilution of the column effluent when the stream was split was about 10:1.

Blue dextran, which was totally excluded from the G50 gel, and 250 mIDU (milli Ivy Dog Units) of cholecystokinin were used to calibrate the column, these marked the position of elution of the void volume and molecules of molecular weight

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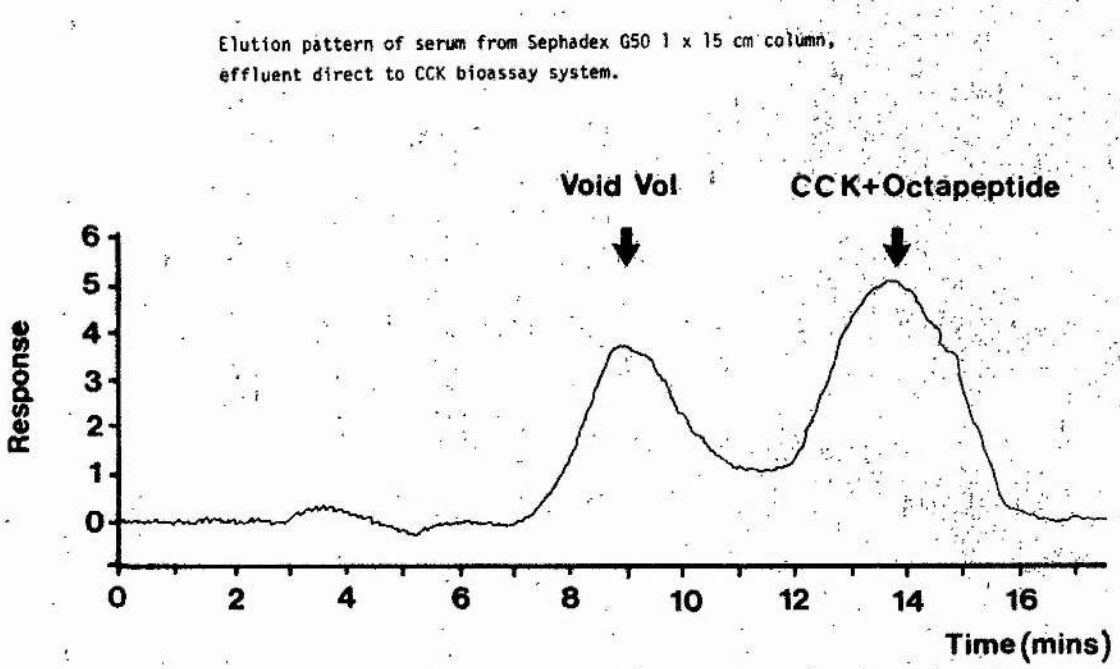

Fig B9.2 Elution pattern of cholecystokinin activity in serum from a Sephadex G50 1 x 15cm column with the effluent fed directly to the bioassay system.

around 4,000 - that of cholecystokinin, - respectively. The position of elution of the blue dextran was observed visually, that of cholecystokinin was marked by the response of the gall-bladders.

B9.3.2 Behaviour of cholecystokinin octapeptide on the column

Bioactivity from cholecystokinin applied to the column as G.I.H. Karalinska Cholecystokinin, Boots Pancreozymin or the synthetic C-terminal octapeptide of cholecystokinin all eluted from the column in the same position. This small column was therefore unable to separate the small octapeptide from the full molecule.

B9.3.3 Separation of Serum

Two millilitres of serum were applied to the 1 x 15 cm column and the elution continued with Krebs solution. The traces from the gall-bladders were examined after 15 minutes - the time calibrated for complete elution of the column.

Two areas of cholecystokinin bioactivity were observed (fig. B9.2), one was in the expected position for cholecystokinin the other appeared with the void volume.

A second serum sample gave the same result, and elution on a longer 1 x 60cm column simply enhanced the separation.

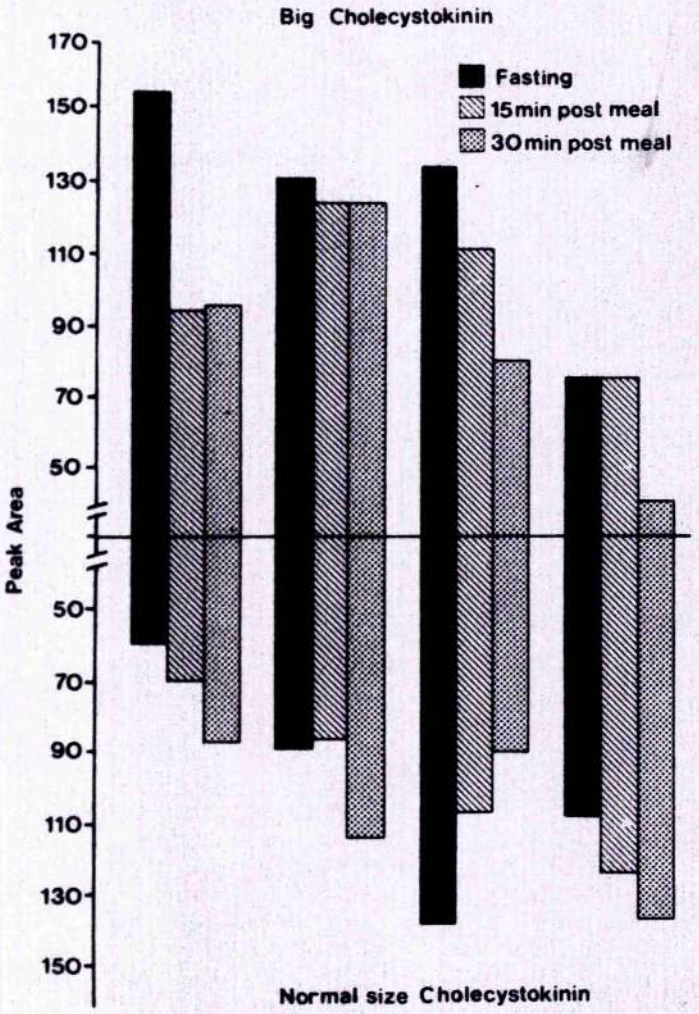

Fig B9.4 Proportion of 'big' and normal sized cholecystokinin-like activity eluted from a Sephadex G50 1 x 15cm column, showing differences in the serum content of the two 'cholecystokinins' when fasting and after ingestion of a meal.

B9.3.4 Effect of feeding

Volunteers were asked to undertake an overnight fast and then exchange some blood for our standard fatty breakfast (Fig. B1.4). Blood samples were taken fasting, and 15 and 30 minutes after completion of the meal. The blood was dispensed into tubes containing 10,000 K.I.U. Trasylol per 10ml blood, and after clotting the serum was collected.

2ml samples of each serum were then applied to the 1 x 15cm column, and the areas - since solutions of constant strength were not being dealt with - of the peaks determined. Further serum samples containing added cholecystokinin were also applied to the column.

B9.3.5 Effect of Trypsin

Samples of serum were digested with Trypsin for 30 minutes at 37°C, these digests were applied to the longer 1 x 60 cm column of Sephadex G50 eluted with Krebs solution directly on to the bioassay system as before.

B9.4 Results and Discussion

Fig. B9.4 shows the proportions of the two cholecystokinins found in the serum of 4 normal healthy volunteers, the height of the columns indicating the peak areas (in arbitrary units) found for 'big cholecystokinin' (above the line) (that area of cholecystokinin

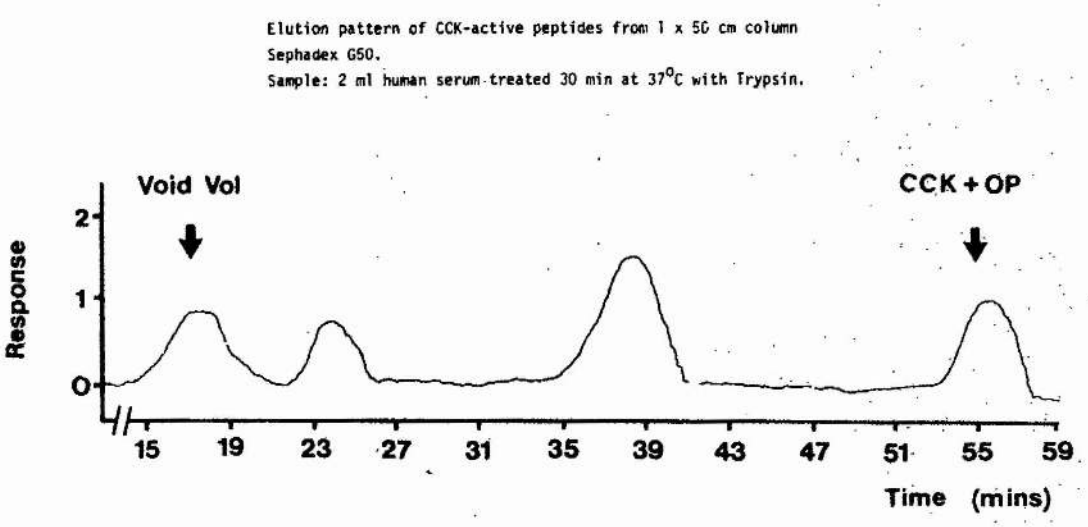

Fig B9.5 Elution pattern of cholecystokinin - like activity, in serum treated with trypsin, from a Sephadex G50 1 x 50cm column.

bioactivity eluted with the void volume of the column) and normal size cholecystokinins (below the line) in the fasting, 15 and 30 minutes post-meal serum samples.

It appeared that in response to feeding there was a reduction in the amount of 'big cholecystokinin' and, in most cases, an increase in the amounts of the normal sized cholecystokinins. At this time it was impossible to say whether this was due to the release of new normal cholecystokinin with a consequent reduction in the amount of 'big cholecystokinin', or if the 'big cholecystokin' was a means of storage or a precursor.

Boots Pancreozymin, the Karolinska Cholecystokinin and the synthetic C-terminal octapeptide do not alter their position of elution when added to serum, it was therefore unlikely that 'big cholecystokinin' is simply normal cholecystokinin attached to a carrier since at least a small increase in 'big cholecystokinin' would be expected after the addition of exogenous cholecystokinin.

Four areas Fig. B9.5 of cholecystokinin-like activity were eluted from the column to which tryptic digests of serum had been applied. Two in the positions already described with the other two between them.

Since it was totally excluded from the G50 gel 'big cholecystokinin' must have a molecular weight in excess of 30,000. The experiment with trypsin encouraged the belief that 'big cholecystokinin' was a protein in its own right with a molecular weight in excess of 30,000 although the possibilities of a polymer or aggregate cannot be ruled out at this stage.

Attempts were made to achieve a separation on a 1 x 15cm column of Sephadex G100, but the flow rate through this column was too slow, this resulted in the final dilution of the column effluent being too great for any cholecystokinetic effects to be seen in terms of a contraction of the gall-bladder. This particular problem may be overcome by collecting fractions and this possibility is being examined.

Chapter B10 Biological Measurement of Cholecystokinin in Clinical Practice

B10.1 Introduction

Much of the physiology of cholecystokinin is known and documented (see Chapter A4); it is known that the main source of cholecystokinin is the upper small intestine, in particular the duodenum, the main target organs are the gall-bladder and the pancreas, and it is firmly established that amongst the stimuli for cholecystokinin release are the presence of acid or fat in the duodenum.

Therefore, since these things were known, it was of interest to look at patients whose metabolism might be altered by disease of, or a surgical operation on, one of the target organs, or a portion of the gut manufacturing cholecystokinin, and to compare these patients with other normal controls.

B10.2 Protocol for Study

Patients were selected and grouped only according to the operation to be performed. Early patients were given a full work up - overnight fast, then blood samples taken while fasting and then 10 min, 20 min, 30 min, 60 min and 120 min, following a standard fatty breakfast consisting of; 1 fried egg, 2 rashers of fried bacon, 2 slices of bread, butter and marmalade, and 1 or 2 cups of tea or coffee with sugar to taste (see Fig. B1.4). This procedure was performed

preoperatively and repeated postoperatively, or postoperatively only (and compared with a control series) at least one month after the operation. In later patients only fasting samples were taken at the two specified times.

B10.3 Controls

B10.3.1 Experimental

To establish the normal range for serum cholecystokinin volunteers assumed to be normal, healthy and with no history of gastrointestinal disease were asked to undertake an overnight fast. A single fasting blood sample was taken from some of the volunteers, others were given a full 'work up' with blood samples also being taken 10 min, 20 min, 30 min, 60 min and 120 min. following a standard fatty breakfast. In a second series fasting blood samples were taken from a different set of volunteers and the blood dispensed into tubes containing 10,000 KIU Trasylol per 10ml of blood.

B10.3.2 Results

Of the two series of controls obtained, only the first series which did not use Trasylol was applicable to the trials described below. The series using Trasylol may be taken as the normal fasting control value expected in the trial detailed in chapter B11.

The mean fasting value of circulating cholecystokinin for normal controls in the first series was 91 ± 27mIDU/ml serum, obtained from 19 volunteers consisting of 10 males and 9 females (range 46 - 135mIDU/ml).

In the second series the mean fasting value was found to be 28 ± 16mIDU/ml serum from a series of 13 volunteers consisting of 7 males and 6 females (range 10 - 75mIDU/ml).

There was no significant difference between the values obtained for male and female volunteers in either of the two groups.

B10.4 Operations By-passing the Duodenum

B10.4.1 The short loop Polya Partial gastrectomy

B10.4.1.1 Operation and method

This operation (see Fig. B10.1) removes the antrum of the stomach then lifts the jejunum and anastomoses this near its junction with the duodenum to the remains of the stomach. This leaves a loop of duodenum into which food will not pass except by reflux. Patients in this group would therefore be expected to show only a small response to food.

Five patients were studied postoperatively as described.

B10.4.1.2 Short loop Polya partial gastrectomy; results

The five patients in this group had fasting cholecystokinin levels below that established as normal and all had only small

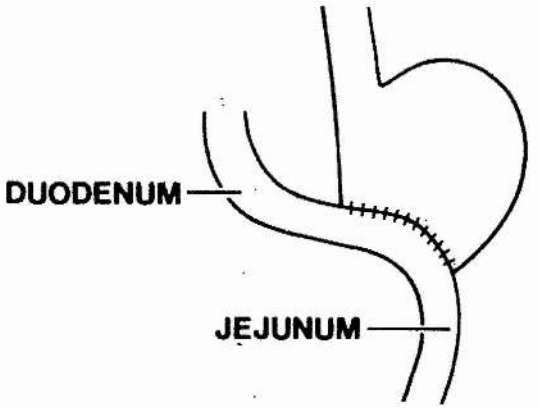

Fig B10.1 Detail of the surgical procedure for short loop Polya partial gastrectomy.

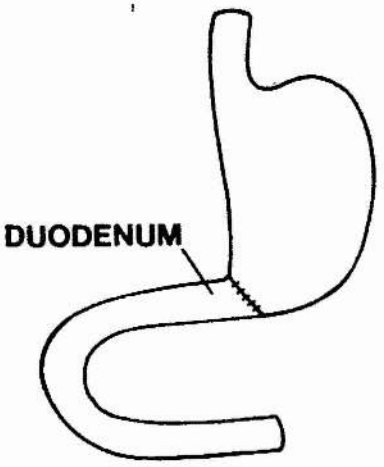

Fig B10.2 Detail of surgical procedure for Billroth 1 gastrectomy.

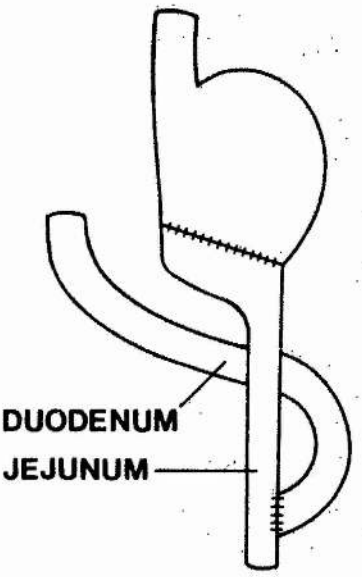

Fig B10.3 Detail of surgical procedure for Roux - en - Y gastrectomy.

increases; (one case no increase) in serum cholecystokinin following a meal (Table B10.1)

B10.4. 2 The Billroth I Gastrectomy

B10.4.2.1 Operation and method

This operation (see Fig. B10.2) removes the same anatomical parts as the short loop polya, but the gastric remnant is connected differently. In this case the antrum and pyloric sphincter are removed from the stomach leaving the fundus and at least part of the body anastomosed to the duodenum. Although the source of gastrin has been removed, and acid secreted from the fundus of the stomach has no real barrier to its passage into the duodenum, it would be expected that these patients should have a more normal response to food than after a polya gastrectomy. A total of seven patients were studied postoperatively as described.

B10.4.2.2 Billroth I Gastrectomy; results

Mixed results were obtained from the seven patients studied in this group (Table B10.2), two patients (1 and 2) had serum cholecystokinin levels above the normal range (see section B10.3.2), three were at the upper limit of normal (3, 4 and 6) and one displayed no activity at all in the fasting state but showed a steady rise in activity to 48mIDU CCK/ml in the first hour following a meal but

Table B10.1

Short loop Polya Partial Gastrectomy

CCK in mIDU/ml serum at time

Patient	Fasting	10 min	20 min	30 min	60 min	120 min	Peak fast
1	58	49	47	40	43	32	0
2	37	34	32	38	24	25	1
3	39	32	36	55	41	39	16
4	41	35	30	54	49	24	13
5	49	73	64	93	93	83	44

Table B10.2

Billroth 1 Gastrectomy Results.

Patient	mIDU/ml serum post-operatively						
	fast	10 min	20 min	30 min	60 min	120 min	peak-fasting
1	269	341	169	162	131	78	75
2	155	200	162	153	189	193	45
3	80	69	99	71	121	99	41
4	53	90	100	50	60	53	47
5	0	28	22	38	48	32	48
6	91	26	101	72	35	38	10
7	23	18	33	29	46	30	24

all showed a significant rise in cholecystokinin activity. The mean rise in cholecystokinin activity following a meal was 41mlDU/ml serum. The variation between patients may reflect different rates of gastric emptying, but the rise in activity contrasts with the polya and is to be expected since in this case the duodenum is stimulated.

B10.4.3 The Roux - en - Y Gastrectomy

B10.4.3.1 Operation and method

This operation (see Fig. B10.3) again removes the antrum, most of the body of the stomach and pyloric sphincter from the patient, but the method of reconnection follows another pattern; the patient is left with the fundus of the stomach anastomosed to the jejunum and with the duodenum, through which bile and pancreatic juice may enter the jejunum, as a closed side piece anastomosed end to side to the jejunum.

The food of patients who have had this operation, and any stomach acid will therefore pass directly to the jejunum and very little will enter the duodenum which is the main source of cholecystokinin and the point of stimulus for its release. These patients would therefore be expected to have low levels of cholecystokinin circulating postoperatively

and a small, delayed response to food.

Three patients who had had this operation were studied postoperatively.

B10.4.3.2 The Roux - en - Y Gastrectomy; results.

The three patients studied in this group had fasting cholecystokinin levels at the upper end of the normal range. They all showed only a slight and very delayed increase in serum cholecystokinin activity at one hour or later following the fatty breakfast (Table B10.3).

B10.5 Operation Affecting Stimulation

B10.5.1 Highly Selective Vagotomy

B10.5 .1.1 Operation and method

This relatively new operation is now commonly performed to treat a duodenal ulcer. The operation involves severing the vagus nerve at specific points so that the acid secreting cells (parietal) of the fundus and body of the stomach are denervated while the nerve supply to the antrum and pyloric sphincter remains intact.

Both fasting and stimulated gastric acid output is reduced, but gastric emptying is nearly normal.

Nine patients were studied preoperatively and five of these were re-examined after their operation.

Table B10.3

Roux - en - Y Gastrectomy

Patient	mIDU/ml serum post-operatively						
	fast	10 min	20 min	30 min	60 min	120 min	peak-fasting
1	81	64	65	51	80	61	0
2	106	88	94	94	112	88	6
3	91	63	38	63	0	115	24

B10.5.1.2 Highly Selective Vagotomy; results.

The complete study was performed on five patients in this group; preoperative fasting serum cholecystokinin levels were all at or above the upper limit of normal and the response to the meal was in some cases excessive (Table B10.4)

Following the operation fasting serum cholecystokinin levels had dropped to well within the normal range, and the response to the meal was also more normal. This difference in fasting cholecystokinin levels was significantly different ($P<0.01$, paired t-test) and this is exactly as would be expected from the performance of this operation.

B10.6 Target Organ Effects

B10.6.1 The Cholecystectomy Operation

B10.6.1.1 Operation and method

This operation simply removes the diseased or stone filled gall-bladder. It was of particular interest to study patients undergoing this operation since the removal of a target organ was most likely to have an effect on serum cholecystokinin levels. In particular an increase in activity of considerable size could be expected to be generated if the observations and theory of Caroli, Plessier and Plessier (1960), that there is an anti-cholecystokinin substance which probably originates in the neck of the

Table B10.4

Highly Selective Vagotomy.

Fasting

Patient	mIDU/ml serum				
	pre-op	peak	post-op	peak	% of pre-op value
1	250	575	75	98	30
2	114	212	49	176	43
3	69		21		30.5
4	77	281	54	161	70
5	75	88	26	45	34.5
6	138	308	90		65

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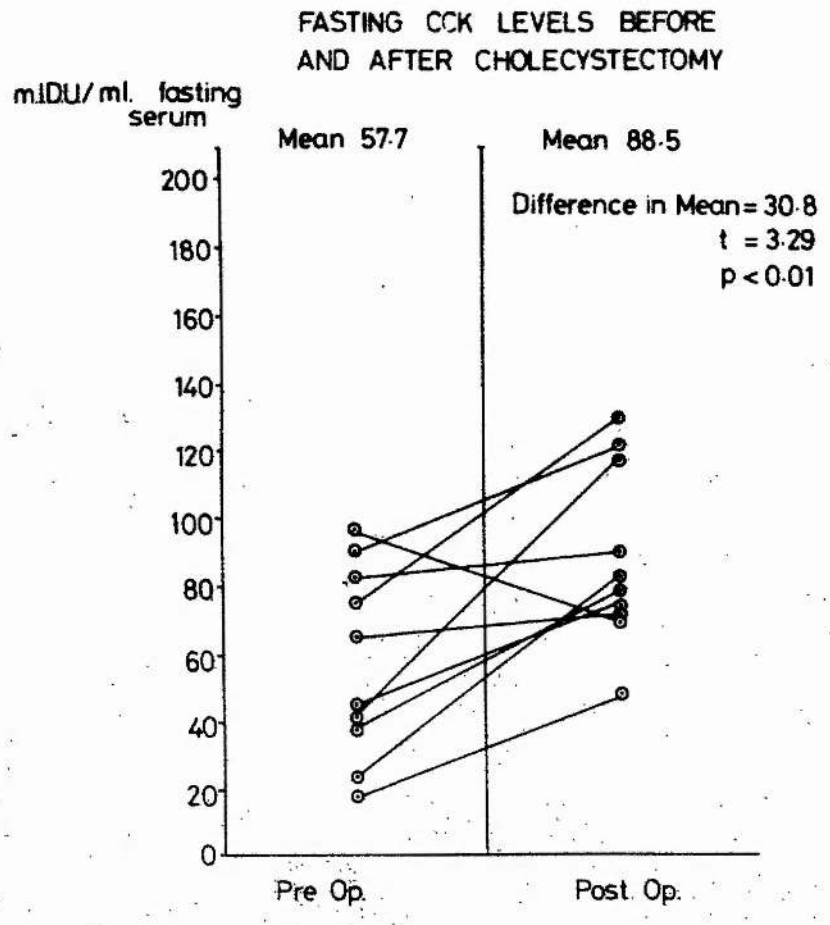

Fig B10.4 Pre- and post - operative fasting serum levels of cholecystokinin in patients having undergone a cholecystectomy operation.

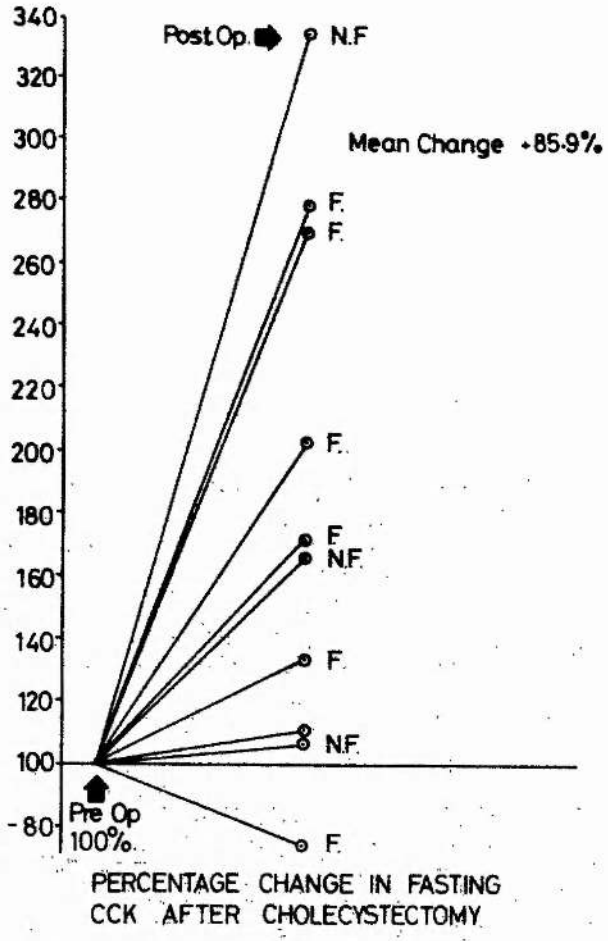

Fig B10.5 Post-operative fasting serum levels of cholecystokinin in patients having undergone a cholecystectomy operation, expressed as a percentage of the pre-operative fasting level. (F = functioning gall-bladder, NF = non-functioning gall-bladder).

gall-bladder, are correct. 17 patients took part in this experiment and were treated as described in section B10.2.

B10.6.1.2 Cholecystectomy; results

In the majority of cases (16 out of 17) an increase in the fasting level of serum cholecystokinin was observed following the operation, the one patient showing a postoperative drop in serum cholecystokinin had cholecystokinin levels within the normal range (section B10.3.2).

The results of this experiment are shown in Figs. B10.4 and B10.5 and also Table B10.5, a statistically significant difference was found ($P<0.001$) for the pre and post operative observations using Students t-test for paired data.

B10.6.2 Chronic Pancreatitis

It would be expected that disease of the pancreas would also have some effect on circulating cholecystokinin levels, and indeed Harvey, Rey et al. (1976) have demonstrated raised serum levels of cholecystokinin in patients with pancreatic disorders associated with deficiency of the pancreatic exocrine system, and they have suggested that this could be of some use in diagnosis.

Table B10.5

Cholecystectomy Results

Patient	mIDU/ml serum	
	pre-op	post-op
1	83	90
2	75	115
3	90	121
4	39	79
5	18	48
6	42	118
7	96	69
8	45	75
9	24	83
10	105	197
11	43	135
12	28	41
13	30	51
14	33	146
15	95	150
16	102	237

Insufficient patients with proven chronic pancreatitis without other complications were available for our study and it has not yet been possible to confirm or refute the findings of Harvey and his co-workers, but Planche, Chau-Huu, Lai and Sarles (1977) could not demonstrate a change in circulating cholecystokinin in chronic alcoholic dogs.

B10.7 Discussion

In these trials it has been demonstrated that following a cholecystectomy operation the fasting level of serum cholecystokinin rises significantly, conversely following a highly selective vagotomy operation fasting serum cholecystokinin levels fall significantly. Results from gastric operations diverting the food supply to other parts of the small bowel were as expected from the anatomical rearrangements tending to show a diminished response but with serum cholecystokinin levels about normal.

We can speculate that this rise in serum cholecystokinin following cholecystectomy is due to the removal of the inhibitor postulated by Caroli et al. (1960) but an alteration to bile salt metabolism could also contribute, while the fall following highly selective vagotomy is clearly a consequence of reduced acid output by the stomach.

These results are interesting and should be persued further, the results demonstrate the extremely complex nature of the hormonal control of the gastrointestinal tract, but are very much as expected and demonstrate that it is possible to detect the predicted physiological changes despite the minute quantities of hormone involved. However one of the major problems with this study is the rate of gastric emptying, this can neither be controlled nor predicted and as a result a late peak from a slow emptier could be missed, although it is felt that the protocol used gave the best chance of seeing a cholecystokinin peak should one occur.

Chapter B11; Cholecystokinin and Obesity

B11.1 Introduction

This experiment arose from the observations of Young, Gibbs et al (1974), that injections of cholecystokinin to rats with gastric fistulae that are sham feeding caused the rats to exhibit all the signs of satiety. It was therefore of interest to study obese patients and look for any significant differences in their fasting serum cholecystokinin levels or the pattern of cholecystokinin release following a stimulus.

Our hypothesis was that hyperphagic obese patients ate continuously and to excess because there was a failure in cholecystokinin output and therefore no satiety signal to tell them to stop eating.

B11.2 Method

Patients were selected and divided into three equal groups, these were: (A)* Normal lean, people with no known gastrointestinal disorders and no previous history of such disorders and of normal weight. (B)* Normal obese; people who were overweight but had a normal appetite and again no knowngastrointestinal disorders or history of such disorders. (C)* Hyperphagic obese; people overweight and with an abnormal appetite, that is compulsive eaters, again no known history of gastrointestinal disease.

* For definition of A,B and C see Appendix E p. 159.

Table B11.1

Cholecystokinin release patterns in normal and obese patients.

Patient	-5	mean	0	mIDU at time - blank +5	10	15	30	45	60	blank	P-F/mn	% rise
Hyperphagic obese												
M	36	44	52	NO sample	56	21	49	37	37	17	5	11
N	60	71.5	83	66	98	68	103	76	145	48	73.5	103
O	92	86.5	81	45	110	90	89	74	68	38	23.5	27
P	126	120.5	115	79	144	124	124	109	102	4	23.5	20
Q	181	167	153	104	173	154	204	196	115	5	37	22
R	188	172.5	157	206	213	154	158	174	184	17	40.5	23
mean	114	110	107	100	132	102	121	111	109	22	33.8	34

Table B11.1

Cholecystokinin release patterns in normal and obese patients.

Patient	mIDU at time - blank											
	-5	mean	0	+5	10	15	30	45	60	blank	P-F/mn	% rise
Normal obese												
G	14	9	4*	37	56	66	19	4*	21	16	57	633
H	14	16	18	102	0	38	23	37	17	16	86	538
I	70	61	52	78	68	52	41	25	60	11	17	28
J	74	66.5	59	151	40	212	161	53	83	29	145.5	219
K	199	180	161	109	125	201	111	69	81	11	21	12
L	234	204	174	263	239	183	254	202	194	5	59	29
mean	101	89	78	123	88	125	102	65	76	15	64.25	243

*single estimate

continued

Table B11.1

Cholecystokinin release patterns in normal and obese patients.

Patient	mIDU at time - blank											
	-5	mean	0	+5	10	15	30	45	60	blank	P-F/mm	% rise
Normal lean												
A	16	13.5	11	20	30	30	33	35	38	15	24.5	181
B	18	20	22	18	31	18	25	19	11	1	11	55
C	75	59.5	44	60	54	103	71	56	48	1	43.5	73
D	112	110.5	109	122	79	120	66	135	100	2	24.5	22
E	133	128.5	124	144	188	145	66	74	269	60	140.5	109
F	284	232.5	181	263	258	232	254	247	212	64	30.5	13
mean	106	94	82	105	107	108	86	94	113	24	45.75	76

continued

All these patients were treated in the same manner; following an overnight fast (nothing to eat or drink or smoke from midnight to the time of the test - around 9 a.m.) 10ml blood samples were taken five minutes before and immediately before (zero time) they were given a liquid meal of approximately 450ml 'Build Up' (Carnation). Further blood samples were taken 5 min, 10 min, 15 min, 30 min, 45 min and 60 min. following consumption of the liquid meal.

The blood was dispensed into tubes containing 10,000 K.I.U. Trasylol per 10 ml blood. After clotting the serum was collected and stored frozen until required.

Cholecystokinin bioactivity in each sample was measured using the final automatic system (section B3.4) and one estimation of 'serum blank' (section B6.9) was made for each patient. The analysis was performed 'blind', that is without knowledge of the group to which each patient belonged.

B11.3 --Results

Individual results are recorded in table B11.1. There was no significant difference between the groups in serum cholecystokinin content in either of the two fasting samples, the peak values, the timing of the cholecystokinin peak following the meal nor in the actual rise inmserum cholecystokinin (P-F/mn), and in no between group comparisons with an unpaired t-test did the value obtained for t correspond to a P value lower than 0.2, i.e P 0.2 on all comparisons.

B11.4 Discussion

Each group of patients exhibited the whole range of values for serum cholecystokinin from the lower end of normal through the upper limit and beyond to that which would be classed abnormal. All patients showed a reasonable response to the meal - as raised serum cholecystokinins - generally within 30 mim and of significant order.

Should cholecystokinin be a satiety signal in human beings (although this has yet to be demonstrated) (Goetz and Sturdevant, 1975) failure of hormone production in hyperphagic obese patients clearly does not occur. If cholecystokinin is to be implicated in this connection it must be as a failure of the receptor site to respond to cholecystokinin and not the failure of the body to produce and release cholecystokinin.

SECTION C

RADIOIMMUNOASSAY

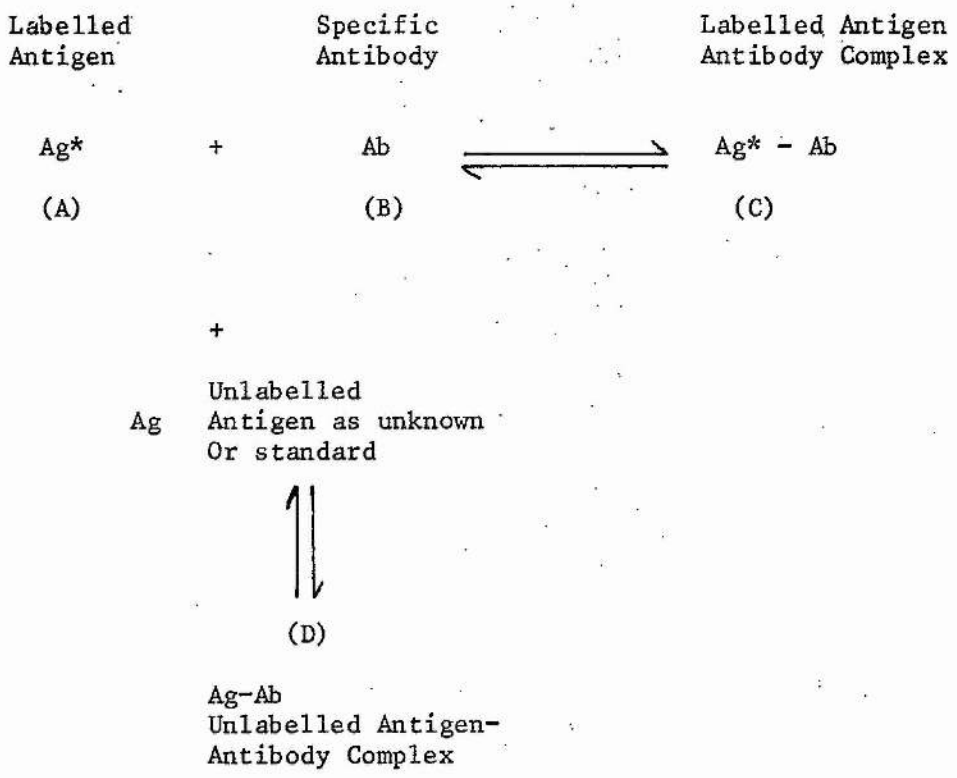
Labelled Antigen
Specific Antibody
Labelled Antigen Antibody Complex
Ag*
+
Ab
Ag* - Ab
(A)
(B)
(C)
+
Ag
Unlabelled Antigen as unknown Or standard
(D)
Ag-Ab
Unlabelled Antigen-Antibody Complex

Fig C1.1

Chapter C1 Introduction

C1.1 Background

Radioimmunoassay is a much more recent arrival in the field of methods for measuring biological molecules - in particular human serum or plasma - than bioassay. The technique appeared as a spin-off from Mirsky's (1952) hypothesis that maturity-onset diabetes might be due to rapid degradation of insulin rather than a secretory deficiency. This led directly to work by Berson, Yalow, Bauman, Rothschild and Newerly (1956) and a demonstration of an insulin-binding globulin in the circulation of insulin-treated subjects. (Yalow and Berson, 1959). There are many radioimmunoassays for other gastrointestinal hormones some of which are commercially available, cholecystokinin is a most notable exception, and has rarely yielded to radioimmunoassay (see Chapter A3.2).

The simplicity of the reaction involved in a radioimmunoassay is summarised below in Fig. C1.1.

These are competing reactions and the amount of labelled antigen that will be bound to the specific antibody depends upon the amount of unlabelled antigen with which it is competing for the antibody binding sites.

The antigen-antibody complex may then, depending on the valencies of the two contributing components (antigen and antibody), formaggregates, with the complexes eventually forming a matrix of sufficient size to precipitate spontaneously. Generally the antigen is multivalent, the valency of antibody being limited to 2 (Eisen and Karush, 1949). This permits the formation of three-dimensional complexes with resultant precipitation. However in certain special cases the antigen may be so small as to be only mono-or di-valent, in these cases it is unlikely that the antigen-antibody complexes would form aggregates of sufficient size to precipitate spontaneously.

Cholecystokinin is one antigen which by its size would not be expected to be more than di-valent and is probably mono-valent. Since separation of A from C (Fig. C1.1) is required for a working radioimmunoassay further treatment of the reacting solutions is necessary. Three main methods of achieving this separation are available (Harvey, Dowsett, Hartog and Read 1974).

(a) Charcoal Separation

Charcoal will absorb free labelled antigen (A) but not antibody-bound labelled antigen (c). Separation of

free and bound labelled antigen can therefore be achieved by mixing a little dextran-coated charcoal (chapter B6.5) with the incubation mixture, removing the charcoal by centrifugation and testing both the charcoal and the supernatant for radioactivity.

(b) Ion exchange resin

A similar effect may be obtained using Amberlite CG-400 ion-exchange resin, this absorbs the free but not the antibody bound labelled antigen. The resin is removed by centrifugation and both resin and supernatant are counted for radioactivity as before.

(c) Use of a second antibody

In this case a second antibody directed against the gamma-globulin of the species used to raise antibodies against the antigen under test is added to the incubation mixture. This may be done either before or after addition of the labelled antigen and the test sera or standards.

In practice methods (a) and (b) have been found unsuitable for cholecystokinin investigations (Harvey et al 1974) and method (c) was adopted for this study.

C1.2 Principle of the working assay

To establish a radioimmunoassay for a given compound antibodies against that compound must first be generated by

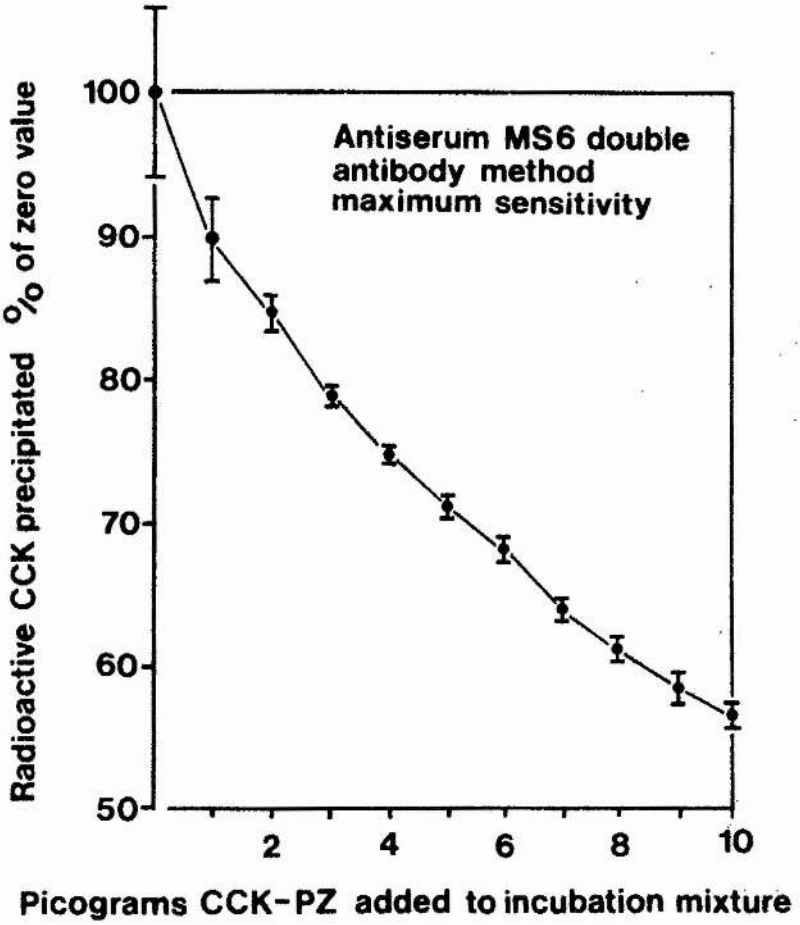

Fig C1.2 Standard curve using a rabbit antiserum with a double antibody separation and ^{131}I - CCK of high specific activity (from Harvey et al., 1974).

injecting the compound into an animal, either in pure form or coupled to a larger modecule e.g. Bovine Serum Albumin to enhance its antigenicity, and time allowed for the immune response to occur.

Blood is taken from the animal, the serum collected and diluted to a concentration suitable for use. To portions of this antiserum are then added a specific amount of the test compound, to which a radioactive tracer has been attached, and graded amounts of the natural (or cold) compound.

After separation of the free radiolabelled compound the amount of radioactivity bound to antibody in each case is determined. A calibration line for the amount of radioactivity bound in the presence of graded amounts of the cold compound is thus obtained.

The amount of the test compound in any unknown test sample can now be determined by reading the amount of cold compound that corresponds to the amount of radioactivity bound by the test samples (see fig. C1.2 for an example).

Chapter C2 Immunisation Procedure

C2.1 Selection of animals

Initial procedures were carried out using young New Zealand White Rabbits. These were eventually replaced with rabbitsof the Old English strain. One series of immunisations was also carried out late in the programme using eight CFY rats and 6 Dunken and Hartley guinea pigs.

C2.2 Treatment of Animals

C2.2.1 Material Injected

At each injection the animals received either 25 Ivy dog units of cholecystokinin (Pancreozymin, Boots Pure Drug Co.) or 75 Ivy Dog Units of cholecystokinin (Karolinska Institute for Gastrointestinal Hormone Research) dissolved 0.5 ml Krebs solution and emulsified with 0.5ml Freund's complete adjuvant (Difco). On some occasions the cholecystokinin was coupled to polyvinylpyrrolidone (M.W. 400,000/British Drug Houses Ltd) to increase the immunogenticity. The coupling was performed according to the method of Assan, Rosselin, Drouet, Dolais and Tchobroutsky (1965): the Cholecystokinin was dissolved in 0.75ml distilled water, mixed with 0.75ml of a 25% solution of polyvinylpyrrolidone (P.V.P.) and after incubation for one hour at +4°C this viscous mixture was injected into the animals.

C2.2.2 Method of Injection

C2.2.2 .1 Rabbits

These animals received (one of the above preparations at each injection;) after the first priming challenge the animals were given a boosting injection 10-14 days later and thereafter further boosting injections at monthly intervals. Initially the animals were injected intradermally at multiple sites - cleared of fur - along the back, approximately 0.2ml being injected at each site, later animals received 0.25ml under each scapula and 0.25ml intramuscularly to both hind legs.

C2.2.2.2 Guinea Pigs

Each guinea pig was injected subcutaneously with Boots or Karolinska Cholecystokinin not coupled to P.V.P., at multiple sites, 0.2ml being injected at each site. Following the initial priming injection a booster injection was given 10-14 days later; thereafter the animals were given booster injections at monthly intervals. Individual animals were recognised by colour code with dye sprayed on their fur.

C2.2.2.3 Rats

The whole 1ml sample of uncoupled Boots or Karolinska Cholecystokinin was injected into the peritoneal cavity of

each rat. Again, following the initial priming injection, a booster injection was given after 10-14 days and thereafter further boosting injections at monthly intervals. Individual animals were identified by coded ear punches.

C2.2.3 Withdrawal of blood from animals

C2.2.3.1 Rabbits

Fourteen days after the fourth injection - to try to catch the peak of the immune response - 10 to 25ml of blood was taken from each animal by ear vein puncture. When the animals were killed they were first anaesthetised with a sub-lethal injection of "Expiral", 80 - 100ml blood was then extracted from each animal by cardiac puncture. Samples of blood from unimmunised control animals were taken at the same time.

C2.2.3.2 Guinea Pigs

Fourteen days after the fourth injection the animals were exposed to CO_2 and 5 ml of blood taken by cardiac puncture while the animal remained unconscious. At death the animals were again made unconscious with CO_2 and 25ml blood taken by cardiac puncture.

C2.2.3.3 Rats

Fourteen days after the fourth injection the animals were anaesthetised with either ether or halothane. Approximately 2ml of blood was taken from each animal via the orbital sinus. At death, up to 8ml of blood was obtained from each animal by cardiac puncture.

Chapter C3 Preparation of radiolabelled cholecystokinin

C3.1 Incorporation of 125 I into the cholecystokinin molecule

C3.1.1 Chloramine T method

Very highly purified (99% pure) cholecystokinin (a gift from Professor Victor Mutt, Gastrointestinal Hormone Research Laboratory, Karolinska Institute, Stockholm) was used for the labelling procedure. The procedure used was a modification of Hunter and Greenwood's (1962) Chloramine-T method, as described by Harvey el at (1974).

1 μg cholecystokinin was dissolved in 200 μl Sorensen's phosphate buffer (see appendix for composition of buffer), 0.05M, pH7.4, the glass vial that had contained the dried cholecystokinin being used as the reaction vessel. 500 μC radioactive iodide as $Na^{125}I$ (Radiochemical Centre Amersham, product IMS 30) and 20 μg of chloramine-T in 10 μl of buffer were added and mixed. The reaction was stopped after 10-20 seconds by the addition of 50 μg sodium metabisulphite in 10 μl phosphate buffer. The reaction mixture was diluted with 200 μl of a solution containing 10mg/ml potassium iodide and was then applied to the top of 1 x 15 cm column of Sephadex G10, or G15, or Biogel P.4 equilibrated with the same phosphate buffer. The column was eluted with the

phosphate buffer and the effluent fed through a coil of microbore tubing attached to a ratemeter detector, the ratemeter itself was attached to an ultraviolet recorder. Two peaks of radioactivity were eluted from the column, one just after the void volume - assumed to be radioactive cholecystokinin - and the other, larger peak - assumed to be unreacted iodide - much later.

C3.1.2 Use of Lactoperoxidase

Some attempts were made to attach ^{125}I to cholecystokinin using lactoperoxidase. The method used was as follows: 200 μC of ^{125}I (as Na ^{125}I, Radiochemical Centre, Amersham, I.M.S.30) was placed in a small plastic tube. 1 μg of cholecystokinin in 400-500 μl Veronal Buffered Saline (V.B.S. see appendix for composition) was added, together with 5 μl Sodium iodide solution 10.6 mg/100ml V.B.S.), 5 μl lactoperoxidase (1 mg/ml V.B.S.) and 5 μl hydrogen peroxide (30% (100 volume) diluted 1:10,000). The tube was shaken and left to stand for 15 minutes when 10 μl of a solution containing 6 mg/ml sodium iodide and 0.03 mg/ml sodium azide were added to stop the reaction.

Since cholecystokinin passes through dialysis tubing the bound and free iodide were separated by applying the reaction

mixture to the top of a 1 x 15 cm column of Sephadex G10 or G15 and eluting the column with V.B.S., Fractions were collected and the positions of elution of radioactive peaks determined by passing the column effluent in front of a detector attached to a ratemeter.

However in a control experiment with no added cholecystokinin, it was found that the same amount of ^{125}I was being incorporated into protein. It was therefore concluded that the lactoperoxidase was attaching the label to itself rather than cholecystokinin.

C3.2 Addition of Tritium to Cholecystokinin

Lack of success with the above two methods was attributed to (a) steric hindrance of iodine addition by presence of the sulphate group on the only tyrosine residue in the cholecystokinin molecule. Substitution of iodine in the positions ortho to the sulphate group - the normal point of substitution for these iodinating reactions would appear to be impossible; (Fig C3.1) (b) breakdown of the cholecystokinin molecule in the severe conditions associated with Chloramine-T oxidation. An alternative milder method of incorporating a radioactive label into the cholecystokinin molecule was therefore sought.

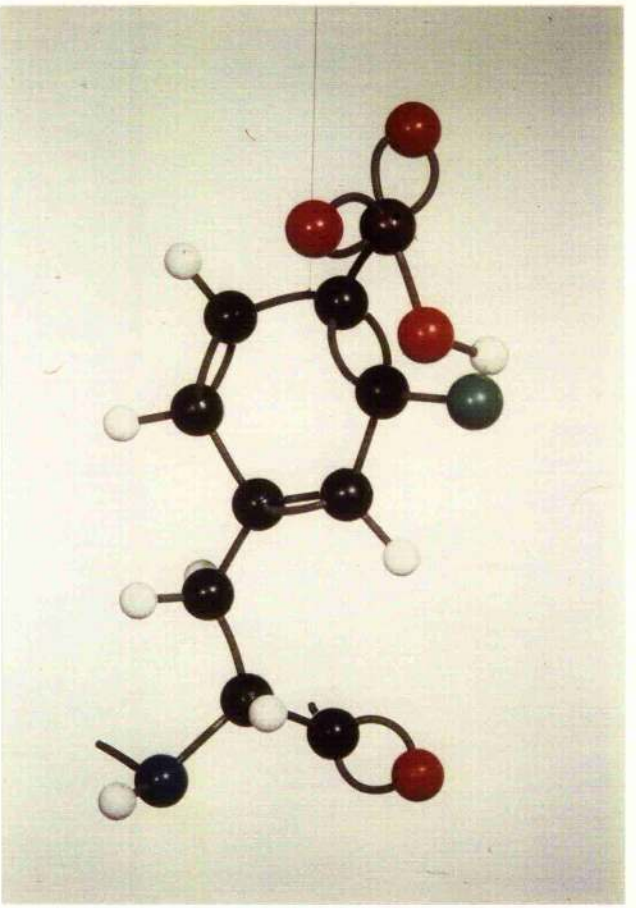

Fig C3.1 Sulphated tyrosine residue showing the theoretical problem of attaching a ^{125}I atom in positions ortho to the sulphate group. Colour code: black = carbon; white = hydrogen; red = oxygen; blue = nitrogen; brown = sulphur; green (not to scale) = iodine.

Chemical modifications of proteins was found to be well described by Means & Feeney (1971) and the method of reductive alkylation of proteins fully described by them three years earlier (Means & Feeney 1968).

They described methods for the addition of several alkyl groups and reported that the reaction appeared to be highly specific for amino groups. Careful examination of acid hydrolysates by amino acid analysis failed to demonstrate the loss of any amino acids apart from those which are N-terminal and also lysine. Although cholecystokinin has an N-terminal lysine, Means & Feeney (1968) state that alkylation of N-terminal lysine was incomplete.

Cholecystokinin has two lysine residues (at positions 1 and 11 - conventional count from N-terminus) and it would seem possible that tritiated isopropyl groups could be added to these positions without too great a loss of antigenic specificity.

Reductive alkylation of cholecystokinin was therefore carried out in the following manner: 1 μg cholecystokinin was dissolved in 500 μl borate buffer at $0^{o}C$ (0.18M, pH9.0, see appendix for composition) containing 10% v/v acetone. The solution was maintained at $0^{o}C$ and a few crystals of tritiated sodium borohydride $NaB(^{3}H)_{4}$ were added directly to the reaction

mixture at approximately ten minute intervals for thirty minutes.

After 40 minutes the reaction mixture was applied to the top of a 1x15 cm column of Sephadex G15 or Bio-Gel P4, and the column eluted with 0.2M acetate buffer pH3.4 (see appendix). 3ml fractions were collected and these were tested for radioactivity by mixing 5 μl of each fraction with 10 ml 'Aquasol' (New England Nuclear Ltd) scintillation mixture and counting on a scintillation counter (Phillips or Nuclear Enterprises). Most of the activity was found to be contained in fractions 3→5. Treatment of these fractions with 3 drops of glacial acetic acid had no effect on the count from these fractions. As any tritium present as borohydride would have been released as tritium gas, it was therefore concluded that all the tritium present in these fractions was bound to cholecystokinin.

Chapter C4 Testing of Antisera.

C4.1 Test using Cholecystokinin Labelled with ^{125}I

C4.1.1 Establishment of amount of second antibody required

Cholecystokinin as already discussed (1.1), is a small molecule, and, as an antigen, is probably only mono-valent and as such would not precipitate spontaneously when mixed with a specific antibody. In order to precipitate the antibody-antigen (CCK) complex a second antibody directed against the -globulin of the species in which the antiserum to cholecystokinin was raised was required.

Before any experiments were performed it was necessary to determine the amount of this second antibody required to precipitate all the γ-globulin in the incubation mixture, since the addition of too little of the second antibody would result in incomplete crosslinkage, while too much of the second antibody would result in formation of complexes of type Ab_x Ag and resolubilization of the precipitated complex.

Dilutions of rabbit serum were prepared at strengths of 1 in 10, 1 in 50, 1 in 100, 1 in 400 and 1 in 800. To tubes containing 100 1 of these solutions were added increasing amounts of donkey anti-rabbit precipitating serum (Wellcome Reagents Limited); these were 5 μl to 30 μl in 5 μl steps and thence in 10 μl steps to 200 μl.

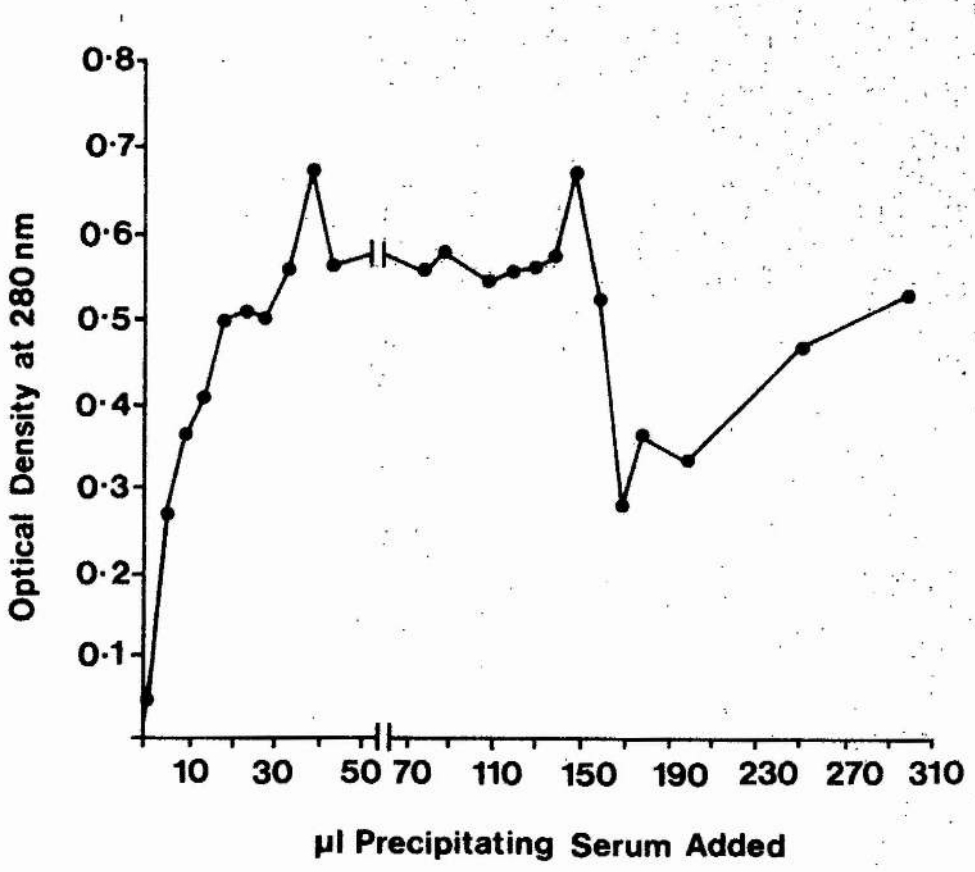

Fig C4.1 Precipitation curve for determination of optimum amount of second antibody to be added to the incubation mixture to ensure complete precipitation of all the rabbit γ-globulin.

These solutions were mixed and incubated at 37°C for two hours and then at 4°C overnight. The tubes were centrifuged in a Microfuge (Beckmann 152A) for 10 minutes. The supernatant was removed and the precipitate washed once with 100 µl distilled water. The tubes were centrifuged again and the supernatant removed.

The precipitate was dissolved in 400 µl of 0.2M sodium hydroxide and the optical density of the solutions at 280 nm determined using 500 µl microcuvettes in an SP1800 ultraviolet spectrophotometer (Pye Unicam Ltd.) The results are shown in Fig. C4.1.

From these results it can be seen that 40 µl of precipitating serum was necessary to ensure complete precipitation of all the rabbit γ-globulins, and that this was sufficient for all dilutions tested.

C4.1.2 Testing Sera for Antibodies using (^{125}I) - CCK and ^{22}Na Volume Marker

A double isotope technique based on that of Gaze, West & Steward (1973) was adopted as standard. All incubations were carried out in the 400 µl Microfuge tubes (Gelman Hawksley Limited) used as reaction vessels.

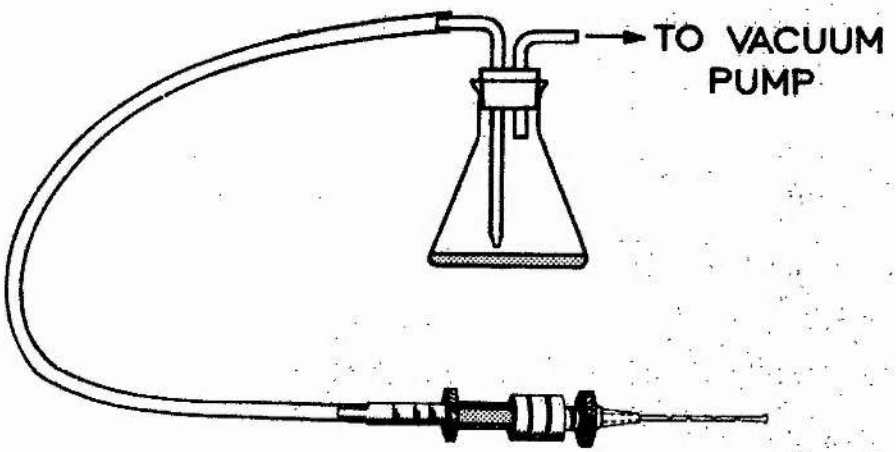

Fig C4.2 Device for removing the majority of the supernatant solution from the incubation mixture.

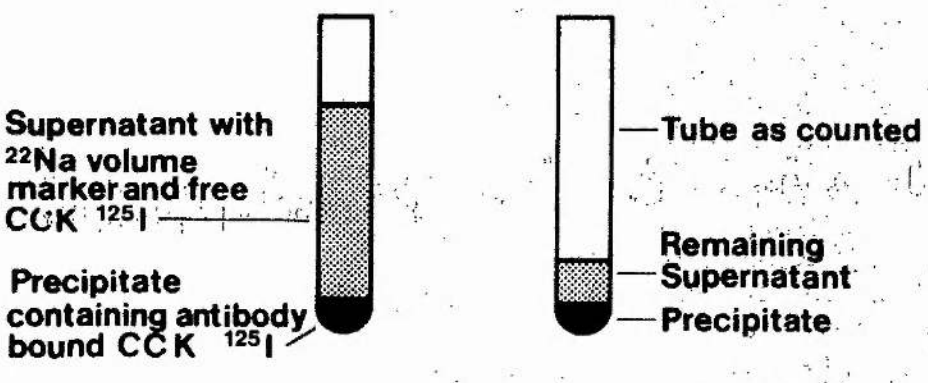

Fig C4.3 Contents of the reaction vessels before and after removal of some of the supernatant solution showing the final contents of the reaction vessel which are then counted for radioactive content.

The tubes for the assay were prepared as follows:

1) 5 tubes containing 10 μl ^{22}Na as $^{22}NaCl$ with an activity of approximately 30,000 counts/minute.
2) 5 tubes containing 10 μl ^{22}Na and 10 μl CCK-^{125}I approximately 10,000 counts/minute.
3) 3 tubes containing 10 μl ^{22}Na, 10 μl CCK-^{125}I plus 100 μl diluted serum from an unimmunised rabbit.
4) 3 tubes containing 10 μl ^{22}Na, 10 μl CCK-^{125}I plus 100 μl diluted test serum.
5) Repeat of (4) for all dilutions of test sera from all animals.

The tubes were capped, mixed on a vortex mixer and incubated at 37°C for 2 hours then overnight at 4°C. Tubes, (3), (4) and (5) were then opened and 40 μl donkey anti-rabbit precipitating serum added, they were then capped and incubated as before.

The tubes were centrifuged in the Microfuge for 10 minutes, the majority of the supernatant was withdrawn by vacuum sucking through the device shown in fig. C4.2. This allowed quick, easy removal of the supernatant with just a small standard amount remaining in the tubes (Fig. C4.3). The tubes were recapped and transferred to the γ-counter (Nuclear Enterprises Limited), the results were printed, and the data recorded on paper tape.

This data was then transferred to the Imperial College London C.D.C. 64,000 computer for processing on its Kronos system. The computer program is recorded in Appendix B, and examples of data presentation are shown in tables C4.1 and C4.2.

C4.2 Testing antisera using CCK-H^3

All the incubations were performed using 400 μl Microfuge tubes as reaction vessels. The experiment was set up as follows:

(A) 2 tubes containing only 10 μl CCK-H^3 solution.

(B) 3 tubes containing 100 μl of diluted serum from an unimmunised rabbit.

(C) 3 tubes containing 100 μl of diluted test sera from each animal.

A complete experiment of tubes A, B and C was prepared for each dilution of the sera that was tested.

To each of the tubes in B and C was added 10 μl CCK-H^3 - calculated to give approximately 20,000 counts per minute - the solutions were mixed and the tubes were then incubated at 37°C for 2 hours, followed by overnight (16 hours) at 4°C. To each of the tubes in B and C was then added the correct amount for that serum dilution of donkey anti-rabbit precipitating serum, the solutions were again mixed and incubated as before.

Table C4.1

Raw data as fed to the computer

Identity.	time.	^{125}I count.	^{22}Na count.	spare channel.	
J01Y	375.60	10000	160581	160318)	
				)	
J02Y	374.43	10000	162964	162636)	^{22}Na only

J06Y	67.53	10000	32663	32602)	
				)	
J07Y	66.82	10000	25332	25269)	^{22}Na + Total ^{125}I

J11Y	243.94	10000	23026	22989)	
				)	Experimental tubes
J12Y	218.43	10000	23987	23945)	

Table C4.2

Presentation of results by the computer.

NA22 in I125 Channel 1605

NA22 26063

SPill .0616

Total NA22 25705

Total I125 CCK counts per tube 7432

Percent Bound Tube 1 = 8.17

Percent Bound Tube 2 = 7.89

After incubation the tubes were centrifuged for 10 minutes in the Microfuge, the supernatant was removed and the precipitate washed in 200 µl of cold Veronal Buffer Saline. The tubes were again centrifuged for ten minutes and the supernatant removed.

To all tubes B and C was then added 100 µl 'Soluene' (Packard Limited) and the tubes incubated at 37°C for two hours. At the end of 2 hours a visual check was made for the complete solution of the precipitate. The contents of each tube were then thoroughly mixed with 10 ml scintillation fluid (Aquasol, New England Nuclear Limited) and the radioactivity in each tube determined on either the Phillips or Nuclear Enterprises Scintillation Counters.
(Phillips Liquid Scintillation Analyser PW4500, Nuclear Enterprises Automatic Sample Counter NE8312).

Chapter C5 Processing of counter results

C5.1 Results from experiments with ^{125}I and ^{22}Na.

C5.1.1 Introduction

Results were obtained from the Nuclear Enterprises machines (Automatic Sample Counter NE8311 or 8312) in the format shown in table C4.1. The first column carried the tube identification number, the second the time to complete the count, the third the number of counts in the ^{125}I channel, the fourth the number of counts in the ^{22}Na channel, while the fifth column carried no relevant information. All this data was obtained simultaneously on punched paper tape for transfer to the computer.

C5.1.2 Transfer of data to the computer

A special file named CCKDT was created in the NULL mode and the data transfered to it via the teletype tape reader. After transfer the file was listed and checked for any inaccuracies in the transfer. Blank lines and errors were edited from the file which was then stored ready for processing.

C5.1.3 Data processing

The calculation of results was performed as in appendix C by the Fortran program CCK1 (see appendix B). This was called from store, the computer was instructed to fetch and then process the data contained in the file CCKDT.

The results appeared in the format as in table C .2, at no time did the amount of iodine bound by a test serum exceed that bound by the control serum, this would be due to either (a) lack of a specific antibody or (b) failure to obtain a labelled antigen (see chapter C6).

Chapter C6 Problems with the radioimmunoassay

One of the main hindrances to this project has been a lack of material; animals have been injected with one or other of the two commercially available preparations of cholecystokinin (Boots Pancreozymin or Karolinska Cholecystokinin). Neither of these preparations contains more than 10% - 20% of cholecystokinin, a maximum of only 8.3μg or 26 μg per vial respectively. The vials are marked in activity units, and, since it is not known which form of cholecystokinin (i.e. whole molecule or fragments) is contained in the vial, the weight of cholecystokinin contained, and the degree of antigenicity and immunogenicity would be considerably less if the vials contained only cholecystokinin fragments.

To be really sure of obtaining specific antibodies the animals should be injected with milligram quantities (Schlegel and Raptis 1976) of cholecystokinin. These however are just not available, and as the cost of each vial of Karolinska cholecystokinin was just over £11, some financial restriction was clearly on the project.

A limited amount of 99% pure cholecystokinin had been obtained as a gift from Professor Victor Mutt of the Karolinska Institute and it was this material, 1 μg at a time, that was

used to try to obtain the radioactive antigen.

The process of attaching the ^{125}I tracer to the cholecystokinin molecule was carried out according to the method described by Harvey et al (1974), but at the separation stage the first peak of radioactivity was always small, sometimes passing unnoticed. Neither the Chloramine-T nor the lactoperoxidase methods seemed to work properly,,this despite claims to the contrary by other workers (Harvey et al., 1974, Schlegel and Raptis, 1976). On occassions when two peaks of activity were obtained, the activity of the first peak, when rechromatographed only three days later, had all moved to the position of elution of the second, free iodide, peak of activity. Close examination of the atomic structure of the cholecystokinin molecule revealed that with the sulphate on the tyrosine residue it was most likely that steric hinderance would prevent iodination of the tyrosine ring in the positions ortho to the sulphate, and that any iodination that did occur would most likely be unstable.

A similar situation occurred with the attempts to label cholecystokinin with tritium; at the separation stage only one major peak of radioactivity could be detected. This could be explained only by: (a) the reaction having gone to completion or, (b) the reaction not having occurred at all, although the

failure to eliminate free tritium with acetic acid suggests that the reaction did occur.

In neither case could any binding to a cholecystokinin antibody be demonstrated, but it is still not clear whether this was due to (a) a lack of antibody, (b) failure to obtain a labelled antigen, or (c) a combination of both. This problem must be resolved as it is clearly important to have both the radioimmunoassay and the bioassay running side by side to obtain the full picture of any physiological changes that may occur in the system under investigation.

It seem, however, unlikely that the problems will be resolved until there is a plentiful supply of pure cholecystokinin from either synthetic or natural sources. This will facilitate experiments with different labelling techniques and will ensure an adequate supply of cholecystokinin for chemical modification to increase immunogenicity before injection to animals.

SECTION D

GENERAL DISCUSSION

General Discussion

This project started with a working manual bioassay for cholecystokinin capable of producing only a maximum of twelve single results - six standards and six test solutions - each day. This assay has been developed until it is almost fully automatic and capable of producing up to forty eight results in duplicate - a total of ninety six results - each day.

From being a purely research tool making slow progress, the technique has been developed until it forms a useful routine tool; large numbers of samples can be processed fairly quickly and a series of investigations can now be completed in a relatively short space of time.

A number of anomalies in the original assay have been investigated and the technique modified to take these into account, either compensating for or eliminating them. The first to come to light was the increase in sensitivity of the gall-bladder to standard solutions of cholecystokinin over a period of several hours. This clearly affected the value obtained for any unknown, the first dose-response curve would have a relatively shallow slope, unknowns processed later would thus tend to have a much greater cholecystokinin content attributed to them than was in fact the case.

Almost simultaneously came the discovery that a solution of diluted serum would increase the sensitivity of the gall-bladder to its maximum immediately. This effect had been suspected during the operation of the manual technique, but it was not until the system was automated that its significance was appreciated and a proper trial undertaken.

As a result of these findings on the change in sensitivity of the gall-bladder the technique was modified so that the first samples presented to each gall-bladder were: (i) a 5mIDU/ml dose of standard cholecystokinin which from experience is unlikely to be exceeded by any unknown, (ii) and (iii) 2ml of pooled serum diluted to 100ml. The first gave some idea of the sensitivity of the gall-bladder and whether or not any adjustment was likely to be needed in the transducer's counter balance, the second and third ensured that the gall-bladder was properly primed, that its sensitivity was maximal, before the samples making the calibrating dose-response curve were applied.

While investigating possible reasons for the high values obtained for serum cholecystokinin compared with

those quoted for other gastrointestinal hormones (we had ng/ml instead of pg/ml) it was realised that if any degradation of cholecystokinin took place during processing and storage it was possible that a significant amount of cholecystokinin activity could be generated in vitro since some of the C-terminal fragments of cholecystokinin are considerably more active on a molar basis than the full molecule. This theory was tested by adding Trasylol (aprotinin, Bayer Pharmaceuticals Ltd.) to the blood samples. In many cases where control samples were taken with the experimental sample this had a dramatic effect upon the apparrent cholecystokinin content of a serum sample and was from that time adopted as standard, all blood samples being dispensed into 10,000 KIU Trasylol per 10ml blood.

Finally in an experiment to compare the results from a radioimmunoassay with those from our bioassay it became apparent from the regression line that the bioassay was measuring something the radioimmunoassay was missing. An experiment with dextran-coated charcoal confirmed that a larger, or non-peptide, substance in serum did possess cholecystokinin activity. This activity was found to be

constant for any individual over the period of our tests but to vary widely from individual to individual. It is now standard practice to measure this 'serum blank' and to subtract its value from the total cholecystokinin activity of that sample.

This work has confirmed that cholecystokinin acts directly on the gall-bladder tissue, and shown that the effects of other gastrointestinal hormones are not sufficiently significant to affect the assay thus despite some interferance by the hormones it may still be said with confidence that the system is specific for cholecystokinin.

With the discovery of a 'big cholecystokinin' by chromatographic means we must speculate as to whether or not it is this big molecule that forms the activity we have termed the 'serum blank'. This seems the most likely explanation despite the fact that digestion with trypsin did not release small, more active, fragments. The release of these fragments would in fact only take place if the nature of the large molecule was such that the C-terminal end of the cholecystokinin was free and exposed; under other circumstances, although the trypsin might split the molecule an increase in activity would not necessarily take place.

The idea of a 'big cholecystokinin' falls nicely into place beside 'big' and 'big, big' gastrins (Rehfeld et al. 1974), and although firmly established as existing, the structure and functions of these molecules, apart from 'big' (G34) gastrin, are not yet known. It is possible that 'big' cholecystokinin is a precursor form being attached perhaps to a glycoprotein in a manner similar to ACTH (Eipper, Mains and Guenzi, 1976)* It's apparent reduction during the period following a meal would support the precursor idea, but whether it is a peptidoglycan or a simple polymer is not yet known and requires further investigation.

* or another peptide in a manner similar to proinsulin (Steiner, Clark, Nolan, Rubenstein, Margoliash, Aten and Oyer, 1969).

The theoretical simplicity of setting up a radioimmunoassay for a given substance is well documented; inject an animal with the substance, harvest the antibody containing serum, attach a radioactive tracer atom to the pure substance and react with the antibody containing serum. This simple theory has not worked at all with cholecystokinin; one of the major problems is isolating

which of the above steps has failed. It would indeed appear very like a 'chicken or the egg' situation: to test for an antibody we must have a labelled antigen, but at the same time, to be sure that we have a labelled antigen we must possess a specific antibody to the antigen.

We are not unique in finding problems with a radioimmunoassay for cholecystokinin as similar problems have already been reported (Rehfeld, 1978), and it must be expected that until adequate supplies of cholecystokinin at a reasonable price become available, these difficulties will remain with us.

The results from the clinical investigations, while inconclusive in several cases, have nevertheless been extremely interesting: Thus clear evidence has been obtained that fasting serum levels of cholecystokinin rise following a cholecystectomy operation. The reason for this is not known although Caroli, Lemonnier, Charpentier and Plessier (1963) have suggested that a cholecystokinin inhibitor is manufactured in and released from the mucosa of the neck of the gall-bladder. There is little supporting evidence for this theory which would however provide a very simple explanation for our findings; the removal of the source of

an inhibitor would naturally lead to an increase in the activity of the original molecule.

However it could also be said that with the removal of a target organ the body is trying hard to produce the same effect - perhaps by stimulating bile flow (something cholecystokinin is not believed to do efficiently) - and achieve an efficient emulsification of fat within the duodenum. With the loss of the storage organ, increased normal flow would be the natural way to maintain normal conditions.

That the opposite should occur when a highly selective vagotomy operation is performed is not at all surprising. This operation is usually performed to correct an ulcerative condition, and ulcers have often been caused by irritation and erosion of the gut mucosa by an excess of acid. Acid has already (chapter A4.2) been shown to be an excellent stimulator of cholecystokinin release, thus a person producing an excess of stomach acid would be continuously passing this to the duodenum and stimulating cholecystokinin release.

Severing the vagus nerve at specific points cuts off the impetus for gastrin release and, as a direct result

the amount of stomach acid released is reduced. This reduces the irritation to the gut mucosa and allows the ulcer to heal; at the same time the continuous stimulus for cholecystokinin release is cut off and fasting levels of serum cholecystokinin drop.

In other clinical conditions we have seen how the response to a meal is slow and small following Roux - en - Y operations, but the trend in other gastric operations is as yet unclear. It has been shown in the case of hyperphagic obese patients that their abnormal appetites are not due to failure of cholecystokinin release, but must, if cholecystokinin is to be implicated at all, be due to a failure at the receptor sites.

There is also some evidence (Monk, Johnson and Marshall, unpublished results) that the level of serum cholecystokinin can be correlated with the diameter of the pyloric sphincter. It was often found that patients with low fasting levels of cholecystokinin had relaxed and open pyloric sphincters through which investigative tubes could be pushed with ease. In patients with high fasting levels of serum cholecystokinin the pyloric sphincter was frequently shut and contracted so tightly that it would not permit the passage of investigative tubes.

This thesis has therefore presented two methods of estimating cholecystokinin in human serum and has examined the problems involved in both methods. Some of the problems have been solved, others remain to be solved so that the two assays can be set to work in parallel.

Some clinically interesting conditions have been examined and several indications have been obtained. It is clear from the diverse reports found in the current literature that far from being completely, documented the cholecystokinin story is probably only just beginning, unlike other gastric hormones, e.g. gastrin, cholecystokinin has only been characterised in one species. It will be most interesting to see how much sequence variation occurs in other species.

A few years ago cholecystokinin was credited with only two target organs - the gall-bladder and the pancreas - more recently it has been associated with pyloric control, gut - in particular colonic - motility, and has also been implicated as a sateity signal. Its commercial availability has led to its use in cholecystogram studies.

A lot is still to be discovered about this hormone, its functions and relationship with the other gastrointestinal hormones. It is probable that cholecystokinin will be found to influence several more body organs before the full story is known. In the meantime one of the first priorities must be to establish the tools with which to carry out the investigations, and this means the establishment of a reliable and highly specific radioimmunoassay to work alongside the automated bioassay described in this thesis.

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APPENDIX A - BUFFER SOLUTIONS

1). Krebs Balanced Salts Solution pH 7.4

$MgSO_4.7H_2O$.	0.29g
Glucose	1.00g
NaCL	6.90g.
KCL	0.35g
KH_2PO_4	0.16g
$NaHCO_3$	2.10g
$CaCL_2 6H_2O$	0.55g

The above quantities were dissolved in distilled water to 1 litre.

2). Sorensen's Phosphate Buffer 0.05M, pH 7.4

Stock Solutions: A; 1/15M KH_2PO_4 = 9.08g KH_2PO_4/litre

B; 1/15M Na_2HPO_4= 9.48g Na_2HPO_4/litre

for pH 7.4 buffer add 19.6 ml A to 80.4 ml B

3). Veronal Buffered Saline (V.B.S.)

Stock Solutions: A; Sodium Barbitone 20.62g/litre

B; 0.1N Hydrochloric Acid

C; 0.01M Sodium Chloride

for buffer pH 7.6 add 60 ml A to 30 ml B and make to 1 litre with C

4). Borate buffer 0.2M pH9.0

Stock Solutions: A; 0.4M Boric Acid in 0.4M KCl.

(24.8g H_3BO_3 + 39.84 KCl per litre)

B; 0.1N Sodium Hydroxide

for buffer pH 9.0 add tO ml A to 21.3 ml B and dilute to 100 ml.

5). Acetate Buffer 0.2M pH3.6

Stock solutions A; 0.4M Acetic Acid (24g/litre)

B; 0.4M Sodium Acetate (16.4g/litre

CH_3 COONa, 27.2g/litre CH_3 COONa. $3H_2O$

For buffer pH 3.6 add 46.3 ml A to 3.7 ml B and dilute to 100 ml.

APPENDIX B - COMPUTER PROGRAM

```
 200 PROGRAM CCK1 (INPUT, OUTPUT, CCKDT, TAPE5=INPUT, TAPE6=
                   OUTPUT, TAPE 7=CCDT)
 250 DIMENSION ATOTS (5), SODS (4)
 300 1 FORMAT (5X,*NUMBER OF SETS OF DATA THIS RUN*)
 350 2 FORMAT (I2)
 400 3 FORMAT (1X, I2, 1X,F8.2,F7.0,F7.0)
 450 4 FORMAT (5X,*NA22 IN I125 CHANNEL *, F7.0)
 500 5 FORMAT (5X,*NA22,F7.0)
 550 6 FORMAT(5X,*SPILL*,F6.4)
 600 7 FORMAT(5,*TOTAL NA22*,F7.0)
 650 8 FORMAT(5X,*TOTAL I125 CCK COUNTS PER TUBE*,F7.0)
 700 9 FORMAT(5X,*PERCENT BOUND TUBE*,I3,*=*,F6.2)
 750 WRITE(6.1)
 800 READ(5.2)INOM
 850 IF(INOM.GT.90)GOTO.600
 900 READ(7.3)IL(ATOTS(1),I=1,3)
 950 DO 500 I=1,INOM
1000 IT=0
1050 DO 100 J=1,4
1100 100 SODS(J)=0.0
1150 DO 200 J=1,5
1200 IF(IL.GT.5)GOTO 250
```

```
1250 ATOTS(2)=ATOTS(2)/ATOTS(1)
1300 SODS(1)=SODS(1)+ATOTS(2)
1350 ATOTS(3)=ATOTS(3)/ATOTS(1)
1400 SODS(2)=SODS(2)+ATOTS(3)
1450IT=IT+1
1500 READ(7.3)IL,(ATOTS(K),K=1.3)
1550 200 CONTINUE
1600 250 SODS(1)=60/IT*SODS(1)
1650 SODS(2)=60/IT*SODS(2)
1700 WRITE(6.4)SODS(1)
1750 WRITE(6.5)SODS(2)
1800 SPILL=SODS(1)/SODS(2)
1850 WRITE(6.6)SPILL
1900 IT=0
1950 DO 300 J=1,5
2000 IF(IL.GT.10)GOTO 350
2050 ATOTS(3)=ATOTS(3)/ATOTS(1)*60
2100 SODS(3)=SODS(3)+ATOTS(3)
2150 ATOTS(2)=ATOTS(2)/ATOTS(1)*60-SPILL*ATOTS(3)
2200 SODS(4)=SODS(4)+ATOTS(2)
2250IT=IT+1
2300 READ(7,3)IL,(ATOTS(K),K=1,3)
2350 300 CONTINUE
```

```
2400 350 SODS(3)=SODS(3)/IT
2450 SODS(4)=SODS(4)/IT
2500 WRITE(6,7)SODS(3)
2550  WRITE(6,8)SODS(4)
2600 400 ATOTS(3)=ATOTS(3)/ATOTS(1)*60
2650 ATOTS(2)=ATOTS(2)/ATOTS(1)*60-SPILL*ATOTS(3)
2700 ATOTS(2)=ATOTS(2)*SODS(3)-SODS(4)*ATOTS(3)
2750 ATOTS(3)=SODS(4)*(SODS(3)-ATOTS(3))
2800 ATOTS(2)=ATOTS(2)/ATOTS(3)*100
2825 IL=IL-10
2850 WRITE(6,9)IL,ATOTS(2)
2900 READ(7,3)IL,(ATOTS(J),J=1,3)
2950 IF(IL.LE.10)GOTO 500
3000 GOTO 400
3050 500 CONTINUE
3100 600 STOP
3150 END
```

APPENDIX C - CALCULATIONS

1. Double Isotope technique using ^{22}Na and ^{125}I

With two isotopes the possibility of a spill of counts from one channel to the other must be examined. At the levels of ^{125}I used the spill of ^{125}I counts into the ^{22}Na channel was around 0.3% and not considered significant. The spill from the ^{22}Na channel to the ^{125}I channel was however of the order of 5% and was corrected as follows (based on Gaze, West and Steward, 1973).

Total ^{125}I counts added per tube corrected for spill;

$$I = I' - \frac{NX}{y}$$

where I' = Total counts in ^{125}I channel including spill

N = Total count in ^{22}Na channel

X = Counts in ^{125}I channel in tubes containing ^{22}Na only

Y = Counts in ^{22}Na channel in tubes containing ^{22}Na only

counts in ^{125}I channel of experimental tubes corrected for spill $I = I' - \frac{Nx}{y}$

Where I' = counts in ^{125}I channel of experimental tube including spill

N = counts in ^{22}Na channel of experimental tube.

then% antigen bound = $\frac{(NI - NI)}{I(N - n)} \times 100$

APPENDIX D

ADDITIONAL FIGURES

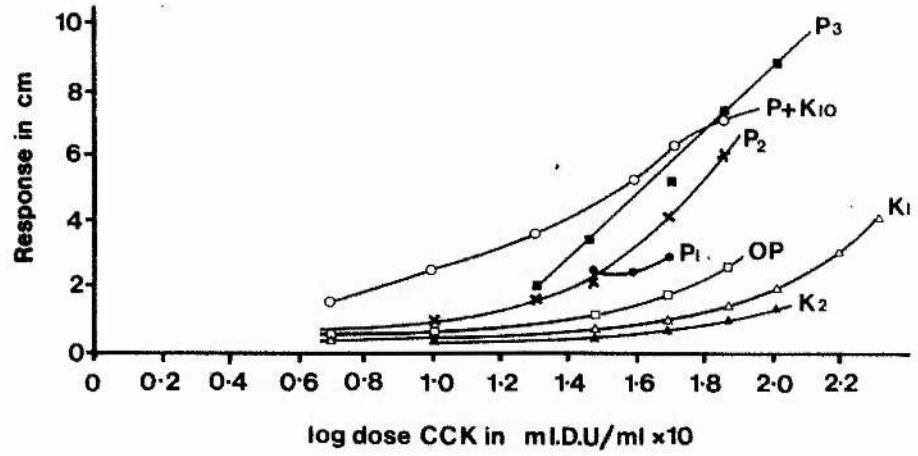

Fig AP1 Dose-response curves on linear axes for different preparations of cholecystokinin

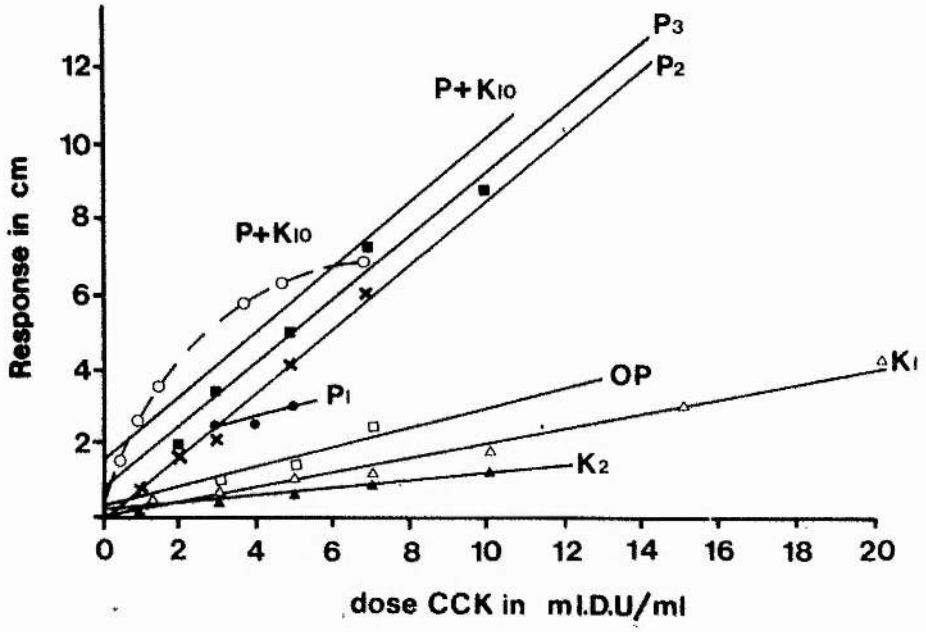

Fig AP2 Dose-response curves on log-linear axes for different preparations of cholecystokinin

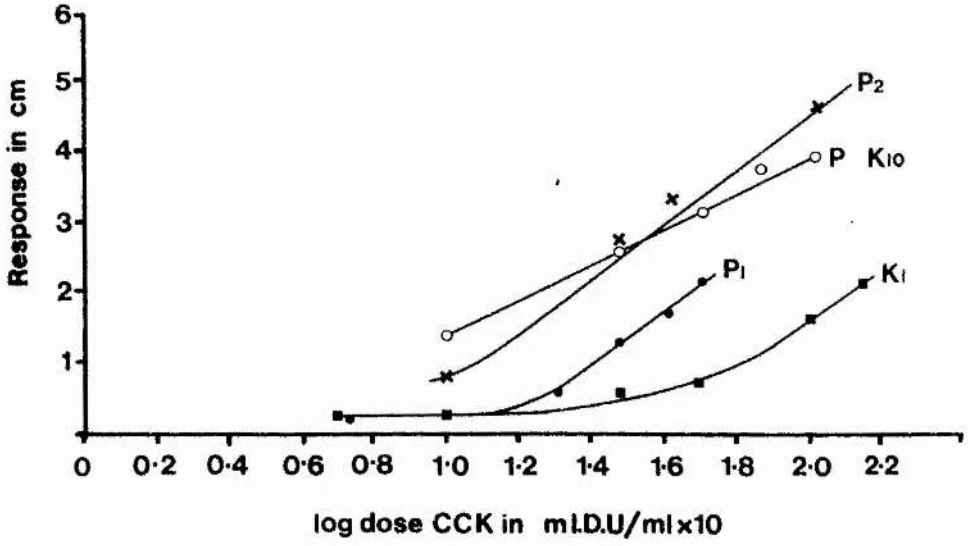

Fig AP3 Dose-response curves on linear axes for different preparations of cholecystokinin

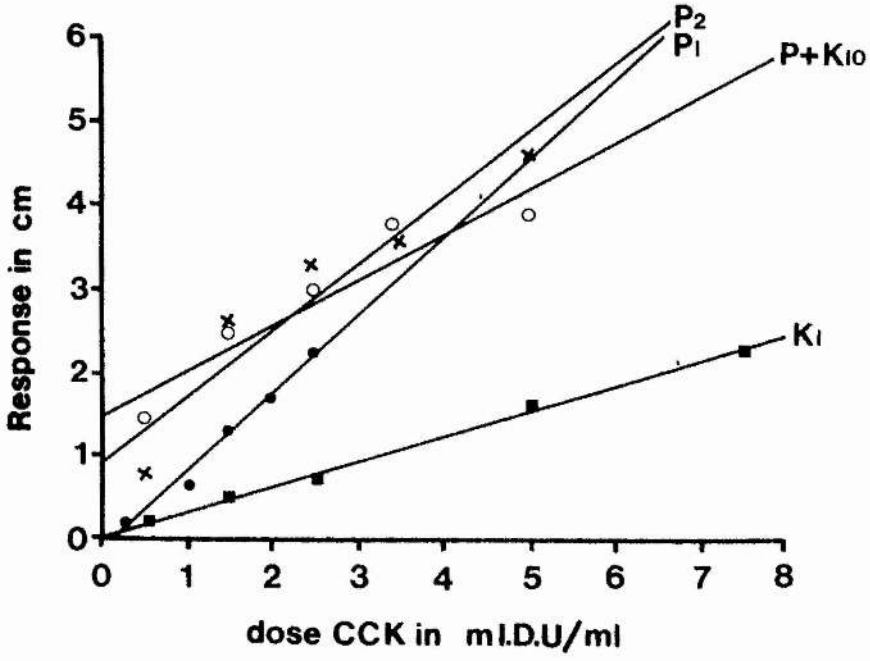

Fig AP4 Dose-response curves on log-linear axes for different preparations of cholecystokinin

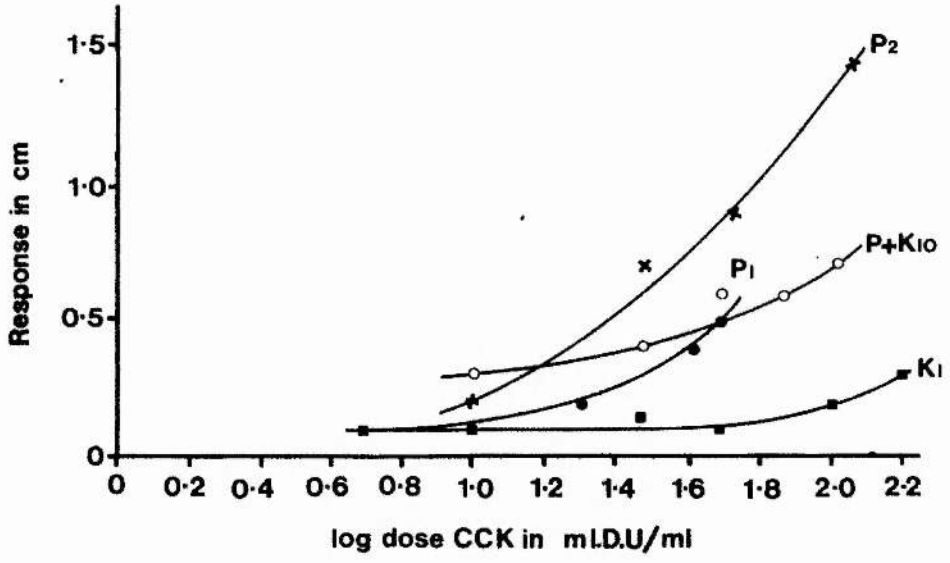

Fig AP5 Dose-response curves on linear axes for different preparations of cholecystokinin

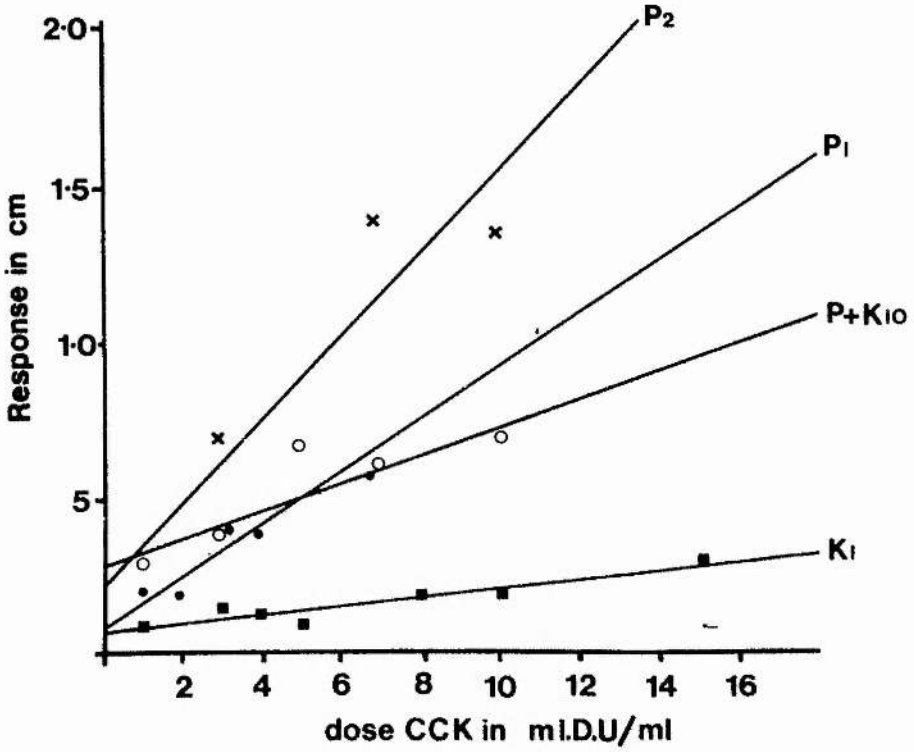

Fig AP6 Dose-response curves on log-linear axes for different preparations of cholecystokinin

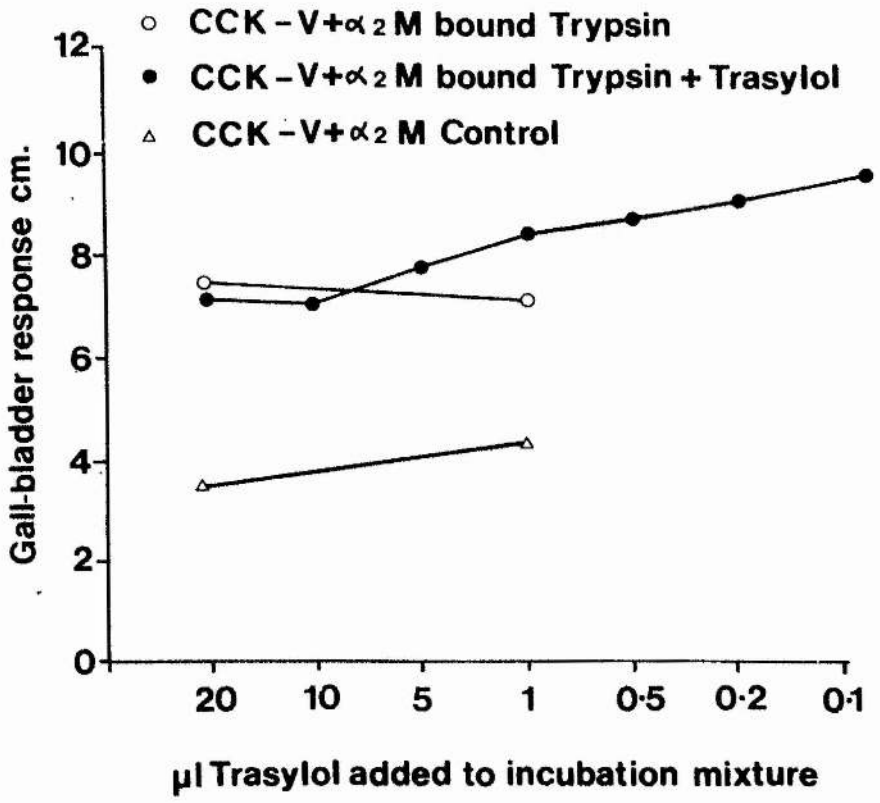

Fig AP7 Effect of aprotinin (Trasylol) on the degradation of cholecystokinin by α_2 - bound trypsin

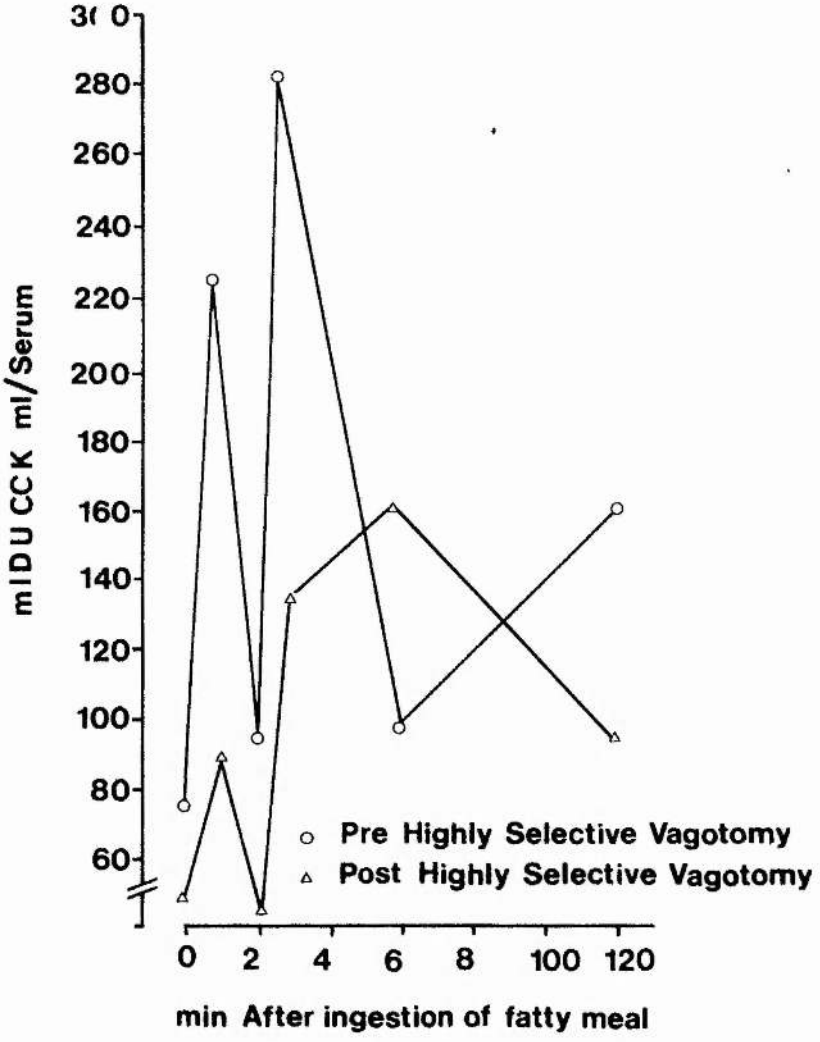

Fig AP8 Time course of release of cholecystokinin following ingestion of the standard fatty meal before and after a highly selective vagotomy operation. Single measurements on one patient.

APPENDIX E

Definition of Normal, Obese and Hyperphagic Patients

1. A normal patient was a normal weight subject with a weight around the ideal i.e. from 20% below ideal weight to 19% above ideal. Ideal body weight was defined as that corresponding to the mid-point of the weight range for medium frame size as listed in the weight for height tables of the Metropolitan Life Insurance Company[1].

2. Obese subjects are those with a weight 20% or more above ideal weight.

3. Intake of food was assessed by dietary recall with weighing of food once per week. A normal appetite was based in the recommended figures for intake as listed in the Government figures: FAO Standards[2]. Abnormal intake was a two fold or greater intake than the standard figure for age, sex, occupation and height.

References

1. Metropolitan Life Insurance Company, New York (1960) Statistical Bulletin 41, Feb. p.6, March p.7.

2. Energy and Protein Requirements: W.H.O. Technical Report Series, No. 522 1973. Report of a joint FAO/WHO Ad. Hoc Expert Committee: Geneva.