A METHOD OF BIOLOGICAL ASSAY OF GASTRIN AND ITS APPLICATION TO THE STUDY OF HUMAN TISSUES

by

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PART I

GENERAL INTRODUCTION

1.1 The 'Gastrin' Hypothesis

In 1905, Edkins put forward the concept of a humoral mechanism in the physiological control of gastric secretion. He suggested that food in the stomach led to the release of a substance from the mucosal cells into the blood stream through which it reached the gastric glands and stimulated them to secrete. The following observations were cited in support of this hypothesis: (Edkins, 1906).

(a) Simple extracts of pig's or cat's antral mucosa, when given intravenously to anaesthesized cats, provoked gastric secretion.

(b) These extracts also possessed vasodepressor activity.

(c) The active principle, which he termed 'gastrin', was found in the antral and cardiac but not the fundic regions of the stomach. It resisted boiling and was therefore not a ferment.

This report was followed by a series of investigations by various workers, aimed at (a), elucidation of the physiological mechanism involved and (b), further clarification of the nature of 'gastrin'.

Sokolov (1904, quoted by Babkin, 1928, 1934) worked on conscious dogs provided with a vagally

innervated Pavlov pouch, a duodenal and a gastric fistula, and, in addition, a mucosal septum at the pyloro-duodenal junction, the myenteric nervous plexeuses remaining intact. Secretion from the Pavlov pouch occurred when meat extract was introduced via the gastric fistula into the 'obstructed' stomach, but not when a similar extract was introduced into the duodenum. Gross (1906), working with a similar animal preparation but with the stomach divided at the junction between the fundic and antral regions instead, found that the reverse happened: meat extract given through the duodenal but not the gastric fistula excited secretion from the pouch. These findings were taken to indicate that the gastric glands were stimulated by an antral mechanism assumed to be nervous; the meat extract was supposed to have refluxed from the duodenum into the antrum in Gross' experiments. Similar results were also obtained by Krzyszkowski (1906) and Orbeli (1907).

Edkins (Edkins and Tweedy, 1909) later presented evidence in support of his own thesis. He showed that, when the fundic and antral regions of the stomach in anaesthesized cats were separated by a balloon ligated in position, the introduction of 0.2% HCl, meat extract, or 5% dextrose into the antral compartment for 1-2 hours led to acid secretion from the fundus. Similarly, Savich and

Zeliony (1913) and Savich (1922 a) demonstrated definite secretogogue action of certain chemical agents when applied to the antral mucosa; and Zeliony and Savich (1914) and Savich (1922 b) further showed that distension of an isolated (vagally innervated) antral pouch was followed by secretion from the main stomach. This mechanical method of stimulation was eventually to play an important part in the final proof of the existence of gastrin. The earlier results of Edkins were also confirmed by Maydell (1913) when he demonstrated that extracts of antral mucosa (but not pancreatic secretin, normal saline or extracts of fundic mucosa) excited gastric secretion when given subcutaneously to dogs with chronic gastric fistulae.

However, opinions still differed as to the part played by nervous reflexes in contrast to this humoral mechanism. Orbeli (1907), for example, noted that when a vagally innervated Pavlov pouch was converted into a vagally denervated Heidenhain pouch, secretion from the pouch in response to a meal was markedly reduced, though histologically there was no atrophy of the gastric glands. He concluded that the secretory response depended in part on the existence of nerve connections. Furthermore, Zeliony and Savich (1911) reported that gastric secretion in response to

irrigation of a vagally innervated antral pouch with secretogogues could be abolished by parenteral atropine or prior application of cocaine to the pouch, strongly suggesting involvement of a nervous mechanism.

Meanwhile, various attempts at isolation of the antral hormone had yielded different results. Popielski (1909, 1911, 1912) in a series of publications ascribed the activity in Edkins' pyloric extracts to a non-specific principle (vasodilatin) presumed to be present in the extracts of various tissues, including the pyloric mucosa. At the same time, a gastric secretory excitant was found in simple extracts of the mucosa of the fundus, duodenum, ileum, jejunum, and oesophagus, as well as the liver, pancreas, brain and thyroid gland. (Ehrmann, 1911-12; Emsmann, 1912; Tomaszewski, 1913; Keeton and Koch, 1915; Luckhardt, Keeton, Koch and LaMer, 1919-1920) so that the specificity of 'gastrin' and hence its hormonal status was seriously questioned.

However, Dale and Laidlaw (1910-1911) had suggested identity between vasodilatin and histamine; and when it was found later that histamine stimulated gastric secretion (Popielski, 1919; Keeton, Koch and Luckhardt, 1920), the confusion cleared considerably, but left behind the doubt as to whether 'gastrin' existed at all. It is

fair to say, however, that Keeton, Koch and Luckhardt (1920) had suspected that histamine and 'gastrin' were different, since the former was more toxic and produced more severe side effects. Ivy & Whitlow (1922) repeated Edkins' experiments (Edkins, 1909) but failed to confirm his findings. They therefore rejected the gastrin theory. Lim (1922-23) however, the only investigator who repeated Edkins' earlier work (Edkins, 1906) on pyloric extracts exactly as he had done them, confirmed his results. In addition, he performed direct and indirect transfusion experiments from cats after a meal to fasting cats but failed to demonstrate 'gastrin' in the blood stream. He concluded that Edkins' 'gastrin' must be an artificial extraction product.

In 1925, a series of 8 papers appeared, all bearing on the physiology of gastric secretion. Lim, Ivy & McCarthy (1925) recorded, amongst other results, that distension of a vagally denervated antral pouch (with sympathetic nerve supply intact) led to acid secretion from the fundic portion of the stomach, and that the effect could be abolished by atropine and topical procaine to the antral mucosa. This came close to proving the existence of a hormonal mechanism, but the results were unfortunately interpreted as indicating that a nervous reflex mechanism was responsible, probably aided by vascular changes.

Thus, 20 years after its first enunciation, the gastrin theory was severely challenged for want of both physiological and pharmacological evidence of its existence. It is clear, in retrospect, that the circumstances arose from (1), the unfortunate confusion with histamine; and (2), misinterpretation of the experimental findings owing to lack of knowledge of the existence of

(a) a nervous mechanism for the release of gastrin and

(b) the possibility that both nervous and hormonal mechanisms may be involved at the same time, so that demonstration of the one does not necessarily exclude the other.

It is of interest then to note that Edkins' original observations, despite what has been said to the contrary, were essentially correct, though some of the conclusions drawn from them, when viewed in the light of present knowledge, were unwarranted.

1.2. Proof of Existence of Gastrin

The essential elements constituting the final proof of existence of a hormone in general, and of gastrin in particular, have been well summarised by Grossman (1950). The evidence relating to gastrin will be considered as follows:

(A) Physiological evidence

(B) Pharmacological evidence: extraction and isolation of gastrin.

(A) Physiological Evidence.

In 1925, in the 8th and last of the series of publications concerning the physiology of gastric secretion, Ivy and Farrell (1925) described the auto-transplanted gastric pouch which was to become a classical method in the study of humoral mechanisms. A part of the stomach was transplanted in 3 stages to the mammary tissue of a bitch, thereby interrupting all nervous and vascular connections with the main stomach, the pouch deriving its blood supply entirely from the surrounding tissues. By this method, any stimulant reaching the pouch must travel by way of the circulation. It was found that the pouch secreted with a meal.

Nevertheless, the presence of a humoral mechanism in the gastrointestinal tract did not provide conclusive evidence for the existence of a hormone, since absorbed food substances or products

of digestion could also stimulate secretion. Kim and Ivy (1933) presented evidence suggestive of a hormonal mechanism when they showed that histamine-free liver extract was four times more effective in stimulating gastric secretion when the extract was perfused through a gastric pouch than when the same amount was given intravenously. These results were later confirmed by Butler, Hands and Ivy (1943). Meanwhile, Gregory & Ivy (1941) made a classical study in dogs provided with an auto-transplanted gastric pouch, a vagotomized pouch of the remainder of the stomach (the main pouch), and an oesophago-duodenostomy. They confirmed the existence of a humoral mechanism by showing that the transplant as well as the main pouch secreted when the latter was irrigated with liver extract. They further observed that (1), prior application of procaine to the main pouch abolished the response from both pouches, and (2), procaine did not interfere with absorption of alcohol, and hence probably of other substances also, from the main pouch, nor did it influence the responses of the gastric glands directly. The procaine then probably produced its effect by preventing the liberation of a hormone from the mucosa of the main pouch. This constituted the first strong evidence of the existence of gastrin, though the evidence was circumstantial.

Crucial evidence finally came from Grossman, Robertson & Ivy (1948) who demonstrated in dogs (1), that distension of a vagotomized antral pouch led to acid secretion from a transplanted fundic pouch; and (2), that distension of a transplanted antral pouch caused similar secretion from a pouch of the main stomach which was vagally innervated. Since there was no question of the stimulating agent being absorbed, the humoral agent must have been a hormone. The evidence was conclusive.

(B) Pharmacological Evidence: extraction and isolation of gastrin.

The earlier confusion with histamine culminated in the paper by Sacks, Ivy, Burgess and Vandolah (1932) when they isolated histamine from dog antral mucosa as the sole gastric secretory stimulant. However, they also noticed certain differences between the physico-chemical properties of histamine and crude gastrin; and the next year, Gavin, McHenry & Wilson (1933) found that the fundic mucosa contained more histamine than the antral mucosa, which did not seem to fit in with the site of origin of the humoral agent demonstrated earlier by Lim, Ivy and McCarthy (1925). These clues were overlooked.

It is now obvious that this misconception of the possible nature of the antral hormone had caused failure in its isolation. Purification

procedures were designed to remove all proteins (hence also gastrin) and retain smaller molecules (including histamine) rather than the reverse. In 1938, Komarov demonstrated for the first time that a protein fraction could be obtained from simple acid extracts of pyloric mucosa by trichloroacetic acid precipitation and 'salting out' procedures. The final material was histamine-free and stimulated acid gastric secretion when given intravenously or intramuscularly, but not subcutaneously, to conscious dogs or anaesthetized cats. This work started a new era in the purification of gastrin, and later other investigators introduced various modifications of the method. Recently, Gregory (1926b) announced the isolation of gastrin in pure form. A more detailed review of this phase will be given in Section 3.1.

The present status of gastrin can now be summarized: there is indisputable evidence of its existence as well as of its physiological role in the control of gastric secretion; its purification and final isolation have recently been achieved.

1.3. The Physiological Role of Gastrin

The available data related to gastrin and associated subjects have been critically reviewed by Grossman (1950), and Gregory (1962a) has summarized knowledge in the field to date with authoritative comments, and made valuable suggestions for future work.

Site of Formation

There is abundant evidence that the gastric antral mucosa is an important site of formation and release of gastrin (Lim, Ivy and McCarthy, 1925; Grossman, Robertson & Ivy, 1948). The superficial part of the mucosa was found to contain more gastrin-like activity than the deeper parts (Lim, 1922-23). The fundic mucosa, however, has not been shown convincingly to possess such properties. Distension of a fundic pouch alone caused secretion of acid (Lim et al., 1925) as well as pepsin (Grossman, 1960) but these findings alone do not constitute evidence for a hormonal mechanism. Indeed, the pepsin secretion pointed strongly to a cholinergic mechanism since vagal stimulation is the only known strong stimulant of peptic cells, and gastrin released from antral pouches has been shown not to stimulate pepsin secretion (Grossman, Woolley & Ivy, 1944; Grossman & Slezak, 1950).

The upper small intestine has been shown to

possess a humoral mechanism for the stimulation of gastric secretion (Lim et al., 1925; Gregory & Ivy, 1941). According to Sircus (1953), the substance presumed to be released from the intestine has the properties of a hormone, but there is no clear evidence that the hormone is gastrin.

Mechanism of Release

(a) Mechanical Stimulation - Local distension of the pyloric antrum has long been known to stimulate gastric secretion (Zeliony and Savich, 1914). That this was due to gastrin release was suggested by Lim & Hou (1929) and proved by Grossman et al., (1948).

(b) Chemical Stimulation - A large variety of substances when applied to the antral mucosa will excite gastric secretion, e.g. food, peptone, glucose (Edkins & Tweedy, 1909), meat extract (Lim et al., 1925), protein hydrolysate, choline, and certain amino acids (Ivy & Javois, 1924-25). But none of these have been shown definitely to act by causing gastrin release, for want of knowledge of the exact constituent responsible for the stimulation. However, there is strong indirect evidence that liver extract (Kim & Ivy, 1933; Gregory & Ivy, 1941) and acetycholine (Robertson et al., 1950) do act, at least in part, by liberation of gastrin from the antrum.

(c) Vagal Stimulation - A possible link between nervous and humoral mechanisms controlling gastric secretion was first shown by Straaten (1933) and clearly put forward by Uvnas (1942) who suggested that vagal stimulation could (1), lead to gastrin release from the antrum and (2), condition the response of the gastric glands to it. Earlier investigators could not repeat Uvnas's findings (Jemerin, Hollander & Weinstein, 1943 and others), but when the cause of failure was found in the phenomenon of inhibition of gastrin release by acid in the antrum, his results were amply confirmed (Woodward, Robertson, Fried & Shapiro, 1957; Pe Thein & Schofield, 1959).

(d) Local Nervous Mechanism - The probable existence of this mechanism could be inferred from the inhibitory effect of topical atropine and local anaesthetics on gastrin release from a vagally denervated antrum by chemical and mechanical stimulation (Woodward, Lyon, Landor and Dragstedt, 1954) and vagal excitation. Such a mechanism may provide a common 'pathway' to which all known stimuli converge to effect release of gastrin. There is so far no histological evidence of its existence.

Inhibition of Release

(a) Central - Elimination of the 'cephalic' phase of gastric secretion impaired digestion and gastric emptying (Pavlov, 1910, p. 99). This,

however, could be accounted for by the absence of vagal effects; there is no evidence of active inhibition of gastrin release.

(b) Gastric - There is ample evidence that acid in the gastric antrum inhibits gastric secretion (Sokolov, 1904; Wilhelmj, O'Brien & Hill, 1936) by antagonizing the release of gastrin in response to chemical and mechanical stimuli (Oberhelman, Woodward, Zubiran & Dragstedt, 1952; Kim, 1955) and vagal stimulation (Pe Thein & Schofield, 1959). The nature of this antagonism remains uncertain. It was probably effected without the intervention of a local nervous mechanism (Redford & Schofield, 1961), or the liberation of an anti-hormone (Longhi et al., 1957) though the contrary has been suggested (Harrison, Lakey & Hyde, 1956; Jordan & Sand, 1957). The critical pH in the antrum at which this inhibition occurs has not been clearly established, but probably lies between pH 2-5 (Gregory, 1962a, p. 48).

(c) Intestinal - A humoral agent, enterogastrone, has been shown to originate from the duodenum and upper jejunum and to produce the well known inhibitory effect of a fatty meal on gastric secretion and motility. (Feng, Hou & Lim, 1929). The evidence in support of its hormonal status is strong. It probably acts by antagonizing the effect of gastrin on the parietal cells

(Andersson, 1962).

Mode of Action

The response of the parietal cell to gastrin is no doubt profoundly influenced by concomitant cholinergic excitation. Thus subthreshold doses of urecholine (carbamyl β-methyl choline) potentiated the response of a transplanted or denervated fundic pouch to endogenous gastrin (Grossman, Robertson & Ivy, 1948; Gregory & Tracy, (1958, 1960a); distension of a denervated fundic pouch (thereby exciting the myenteric nervous plexuses) markedly increased its response to circulating gastrin (Grossman, 1961); and vagotomy decreased the parietal cell response to histamine and pilocarpine but not urecholine (Oberhelman & Dragstedt, 1948; Hood and Code, 1957, and others).

Atropine promptly reduced by 50% a near-maximal response of the parietal cells to injected gastrin (Gregory & Tracy, 1959b) and completely inhibited a weaker one. (Gregory and Tracy, 1960). These results were confirmed by Grossman & Gillespie, 1962. Komarov (1942b) and Blair, Harper, Lake, Reed & Scratcherd (1961), however, reported no inhibition by atropine with their gastrin preparations. These discrepancies probably arise from differences in the gastrin extracts, in the methods of testing, and in the doses used. Local application of 1% atropine to

the mucosa of a vagotomized fundic pouch, however, did not impair its response to endogeneus gastrin (Woodward, Lyon, Landor & Dragstedt, 1954). Thus, the question whether the parietal cell can respond to gastrin in the complete absence of cholinergic effects remains unsettled.

1.4 The Role of Histamine

Histamine is distinct from gastrin. Its mode of action on the parietal cell is different (Grossman, 1961); the antagonism of its effects on the parietal cell by atropine is different (janowitz & Hollander, 1956; Gregory & Tracy, 1961) and it differs chemically from gastrin.

Babkin (1938, 1944) had originally suggested a physiological role for histamine as the final mediator for all stimuli bearing on the parietal cell. Work on histamine contents of gastric juice from man and animals (Emmelin & Kahlson, 1944; Code, Hallenbeck & Gregory, 1947) yielded results compatible with Babkin's hypothesis but also explicable by other theories. The relevant data have been summarized by Code (1956). Furthermore, the response of the parietal cell to injected gastrin was not accompanied by detectable rise in plasma histamine (Gregory & Tracy, 1961) Blair et al., 1961), and histamine injected into the portal circulation could not survive passage through the liver in quantities adequate to

stimulate gastric secretion (Gregory & Tracy, stated by Gregory, 1962, p. 91). However, the possibility remains that, with various stimuli, histamine may be liberated in minute amounts in close proximity to the parietal cell thereby exciting secretion.

1.5 Biological Assay of Gastrin

For want of more exact knowledge of the physical and chemical properties of gastrin, the hormone has hitherto been defined and measured solely in terms of its biological effect, which appears to be specifically that of stimulating the parietal cell to secrete hydrochloric acid. Thus all attempts at quantitative estimations of gastrin activity have been expressed in terms of the acid secretion from the stomach of an experimental animal or man, in response to the presence of the hormone.

The more important method for measuring gastrin activity, from the point of view of its bioassay, is that of estimating the acid secretion from part or whole of the stomach with or without vagal innervation in a conscious or anaesthetized preparation, in response to injected gastrin. Edkins (1906) employed cats under ether-chloroform anaesthesia and with their stomachs ligated at the cardiac end (including the vagi) and cannulated at the pyloric end. The cannula was connected to a reservoir and 20-160 ml of saline introduced into the stomach under low pressure, retained there during the period of response, and then drained and tested for HCl and pepsin contents. The gastrin extracts were injected intravenously every 5-10 minutes. He stated

that the basal secretion from these stomachs was negligible, so that any titratable acid in the gastric contents was taken as a positive response, and the activity of the extracts graded according to the amount of acid secreted. The phenomenon of 'exhaustion' of the animal with decreasing response to multiple doses was noticed.

Keeton and Koch (1915) tested their gastrin extracts on conscious dogs with Pavlov pouches and gastric fistulae. The animals were starved for 36 hours, and their basal secretion was measured for 1-2 hours before commencing the experiment. Gastrin extracts were injected intramuscularly since the intravenous route caused reaction in the animal. Doses of 1 ml were usually used, and gastric secretion was collected in 15 minute samples and titrated for "free" and "total" acid. The pepsin content was also estimated. They recorded observations on the pattern of response and commented that while dogs with gastric fistulae were more sensitive and hence useful for detecting small amounts of gastrin, those with Pavlov pouches were more 'stable' and less prone to accumulation effects of multiple doses. It was also noted that extracts active in the conscious dog produced no effects in etherized dogs or decapitated cats. The conscious dog provided with various types of gastric pouches has

also been much used by Gregory and Tracy for testing their gastrin extracts.

Lim (1922-23), in his attempt to repeat Edkins' work (1906), used a slightly different technique. The stomach of an anaesthetized cat was similarly drained by a pyloric cannula, but the gastric secretion was obtained pure without gastric perfusion and led through a drop recorder so that any increase in rate of flow was noticed and taken as additional evidence of response to injected gastrin preparations. Lim & Ammon (1922-23) employed the same method in studying the difference in effect between injecting gastrin extracts into the systemic circulation in contrast to the portal circulation.

Komarov in 1942 introduced a unit for the assay of his histamine-free gastrin extracts. He used cats and dogs under chloralose-urethane anesthesia. The oesophagus was ligated in the neck, and bilateral cervical vagotomy and sympathetomy were done. The pylorus was ligated and a cannula inserted into the fundic portion of the stomach. The pancreatic and bile ducts were also cannulated and the flow of the respective secretions studied at the same time. Several hours were allowed to elapse between the operation and the commencement of the assay, since considerable inhibition was noted during that period. Gastrin

extracts were injected intravenously. In another group of experiments conscious dogs each with a Heidenhain pouch, a gastric fistula, and oesophagostomy, were used, and the extracts given subcutaneously, intramuscularly, or intravenously. In both groups the doses were adjusted to give 2-7 ml of gastric secretion in 30-90 minutes. The unit was defined as the amount of gastrin giving rise to 1 ml of strongly acid gastric juice.

Munch-Peterson, Rönnow, & Uvnäs (1944) worked on anaesthetized cats prepared in a manner similar to Komarov's, but introduced more exacting criteria for the method of administration of gastrin extracts and assessment of response. Thus the actual assay started 1 hour after completion of the operative procedures. Gastrin extracts were dissolved in physiological saline made slightly acid to Congo red and given intravenously at 0.4 ml/minute for a period of 30 minutes. Gastric secretion was collected in 15 minute samples and the total volume collected in the hour from the start of the intravenous injection was taken as the response. The secretory rate was allowed to return to the baseline, usually in about 30 minutes, before the next dose was given. The unit was defined as the amount of gastrin causing the flow of 1 ml of strongly acid gastrin juice in 60 minutes in a cat weighing 2-3 kgm. Again, the doses were adjusted

so that the secretory rates fell between 5-15 ml in 60 minutes; outputs of less than 2-3 ml per hour were considered inconclusive. They also remarked upon the phenomenon of 'exhaustion' in responses and stated that it was species specific: thus cats become resistent to pig's gastrin but not to cat's gastrin.

Jalling & Jorpes (1947), in a description of their version of Komarov's assay method, gave detailed consideration to the problems of variation of responses in the same animal and between animals, and suggested methods of overcoming them. Gastrin extracts were contained in 20 ml. of physiological saline and given intravenously over a period of 20 minutes; gastric secretion was collected hourly and titrated for acidity with methyl red as indicator. The secretory unit was redefined as the amount of extract producing 1 ml of 0.1N HCl in the gastric juice in 1 hour. The authors stated that the correlation between acid output and dose was better than that between secretory volume and dose.

The assay procedure started with a standard dose of gastrin extract adjusted to give 4-6 ml of 0.1N HCl in 1 hour. The next dose was doubled and given to test 'proportionality', thus introducing the concept of the dose-response curve and its slope in the bioassay of gastrin. Subsequent

doses were adjusted so as not to 'exhaust' the animal. The standard dose was repeated at the 6th or 7th dose to check possible alteration in responsiveness. It was noted that, in 40 tests with the same dose given at the 1st and 7th injection, 13 gave 'good correlation' in the magnitude of response, from which it was inferred that the responses to the doses given in between truly reflected the size of the doses. Nine showed marked exhaustion and the remaining 18 had definite though reduced secretion with the 7th dose. In another 110 experiments on doses of varying strength, it was found that the dose-response relationship held true in 60% of the experiments; most of the remainder showed exhaustion. When the same dose was given to different cats, the responses differed up to 9-fold (2-18 ml 0.1N HCl/hour). The authors therefore recommended using 5 or more cats for each assay. They also noted that impurities in the gastrin extracts reduced the accuracy of the assay and led to earlier 'exhaustion', and that doses producing responses of more than 10-12 ml 0.1N HCl per hour invariably caused 'exhaustion' in the following hour.

Ghosh (1956) and Ghosh & Schild (1958) perfected a method of assaying gastric secretory stimulants and depressants which depended on perfusion of the cavity of the rat stomach and

measurement of the pH of the perfusate. The method was not designed specifically for assaying gastrin, but has been modified and used for that purpose in the present work. It will be referred to in greater detail in Sections 2.1 and 2.2.

Ferguson (1950) assayed mucosal extracts of human stomachs in the cat. The animal was put under light pentobarbital anaesthesia, and its body temperature was maintained at normal levels. Bilateral cervical vagotomy was done, the vagi were cut in the neck, and the stomach was then isolated by ligatures at the oesophagus and duodenum and drained through a cannula. After collecting the basal secretion for an hour, doses of extracts were injected intravenously over 20 minute periods, and the acid secretion during the hour from commencement of infusion of the dose was taken as the response. A basal infusion of normal saline at 20 ml/hour was maintained throughout the experiment. Proportionality between dose and effect was best achieved with amounts of extracts in the range of 3-8 g. of mucosa. Some assays were repeated 2-3 times on the same or different animals and the results averaged.

In 1961, Uvnäs & Emås published a method of bioassay of gastrin with statistical control. They confirmed that responses varied within the same cat

and between cats when under anaesthesia, but found better correlation between dose and effect in conscious cats with gastric fistulae, and worked out a method for the assay of gastrin extracts against histamine as standard. Doses of either substance were contained in saline at pH 3-4 and given intravenously over 15 minutes at 0.4 ml/minute; the responses were estimated as the total acid (in mEq) secreted in one hour from the start of the gastrin infusion. The principle of the assay method lay in bracketting 2 identical doses of gastrin with 2 graded doses of histamine. Preliminary work had shown that the dose-response curve for the gastrin extract as well as histamine was linear over the range used in the assay. The same gastrin extract was used throughout the series, consisting of 7 experiments in each of 3 cats.

Certain strictly defined criteria were to be satisfied before any assay was considered valid. These concerned the basal secretion, the slope of the dose-response curve for histamine, and the size of the response to the gastrin injections. The activity of the gastrin extracts were expressed in Histamine Units (when 1 mg gastrin/kgm body weight /15 minutes elicit the same secretory response as 0.001 mg histamine dichloride/kgm body weight/15 minutes) and Secretory Units (the amount of gastrin causing secretion of 1 ml of 0.1N HCl. Results

given as Secretory Units/mg gastrin for comparison). Standard deviations were calculated and also expressed as percentage of the mean value (the relative S.D.) for each group. It was found that within the same animal, the values for the relative S.D. were similar whether the results were given in Histamine Units (17%) or Secretory Units (14%); but between animals, the relative S.D. for Secretory Units (20%) became double the other (11%), thereby confirming considerable variation in response between animals and suggesting parallel variation of sensitivity to histamine and the gastrin extracts.

Harper, Blair & Reed (1962) independently devised a method for gastrin assay using the anaesthetized cat. The stomach was perfused with 50 ml isosmolar glycine buffer at regular intervals, and the gastric secretory responses to injected stimulants estimated by titrating the perfusate back to its original pH with N/50 NaOH. A continuous basal secretion was induced by injecting 0.5-1 mg of a standard gastrin (prepared by the method of Jorpes et al. (1952)) every 15 minutes. An unknown to be assayed was given to replace one of the standard doses, and the HCl output in the subsequent 30 minutes was compared with the expected amount had there been no substitution. At secretory rates of 0.05-0.5 mEq/15 minutes, a linear relation existed between responses to the

Standard and the Test preparation when the acid output of the Test was within ± 50% of that of the Standard. A second gastrin preparation was assayed on 27 occasions in 7 cats and its activity found to be 75% (S.E. ± 1.97%) of the Standard. For cross reference, the arbitrary standard was assayed against histamine as the unknown, given under specified conditions; 1 mg of the Standard was found to be equivalent to 19.8 (S.E. ± 0.68)µg histamine base/kg body weight.

In summary, then, Edkins' work (1906) was little more than qualitative. Keeton & Koch (1915) employed dogs with chronic gastric fistulae to eliminate the factor of animal variation, though the condition of the same animal in different tests could not be controlled. The unit of activity introduced by Komarov (1942) allowed of some standardization but was largely nullified by the marked animal variation. Denervation of the stomach, however, removed a possible source of stimulation other than the injected gastrin and was an approach to achieving strictly level basal conditions during assays in the same animal. Restriction of size of the doses also helped to minimize accumulation effect and 'exhaustion'. It was Jalling and Jorpes (1947) who introduced some statistical rigour into the field. They also used dose-response relationships and suggested assays in groups of animals to

overcome individual variations. But the unit of activity, though improved, was still subject to the same criticism as that of Komarov. The rat preparation of Ghosh and Schild (1958) provided a means whereby multiple doses of a gastric secretory stimulant could be assayed in comparison with those of another. Four-point or even 6-point assays could be carried out in a single preparation and Latin square designs completed in 2 or 3 animals. The results analysed statistically by standard methods would detect and separate off any error due to animal variation, and in addition yield information concerning repression and parallelism of the dose-response curves and the error of the assay and hence its fiducial limits. Uvnäs & Emås (1961) were the first to employ a reference standard in the assay of gastrin, though the fact that histamine and gastrin act differently on the parietal cell would nullify the validity of the comparison (Gaddum, 1959). Nevertheless, the strict attention paid to dose-response relationships, the avoidance of cumulative effects, and statistical analysis of the data fulfilled the essential requirements of modern bioassay techniques.

Similar statements can be made concerning the method of Harper et al. (1962), but the introduction of an arbitrary standard with a composition

similar to that of the unknown has removed the major objection on pharmacological grounds. It is obvious that eventually all arbitrary standards must be assayed against the purified hormone as the final reference standard, when it becomes available.

PART II

METHODS

2. The Perfused Rat Stomach Preparation

2.1 Introduction

The rat has been much used for studying acid gastric secretion. It is readily available and easily handled. In order to empty the stomach before use, Roe & Dyer (1939) starved them for 48-72 hours; coprophagia was overcome by putting each rat in a mesh-wire jacket. Gastric juice was collected from the anaesthetized animal by aspirating via a needle through the rumen of the stomach, the duodenum having been ligated. Contamination with saliva was prevented by a cotton plug in the throat. Friedman (1943), in studying the effect of histamine on gastric secretion in the rat, starved them for 24-36 hours in wide mesh false bottom cages. Water was given ad libitum. Komarov et al. (1944) worked on rats with gastric fistulae and ligated pylorus. They recommended starvation for 48 hours with rats under 180 gm and 72 hours with larger ones.

Shay et al. (1945) first introduced the rat with ligated pylorus for acute experiments. Severe ulcerations of the stomach invariably occurred within a few hours. The potency of 'anti-ulcer' agents were studied by noting their effectiveness in the prevention of these gastric ulcers. Madden et al. (1951) showed that

dehydration markedly reduced acid secretion, a point to be noted in starved rats which do not usually drink. Donald & Code (1952) supplied 0.4% saline ad libitum to rat starved for 72 hours. The gastric secretion was tapped by a pyloric cannula, but there was no apparent difference to the rate of secretion whether drainage was intermittent or continuous. Noordwijk & Aarsen (1954) employed a gastric lavage technique on an isolated stomach ligated at the oesophagus and duodenum and drained through a pyloric cannula. The stomach was washed out gently with 20 ml of warm normal saline at 30 minute intervals, and the washings titrated with 0.1N NaOH.

In 1958, Ghosh and Schild published a method for the quantitative study of gastric secretion in the rat, employing the technique of continuous perfusion of the gastric cavity. Rats were anaesthetized with urethane, and their body temperature was kept constant at 34°C. by thermostatic control. The stomach was opened at the ruminal portion and washed clean of its contents. It was then perfused with warm N/4000 NaOH at a constant rate via oesophageal intubation, and the pH of the effluent was recorded continuously. The pH deflections with identical doses of secretory excitants and depressants were reproducible, and multiple doses could be tested in the same animal,

thereby eliminating variation between them. Application of the Latin Square designs provided a method for statistically valid assays with but few animals.

Antonsen (1959) confirmed the importance of body temperature control when he used the dialysis bag method of studying gastric secretion in the rat. Drugs were given intravenously and fluid was replaced by the same route intermittently to combat dehydration.

Lane et al. (1957) studied the dose-response relationship of histamine in rats provided with chronic gastric fistulae. They confirmed that rats have a high basal acid secretion, persisting even after 72 hours fasting; and they further showed that general anaesthesia, including that with urethane, depressed acid secretion. Thornton & Clifton (1959) adopted the method of Hunt and Spurrell (1951) of studying gastric emptying in man to experiments in the rat. A soft rubber catheter was passed down the oesophagus of the conscious animal and 7.5 ml of a sucrose test meal with phenol red was introduced, left for 45 minutes and then withdrawn and titrated, and the acid output was calculated.

The above account has summarized the various methods used in the rat. For the purpose of the bioassay of a gastric stimulant, however, the method of Ghosh & Schild (1958) provided obvious attractions in that most of the variables known to

affect acid gastric secretion in the rat were controlled, and the method of continuous perfusion allowed of reasonably complete collection of acid secreted and uninterrupted information on the secretory responses. It was therefore adopted for the present work.

2.2 The Technique

Male albino rats of the Wistar strain were used throughout the experiments. Their body weight ranged from 180 gm to 320 gm with a mean of 232 gm. All rats were starved for 24-48 hours before use.

Anaesthesia

General anaesthesia was induced by administration of urethane in 25% solution (w/v), the usual dose being 0.7 ml/100 gm body weight of the rat. Rats varied in their sensitivity towards urethane. Since it was desirable to have a constant level of anesthesia throughout each experiment, as well as a comparable plane of anaesthesia from one rat to another, a scheme was followed whereby the dose was adjusted to the individual animal.

The body weight of the rat at the commencement of starvation was taken for calculation of the dose, since an average rat of 250 gm may lose up to 30 gm of its weight after 2 days without food, and the dose of urethane as estimated then may prove inadequate. Half of the dose thus calculated was given intraperitoneally. After 3-4 minutes, the animal was drowsy, with rapid shallow respiration and only corneal and pain withdrawal reflexes present. This indicated that

the dose was correct, and the remainder was then injected subcutaneously at 2 sites. If spontaneous movement of limbs, blinking, etc. persisted 5 minutes after the intraperitoneal injection, the dose was considered to be too small and 0.2 ml was added subcutaneously. Absence of pain and corneal reflexes after the first dose indicated that the rat was unusually sensitive to urethane and 0.2 ml was subtracted from the remainder of the dose. The rat was usually fully under anaesthesia in 30-45 minutes. By then the respiration was slow (about 60/minute) and mainly or wholly abdominal. Thoracic type of respiration was a bad sign and invariably meant a dying animal.

Operative Procedure

The rat was placed on a Rat Operating Stand (Model E 30, C.F. Palmer Ltd.). Its body temperature was kept at 34 ± 0.5°C by a thermostatically controlled heating system consisting of (a), a table lamp with a 60 Watt electric bulb which was on continually and supplied heat and illumination, and (b), a built-in source of heat in the operating stand, with a 40 Watt electric bulb. This was controlled, via a relay unit (Zecol Plug-in Relay Unit, G.H. Zeal Ltd.), by a contact thermometer set at 34°C and introduced into the rectum of the animal.

A tracheotomy was first performed and a polythene airway inserted and ligated in position. The respiratory passages were kept open by suction with a polythene pipette to remove mucus when necessary.

The abdomen was then opened by a transverse incision about an inch long and just touching the tip of the xiphisternal process, so that the end of the incision barely reached the costal margins. This provided a better exposure than a midline longitudinal incision. The lower edge of the liver was gently displaced upwards and the duodenum thus exposed was brought to the surface with the aid of a seeker. Three ligatures were passed round the pyloro-duodenal junction as close to the gut wall as possible to avoid inclusion of any vessel running between the stomach and duodenum in the omentum. The first ligature served for traction. A small duodenostomy was made about one quarter of an inch from the pylorus, choosing an avascular spot, and a polythene cannula inserted towards the pylorus. The second ligature was then tied tightly midway between the duodenostomy and the pylorus. A pair of non-toothed forceps was applied to the duodenum at this ligature, thus holding the duodenum steady, while the cannula was gently pushed with a slight turn through the pylorus until the tip was just in the stomach.

This manoeuvre allowed passage of the cannula through the pylorus even if the latter was contracted, without undue damage to the duodenum. The third ligature was then tied at the pylorus.

A soft rubber urethal catheter was passed down the oesophagus till the tip lay just in the ruminal portion of the stomach. The approximate length was equivalent to that between the teeth and the xiphoid process. The passage of the tube was usually accompanied by deglutition which facilitated the process. A ligature was then placed round the oesophagus at the neck.

The stomach was next mobilized from its bed and delivered through the abdominal wound, as far as the ligaments would allow without undue stretching. This was achieved mainly by gentle traction on the relatively avascular ruminal portion. Handling of the glandular portion was avoided for such invariably resulted in trauma to the secreting mucosa with bleeding. The stomach was then washed by a slow stream of tap water introduced via the oesophageal tube from a reservoir held 50 cm above the rat. This was continued till the effluent was clear and all parts of the stomach especially the ruminal portion, were empty and collapsed. The stomach at the end of 1-2 days starvation usually contained some food but was never full, so that

the washing out was not difficult. It was then returned into the abdomen: a small pad of cotton wool moistened with normal saline was placed against the ruminal portion to keep it collapsed during the experiment. This prevented any considerable collection of perfusate in this dependent portion.

Perfusion of the stomach was carried out with 0.9% saline from a reservoir placed 200 cm above the rat. This head of pressure was reduced by means of a suitable length of capillary glass tube so that the final flow rate was constant at 0.7 ml/minute. The saline was then led through several coils (length 60 cm) of thin polythene tubing (internal diameter .15 mm) placed between the rat and the table to warm the perfusate to the body temperature of the rat before finally introducing it through the oesophageal tube into the stomach. The gastric effluent from the pyloric cannula was led through a length of polythene tubing (24 cm long, 2 mm internal diameter) to a glass dropper placed 5 cm below the level of the rat to improve drainage of the stomach by siphon action. The fluid was collected in 10 minute samples, which measured 7 $\pm$ 0.5 ml in the majority of cases. The variation in volume arose from differences in resistance in the different stomachs

and the occasional gastric peristalsic movement, but the volume tended to be constant during each individual experiment.

The right femoral vein was exposed at its origin and a fine polythene cannula was inserted and ligated in position. A slow continuous basal infusion of 0.9% saline was started, using an electrically driven slow injection apparatus (Model 67, C.F. Palmer Ltd.) and adjusting the rate at 2 ml/hour.

All exposed tissues were covered by pads of cotton wool moistened periodically with 0.9% saline. The whole operative procedure was usually completed in 30-40 minutes and assay procedures could be started immediately afterwards.

Titration

The samples of gastric perfusate were titrated against N/100 NaOH with phenolphthalein as indicator. The NaOH solution was stored in a polythene reservoir connected in close circuit to a 5 ml microburette, the tip of which was narrowed by a polythene tube drawn out at one end so that the drop size was about 0.15 ml. The whole system was protected from atmospheric carbon dioxide by soda lime.

A simplified diagram of the set up of the animal preparation is shown in Fig. 1.

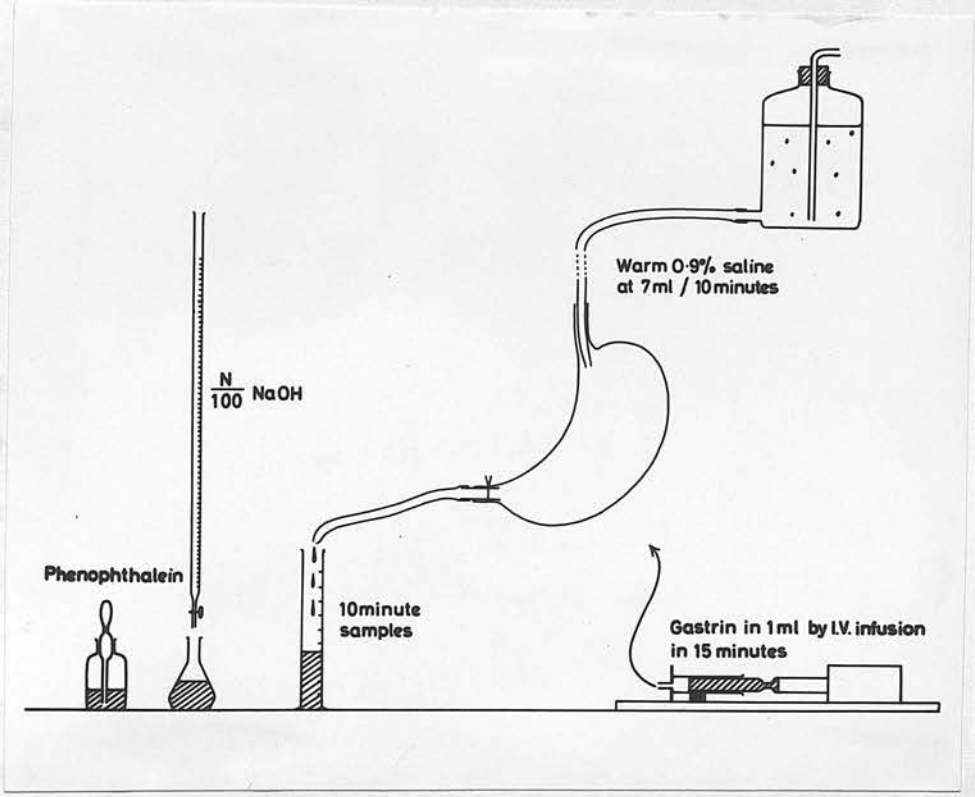

Fig. 1. Simplified diagram of the rat preparation.

2.3 Discussion

Rats of the same strain and sex were used to reduce the error of the assay. Urethane proved to be a suitable anaesthetic, (Ghosh, 1956), since a single dose provided constant anaesthesia throughout the experiment. The scheme described above ensured correct dosage and rapid induction of anaesthesia. The intraperitoneal route has not been found to affect acid gastric secretory responses.

The method of preparing the rat differed from that of Ghosh (1956) in several respects:-

(1) Starvation of the rat and washout of the stomach without gastrostomy, thereby minimising trauma to the stomach. It was important to starve the rat, since otherwise the stomach was usually packed with food which blocked the pyloric cannula and defied complete washout; a thoroughly clean stomach was essential for accuracy in the titration of the perfusate. Periods of starvation up to 48 hours did not impair the vitality of the animal as judged by the easy, regular respiration at the end of 8-hour experiments and the unchanged pattern of gastric secretory responses.

(2) Perfusion of the stomach with 0.9% saline instead of dilute alkaline solution (Ghosh, 1956) or a buffer solution (Rosenoer & Schild,

1962), since the perfusate was to be titrated for acidity.

(3) The rate of perfusion at 0.7 ml/minute was chosen arbitrarily, being fast enough to wash out all the acid secreted from the stomach without stasis, and slow enough to yield a volume convenient for titration.

(4) A basal continuous 0.9% saline infusion intravenously. It was felt that a large proportion of the weight loss during starvation was due to dehydration and loss of electrolytes, since it is well known that rats do not drink readily without food. The saline infusion served to correct this deficiency and to ensure a constant supply of Cl^-, in addition to keeping the vein open for administration of gastrin extracts. A continuous intravenous infusion of saline alone in the rat does not excite gastric secretion (Ghosh, 1956).

(5) Quantitative estimation of acid secreted from the stomach, instead of a continuous recording of changes in pH of the perfusate. This allowed of a simpler apparatus, and by collecting fractions of the perfusate in 10 minute samples, it was possible to follow closely the changes in the total acid output and hence the pattern of response to gastrin extracts.

3. PREPARATION OF GASTRIN EXTRACTS

3.1 Introduction

A brief review of the methods employed in the attempts to extract gastrin will be given. Tables summarizing the physiological and biochemical properties of these extracts are given in the Appendix (I(a) and (b)).

The period under review can be conveniently divided into two parts separated by Komarov's classical work in the demonstration of a histamine-free gastrin extract. The methods used during the first part yielded extracts all of which probably contained histamine; these extracts are therefore not considered in the table of properties.

Edkins (1906), prepared simple extracts of hog gastric mucosa with cold and boiling water, 5% dextrin or glucose, solutions of peptone or glycine, and 0.4% HCl. Of these, boiling water and glycine were found most effective, peptone and 0.4% HCl less so, and the rest doubtful. Pyloric and cardiac mucosa yielded about equal activity; fundic mucosa had none, but possessed considerable vasodepressor action.

Keeton and Koch (1915), later joined by Luckhardt and laMer, made a series of studies on the extraction, distribution and mode of action of

'gastrin bodies' in various animals. Fresh tissues were extracted with boiling 0.4% HCl , and the active fraction recovered by evaporation and tested in aqueous solution. Considerable activity was found in the mucosa of the pyloric, fundic and cardiac regions of the stomach, and to a smaller extent in duodenal mucosa. The oesophagus, pancreas and muscles were negative. There was a fall in blood pressure for 4-5 minutes after each intramuscular injection of the extracts. It was also found (Luckhardt, Keeton & Koch, 1920) that atropine antagonized secretory responses to injected histamine or gastrin extracts, the effect being proportional to the dose, and that the antagonism could be complete with smaller doses of gastrin extracts but never with histamine. Koch, Luckhardt & Keeton (1920) also extracted 'gastrin bodies' from gastric juice. Subsequent studies on the properties of the extracts led to the conclusion that the 'gastrin bodies' were distinctly basic, closely similar to histamine, and might be a group of closely related peptamides.

The work of Sacks et al. (1932) was aimed at isolation of histamine rather than gastrin.

Komarov's (1938, 1942 a) classical work showed that a vasodilatin-free non-toxic extract could be made from hog antral mucosa which stimulated acid but not pepsin secretion. His

extraction procedures included saturation with NaCl, precipitation with 10% trichloroacetic acid (TCA) and fractionation with methanol. The potency of the final product, Purified Gastrin II, was 2.5 - 4 mg/unit. Secretin, being soluble in 10% NaCl (but not in 30%) and insoluble in methane, was separated from gastrin.

Uvnäs (1943a) employed an abridged version of Komarov's method, obtaining crude extracts by boiling mucosal strips in N/10 HCl and precipitating with 10% TCA after partial neutralization, and finally drying the precipitate through acetone and ether. Doses equivalent to 2-5 G mucosa from the cat or 5-10 G mucosa from the hog given intravenously over 30 minutes to anaesthetized cats illicited copious responses with maximal acidity of up to 180 mEq/litre. Activity was localized to the pyloric mucosa.

Munch-Petersen, Rönnow & Uvnäs (1944) described in detail a modification of Komarov's method as applied to extraction of pyloric mucosa of cats and pig. Material from cats was dealt with in a manner identical with that of Uvnas (1943a) above. Pig mucosa was similarly extracted with boiling N/10 HCl. The filtrate was partially neutralized and the activity in it precipitated with 20% NaCl and finally dried to yield 4-7 G of

powder per stomach. This crude gastrin was further purified by (a) the tannic acid method or (b) the trichloroacetic acid method. The activity of the crude powder, the tannic acid precipitate, and the trichloroacetic acid precipitate as tested in the anaesthetized cat was about 6 mg, 2.5 mg, and 2 mg/unit respectively.

Uvnäs (1945a) introduced the isoelectric precipitation of the activity at pH 4 - 5.5 in place of the tannic method. Precipitation was complete at pH 4.4 and selective for gastrin at 5 but with considerable loss of activity.

Harper (1946) applied a modification of Mellanby's method for extraction of secretin to the gastrin problem. Mucosal tissue was extracted with alcohol; the active material was precipitated by saturation with NaCl or addition of bile salt; the latter was finally removed with alcohol. Only extracts from the mucosa of the pyloric antrum and the upper small intestine were active.

Jorpes, Jalling & Mutt (1952) devised a method of extraction with 95% acid methanol, suitable for handling large quantities of material. The main steps included repeated isoelectric precipitation at pH 7 and final dialysis to yield 1.5 G of product per 10 kg mucosa. Its activity as tested in the anaesthetized cat varied, but was about 10 units/mg.

Gregory and Tracy in a series of papers (1959b, 1960b, 1961) described the development of a 3-stage method for the extraction and purification of gastrin from hog antral mucosa. This method has been used for the preparation of crude and purified gastrin used in the present bioassay work, and a detailed 'flow sheet' of the method is given in Appendix II.

The first stage of the method started with extraction of mucosal tissues with aqueous acetone containing 4-5% TCA and subsequent removal of the acetone and TCA with ether to yield an aqueous extract. This was freed of a considerable portion of inert material by precipitation at pH 5-5.5 and then at pH 8.5 in hot solution, when the active fraction was redissolved. Subsequent 'salting out' by saturation with NaCl and repetition of alkaline precipitation yielded a clear supernatant which was then treated twice with TCA (4%) at 10°C, and the precipitate containing the activity was dried through acetone and ether. This powder will be subsequently referred to as "Crude Gastrin" (Stage 1). The yield was 600-700 mg/kgm mucosa, and amounts equivalent to 10-20 gm mucosa provoked definite secretory responses from a dog when injected subcutaneously, intramuscularly or intravenously.

The second stage of the method was aimed at

securing a gastrin solution largely devoid of inert material and suitable for column chromatography. It consisted essentially of dissolving the crude gastrin powder in distilled water, making the solution strongly alkaline, and then precipitating the denatured protein at pH 8.5 by addition of acetic acid in the presence of 80% acetone. The acetone was removed with ether, and the clear aqueous residue was ready for the final step in purification.

In the third stage, the active fraction in the aqueous residue was adsorbed onto a $CaPO_4$ gel column and then eluted by dilute Na_2HPO_4. A sharp peak of biuret-reacting material was associated with the front of the eluate and contained most of the activity. The final solution was clear and colourless with a pH of about 7. Amounts derived from 1 kg of mucosa contained approximately 0.5 mg total N. It could be stored deep frozen for months without loss of activity, and was highly potent. This fraction will be referred to later as "Purified Gastrin" (Batch A nr B).

Grossman, Tracy & Gregory (1961) later published an abridged version of the above method which was employed in the extraction of tumour tissues. The extracts from both the primary pancreatic tumour and the secondary deposits in the liver of a patient with Zollinger-Ellison

Syndrome were active when given subcutaneously to dogs with Heidenhain pouches. This method has been applied to human tissues for quantitative recovery of gastrin-like activity in the present work. It is described in detail in a later section, and a 'flow sheet' of the method is also appended (Appendix III),

Blair, Harper, Lake, Reed & Scratcherd (1961) reported a simple method for isolating crude gastrin from antral mucosa of man, pig, dog, and cat. The tissue was minced, homogenized in water and boiled for 10 minutes. After cooling, the major part of the residue was removed by centrifugation and then prolonged filtration at 5°C. The active fraction in the clear filtrate was precipitated by excess of acetone, washed, and dried, yielding a white powder (about 11 mg/gm mucosa) which has been stored for 4 years without loss of activity.

Fletcher et al. (1961) and later the same group of workers (Anderson et al., 1961) described a 'somewhat novel' method for extraction of gastrin, which differed from all other methods in two main aspects: (1) the pH of the extract at all stages was kept above 4, and (2) the temperature never exceeded 40°C. The mucosa was fixed in acetone, and digested in warm dilute aqueous NaOH (pH 10.5). Subsequent procedures included

repeated precipitation of inert material at pH 7-8 and final recovery of the active fraction at pH 5. For bovine tissue, activity was found with the precipitate at pH 4 and 3 but not at 5. All precipitates were redissolved in dilute alkali (pH 11.0), dialysed against distilled water, and then dried. The yields varied between 0.5-2.0 gm/ 20 antra, and all possessed high activity. In an extensive study of the physico-chemical properties of the dialysed product, the authors found one main potassium-containing protein fraction (40%) and two other smaller protein-like fractions, and one non-protein fraction with 4-7% phosphorus. The product after electrophoresis lost a considerable part of its activity and was less stable. Some differences were noted between bovine and porcine gastrin, e.g. in the latent period between a dose and the onset of response, and in the isoelectric point.

Gregory (1962b) reported the isolation of gastrin in pure form, together with results concerning its amino acid components.

In summary, then, it can now be seen that Edkins' (1906) simple extracts undoubtedly contained histamine, but the recent work of Blair et al. (1961) has shown that similar extracts did contain gastrin-like activity, quite apart from

histamine. The alcoholic extracts of Keeton et al. (1915-20) probably also contained both. Komarov's work (1938, 1942a,b) marked the turning point, and all subsequent methods yielded extracts largely free of histamine. The procedures of TCA precipitation and 'salting out' by saturation with NaCl introduced by him formed the main stay of most methods thereafter. Uvnäs (1943a, 1945a) removed inert material by warming at alkaline solution and precipitating the active fraction at its isoelectric point of 4 - 5.5. Jorpes et al. (1955) obtained their active fraction from methanol extracts by precipitation at pH 7, and purified it by reprecipitation from distilled water and then dialysis. Then Gregory & Tracy (1961) in their three-stage method produced material largely devoid of inert proteins at the end of the second stage, after the combined efforts of isoelectric precipitation, 'salting out' with NaCl, TCA precipitation, and then selective removal of denatured protein by precipitation from strongly alkaline solution. The final fractionation from a $CaPO_4$ gel column yielded active material of high purity. Further extension of the method had apparently produced the hormone in pure form. Fletcher et al. (1961) worked on bovine and porcine tissues with a somewhat different approach and have studied the properties of their products in considerable detail.

3.2 The Reference Standard

The arbitrary reference standard employed in the present bioassay work was prepared by the method of Gregory & Tracy (1961). It was a freeze-dried product of the eluate obtained at the end of Stage III of the method ("Purified Gastrin"). The eluate originally contained about 0.15% Na_2PO_4, and was at pH 7-8. Studies by electrophoresis showed that there was one main active fraction with an isoelectric point of pH 5-6, together with 3 or 4 unidentified substances. Its protein con-tent was equivalent to approximately 200 µg of crystalline albumin/ml of the solution. When freeze-dried, 1 ml of the solution yielded 1100 µg of total solid, in the form of a white powder read-ily soluble in distilled water or 0.9% saline. This powder was reconstituted with 0.9% saline to the original volume of the eluate and then diluted to appropriate concentrations before use.

3.3 The Crude Preparations

(a) "Crude Gastrin" (Stage I)

This powder was obtained at the end of Stage I in the method of Gregory & Tracy (1961). It was usually light brownish, soluble in water or 0.9% saline (aided by warming).

Before being tested in the rat, the solution, containing about 60 mg of powder in 10 ml of 0.9%

saline, was warmed to 70°C; its pH was adjusted to 5 - 5.5 with 0.1 NaOH, and then to 8.5 with dilute NH_4OH, allowing 10 minutes in between the steps. After a further 5 minutes, the solution was cooled and the precipitate removed by centrifugation. The clear supernatant was used for injection.

(b) Crude Gastric Extract (human material) by the method of Grossman, Tracy & Gregory (1961).

The intended quantitative study on the gastrin-like activity in human tissues demanded that a suitable method of extraction be available where maximal recovery of activity could be achieved, even at the expense of the purity of the final product. The method employed by Grossman, Tracy & Gregory (1961) was attractive in that it was relatively simple but yielded extracts which were largely devoid of histamine.

Mucosal tissues, obtained from operative specimens within 30 minutes of their resection, were cut in thin strips and extracted with 10 volumes of 4% TCA in 90% acetone overnight. The brownish supernatant was collected the next morning and the strips re-extracted twice, for four hours each, with 5 volumes of the acid acetone. All extracts were pooled, acidified (with 5 ml of 10 N HCl per litre), and treated with ether.

The pH of the aqueous residue was raised to 3 with 2N NaOH, traces of ether were expelled by gentle warming, and the solution then cooled to 10°C. TCA in solution was added slowly till a final concentration of 4% was achieved. The whitish precipitate was collected, resuspended in distilled water, and reprecipitated with TCA as above. This second precipitate was taken up in a suitable volume of 0.15 HCl, extracted once more with ether, heated to 70° - 80°C, and brought through to pH 5 - 5.5 with 2N NaOH and 10 minutes later to pH 8.5 with dilute NH_4OH. The precipitate was centrifuged off and the clear supernatant injected into the rat after suitable dilution.

3.4 Discussion

From a review on the methods of extraction and properties of gastrin, certain points emerge which are worthy of note.

Firstly, gastrin is apparently a very stable compound, withstanding extremes of temperature (e.g. boiling for 30 minutes) and of pH (e.g. from less than 1 to 11 or 12).

Secondly, despite the apparent multiplicity of methods for extraction of antral gastrin (though in fact they differed only in minor aspects), all products from any one animal have been found active in all animals tested. The usual experimental animals tested were dogs (conscious or

anaesthetized) and anaesthetized cats, and so far, there has been no report of one extract being active in 1 animal and inactive in another, though the magnitude of response varied. Thus, antral gastrin from the hog, dog, cat and cow has no species specificity as demonstrable by these biological tests. This assumption is further strengthened by the recent work of Monaco et al. (1961) who showed that antibodies to a hog gastrin extract could antagonize hog, dog, and human gastrin extracts. Although the gastrin extract used was not in pure form, and the resultant anti-serum contained a mixture of antibodies, the fact that a single anti-serum could antagonize the same activity in all 3 extracts from different sources warranted the conclusion that the active agents were identical or at least possessed identical moeities responsible for their action.

There are, however, apparent contradictions to the above conclusions. Thus Uvnäs (1943a) observed that cats were more easily 'exhausted' when responding to hog antral gastrin than to cat antral gastrin; 'gastrin' from different sources and prepared by different methods have been reported as dialysable and non-dialysable; and Fletcher et al. (1961) noted that bovine and porcine gastrin had different isoelectric points. But before any

interpretation of these findings can be properly made, certain points need be considered:

(1) All these observations were made on relatively crude preparations. As Gregory & Tracy (1961) suggested, the behaviour of 'gastrin' on dialysis might be influenced by its tendency to adsorb onto proteins also present, thereby giving a false impression of its molecular size. A similar effect may be produced on the apparent isoelectric point if other denatured proteins are also precipitated.

(2) The mode of action of gastrin can be greatly influenced by such adsorption onto inert proteins (Gregory & Tracy, 1961).

(3) The possibility remains that different crude extracts might contain, besides gastrin, other fractions which might stimulate or inhibit acid gastric secretion.

It would appear, then, that unless more concrete evidence from studies on purified preparations points to the contrary, it is possible that gastrin is not species specific amongst dog, cat, hog and cow.

Thirdly, in testing the biological effects of gastrin extracts and the influence of certain factors upon them (e.g. atropine, cholinergic excitation, etc.), it is important that conclusion be drawn only when the experimental conditions are

as near as possible to the normal physiological state in man under which it would act. In this context, the conscious dog should be used, for the following reasons:

(1) It has been the most widely used animal in experiments based on the endogenous release of gastrin, upon the results of which conclusions have been drawn and inferences made on the physiology of gastrin in man. So far, there has been no known contradictions to these inferences by the sporadic data obtained direct from man. It would seem wise, therefore, to keep to the same animal species for tests on gastrin extracts.

(2) The conscious dog is much easier to handle than other conscious animals for experimental purposes (e.g. cat, rat, etc.). Stress is to be laid on the conscious state, since anaesthesia could possibly influence the response, both quantitatively and qualitatively.

For the purposes of biological assay of gastrin, however, it is quite permissible to use anaesthetized animals, provided both the chosen standard and the unknown have been shown previously to be active in the conscious dog; the assumption is made that the same active agent stimulated the dog as well as the anaesthetized animal to secrete.

(3) Only one purified gastrin extract has been tested in man and found active (Gregory & Tracy, 1961). The dog is the only experimental animal tested in the conscious state in which this same extract was active.

4. THE METHOD OF BIOLOGICAL ASSAY

4.1 The Basal Secretion

The anaesthetized rat produced a continuous basal acid gastric secretion which, under the conditions of the experiments, ranged from 1 to 2.5 μEq/10 minutes in the majority of cases. The rate was relatively constant for hours when the rat was in a resting state, but it occasionally varied to a slight extent (e.g. up to ± 1 μEq/10 minutes) at the intervals between doses of gastrin extracts.

4.2 The Pattern of Response

The gastrin extracts were given by intravenous injection throughout the assay work, in order to avoid possible variation in the rates of absorption with the other routes of administration. The pattern of responses was studied with the following methods of intravenous injection:

(a) Rapid Injection - The dose was contained in 0.4 ml normal saline, injected within 20 seconds, and flushed in by 0.4 ml of saline.

An example of the response to a moderate dose of "Crude Gastrin" (Stage 1) is shown in Fig. 2. Acid secretion commenced in the first 10 minute period, rose to a peak in the second, and was usually down to the baseline in 40-50 minutes. When the dose was small, demonstrable acid

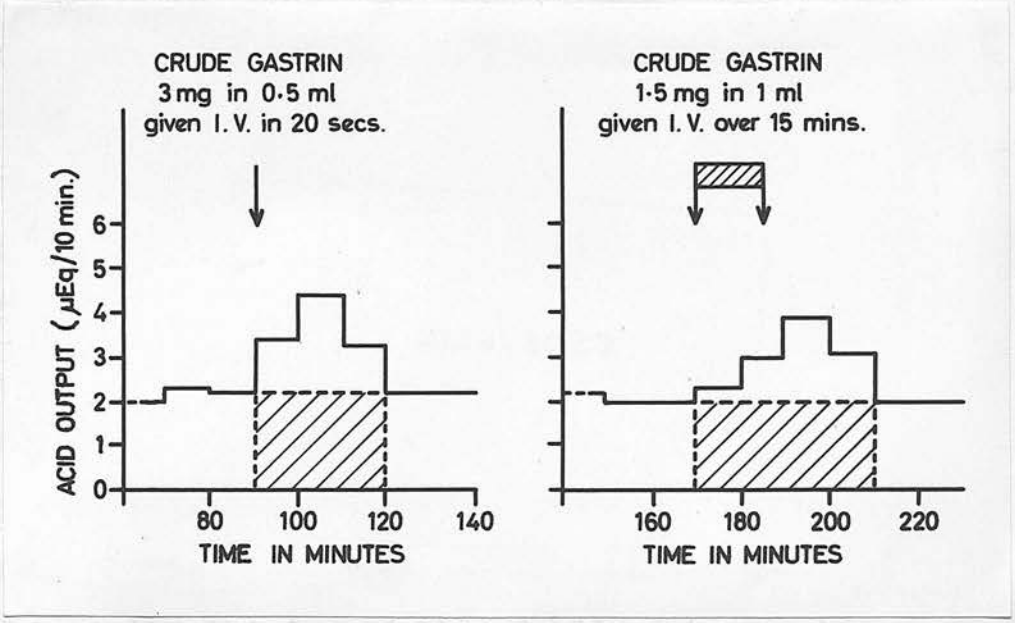

Fig. 2. The pattern and magnitude of response to different modes of intravenous administration of a gastrin extract.

secretory response might not occur until the second 10 minute sample, would persist only in the third, and would be back to basal rates of secretion by the 4th. The peak rate of secretion depended on the dose and the sensitivity of the animal, but ranged from a barely detectable rise over the baseline to about 10 μEq/10 minutes.

The same dose injected over a period of 5 minutes showed a similar pattern of response.

(b) Slow Infusion - The dose was contained in 1 ml of normal saline and injected over a period of 15 minutes.

The response to a dose of the same "Crude Gastrin" (Stage 1) preparation but half of the size of the dose used in (a) above, is also shown in Fig. 2. It will be noted that the overall magnitude of response is similar in the two cases. In a typical example, the acid output was usually detectable at the first sample, increased steadily to a peak at the third, and then declined to reach the baseline again by the 5th or 6th sample. The peak response always fell on the 3rd sample, though its magnitude varied considerably with the dose and the sensitivity of the animal and might reach 16 μEq/10 minutes or higher.

With identical doses of medium size given in succession to the same animal, the total acid output as well as the duration of response varied

from one dose to another, but their ratio remained relatively constant. After the 4th or 5th dose, however, all values including the ratio increased, presumably due to accumulation of effect (See Fig. 4).

With increasing doses, the effect was first seen in a rise in the acid output and in the ratio 'acid output/duration of response', with or without an increase in the duration of response. When the dose reached 3-4 times the threshold dose or above, all 3 parameters increased, but of these three, the ratio 'acid output/duration of response' alone remained roughly proportional to the dose. This proportionality did not hold, however, when (i) the dose given was too large, so that the peak response was 12 μEq/10 minutes or higher, and (ii) more than 4-5 doses were given to each animal, in which case the responses to doses after the fourth or fifth one were disproportionately large in relation to the dose.

The patterns of response to equivalent doses of "Crude Gastrin" (Stage 1) and "Purified Gastrin" were similar.

(c) Continuous gastrin infusion.

In one experiment with a continuous intravenous infusion of "Purified Gastrin" at a medium rate over a period of 2½ hours (Fig. 3) the response rose sluggishly to a maximum by the end of the first hour, and then fluctuated considerably.

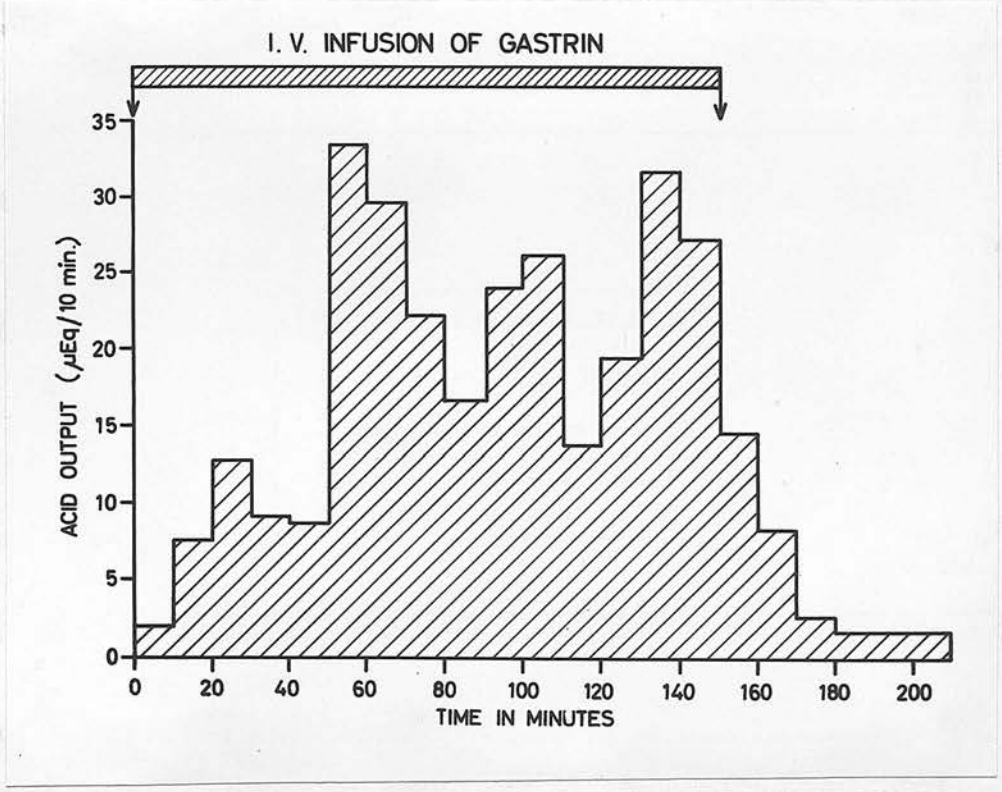

Fig. 3. Response to Continuous Intravenous Infusion of Gastrin.

Male rat, 232 g.

Dose: "Purified Gastrin" Batch B, before freeze-drying, 0.01 ml/100 g body weight in 1 ml saline/15 minutes.

When the infusion was stopped, secretion declined rapidly and was back to the baseline within 30 minutes.

4.3 Measurement of Effect

All doses of gastrin extracts were contained in 1 ml of normal saline at pH 7 and injected intravenously over a period of 15 minutes. The study on the patterns of response with repeated identical doses revealed that the best criterion for assessment of response under the conditions of the experiment was the 'Mean Rate of Acid Secretion', expressed in μEq/10 minutes and defined as:

$$\frac{\text{Total acid output - estimated basal acid output (in μEq.)}}{\text{Duration of response (in periods of 10 minutes)}}$$

where Total acid output = acid output from baseline to baseline

Estimated basal acid output = Basal rate of acid secretion x duration of response.

Basal rate of acid secretion = ½(mean rate of secretion before the dose + mean rate of secretion after the dose.

Each mean rate is based on 3 consecutive similar readings (differing by less than 0.2 μEq/10 minutes)

Duration of Response = Period between commencement of response to a dose and the next baseline reading.

An example of the calculation is given in Fig. 4.

This method of calculation takes into consideration minor changes in the baseline. For the

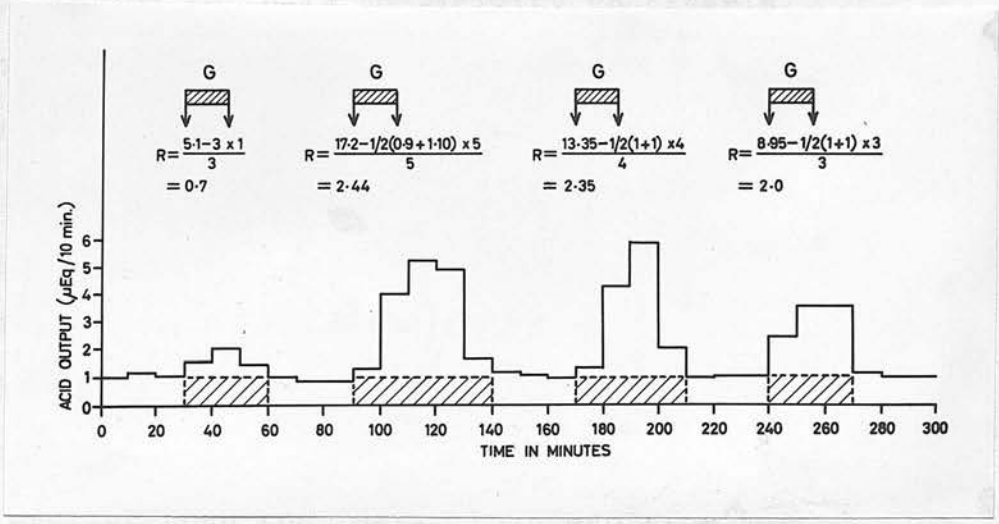

Fig. 4. The Measurement of Effect.

Male rat, 280 gm.

G = "Crude Gastrin" (stage 1) 0.5 mg/100 gm body weight in 1 ml given by IV infusion over 15 minutes.

R = Estimated 'mean rate of acid secretion'.

The response to the first dose was disregarded.

purpose of the assay, however, a 'resting' secretion which persisted at twice the original basal level (or higher) for more than 60 minutes, was taken as evidence of accumulation of the injected material and the assay of subsequent doses was therefore considered inaccurate.

4.4. Dose-Response Relationship

4.4.1 Sensitivity

Rats varied in their sensitivity to gastrin. Differences of up to 8-fold have been noticed, though most of them fell within the range of 4-fold.

4.4.2 Response to Repeated Identical Doses

Equal doses (0.5 mg/100 g. body weight) of "Crude Gastrin" (Stage 1) were given to the same rat. Response to the first dose was irregular and usually smaller than the rest. This was true for histamine (Ghosh, 1956). When subsequent doses were given in rapid succession, i.e. a second dose given as soon as the response to the previous one ceased, increasing rates of secretion were invariably observed, commencing with the third or fourth dose. Prolonged waiting in between doses delayed but did not prevent this tendency of increasing responses, which eventually supervened with the fourth or fifth dose in any one animal. Contrary to most reports on experience with cats or dogs, the effect of accumulation of injected

gastrin extracts in the anaesthetized rat manifested itself in a progressive increase in acid output. The duration of response, however, was not prolonged proportionately.

To overcome this interference of one dose with another, the following rules were observed:

(a) The effect of the first dose was disregarded, though useful in giving a rough guide to the sensitivity of the rat.

(b) After each dose, an interval was allowed which was equal to the period of response to that dose.

(c) The size of the doses was limited so that the peak acid output/10 minutes with any dose did not exceed 10 μEquiv.

With these precautions, the estimated mean rates of acid secretion for the second, third and fourth, and occasionally the fifth dose were closely similar (Fig. 5). In the circumstances of the experiment, then, the number of doses to be assayed in each animal had to be limited to three.

The results of experiments on 8 rats are shown in Table 1. There is no significant difference between the means of the 'mean rates of acid secretion' of the 3 doses. The response to the first dose in each rat has been ommitted.

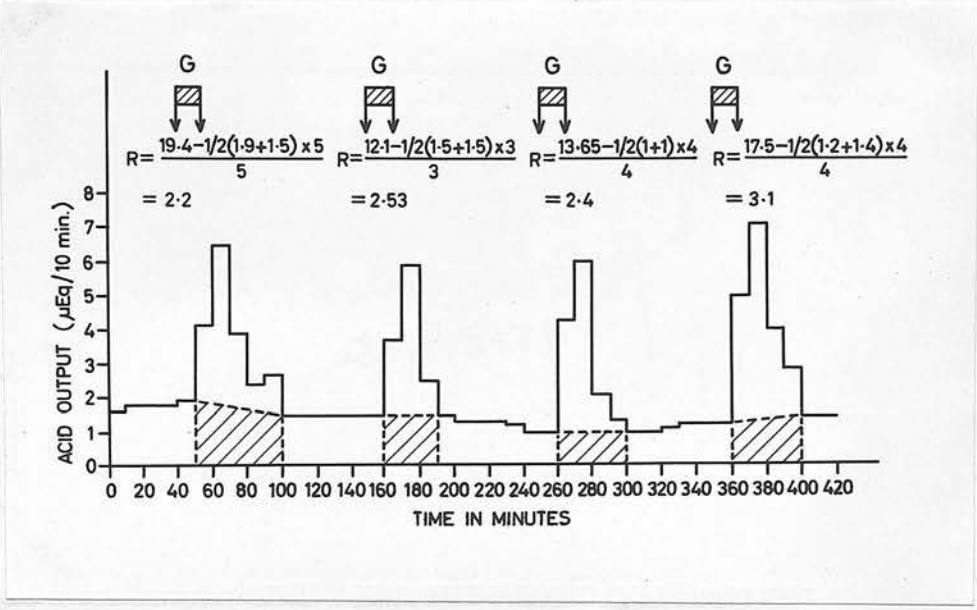

Fig. 5. Reproducibility of Effect with Identical Doses.

Male rat, 260 gm.

G = "Crude Gastrin" (Stage 1) 0.5 mg/100 gm body weight in 1 ml given by IV infusion over 15 minutes.

R = Estimated 'mean rate of acid secretion'.

The response to the first dose has been omitted. Responses to the second, third and fourth doses were comparable, but that to the 5th was increased.

TABLE I

REPEATED IDENTICAL DOSES

"Crude Gastrin" (Stage I material)

Each dose = 0.5 mg/100 Gm body weight of rat

Rat No.	Dose Order 2nd	3rd	4th	Block Total
1	0.65	1.04	1.68	3.37
2	0.85	1.50	1.20	3.55
3	1.23	1.60	1.80	4.63
4	1.20	0.60	1.00	2.80
5	2.20	2.53	2.40	7.13
6	1.64	1.42	1.42	4.48
Column Total	7.77	8.69	9.50	25.96
Mean	1.295	1.45	1.58	

Table of Analysis of Variance

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Between Columns	2	0.249	0.125	1.32	> .2
Between Blocks	5	3.941	0.788	8.34	.01-.001
Error	10	0.944	0.094		
Total	17	5.134			

For Differences between the Mean Responses to the 2nd and 4th doses:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S\bar{x}_1 - \bar{x}_2} = 0.051 \qquad d.f. = 10 \qquad P > 0.5$$

4.4.3 Responses to Graded doses

(1) "Crude Gastrin" (Stage 1).

Initial experiments were carried out with "Crude Gastrin" (Stage 1). Doses were given at 3 levels A, B, and C, graded on a logarithmic scale so that C was double B and 4 times A. Any error arising from bias in dose order was controlled by giving them in random order to a group of 6 rats so that all possible dose sequences were encountered within the group. The same rules as those for studying responses to identical doses were observed. The results are represented graphically in Fig. 6 and analysed in Table 2. It is evident that the individual log dose-response relationship is approximately linear in some animals only, and that in 2 animals, the response to dose A was greater than that to dose B. When the results were analysed as a group, however, there was highly significant regression of response on log dose.

(2) "Purified Gastrin", Batch A, freeze-dried.

Because impurities in the gastrin extracts might influence the response, the same experiments were repeated with material obtained at the end of the last stage of purification by the method of Gregory & Tracy (1961). The preparation was in crystalline form and derived from dessication of the active fraction of the eluate from the calcium phosphate gel column; it was highly potent. The

TABLE 2

DOSE RESPONSE RELATIONSHIP WITH GRADED DOSES

"Crude Gastrin" (Stage I Material).

Rat Number & Dose Order	A	B	C	Block Total
(1) A,B,C	1.96	2.93	4.50	9.39
(2) A,C,B	1.80	3.38	4.40	9.58
(3) B,C,A	3.70	3.50	3.46	10.66
(4) C.B,A	3.55	2.20	5.04	10.79
(5) C,A,B	4.04	5.50	5.87	15.41
(6) B,A,C	2.54	3.45	4.25	10.24
Column Total	17.59	20.96	27.52	66.07
Mean	2.93	3.49	4.59	

Table of Analysis of Variance

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Regression	1	8.217	8.217	14.6	.01-.001
Deviation from Regression	1	0.283	0.283	0.504	N.S.
Between Columns	2	8.50	4.25	7.56	.01
Between Blocks	5	8.264	1.653	3	.05
Error	10	5.624	0.562		
Total	17	22.388			

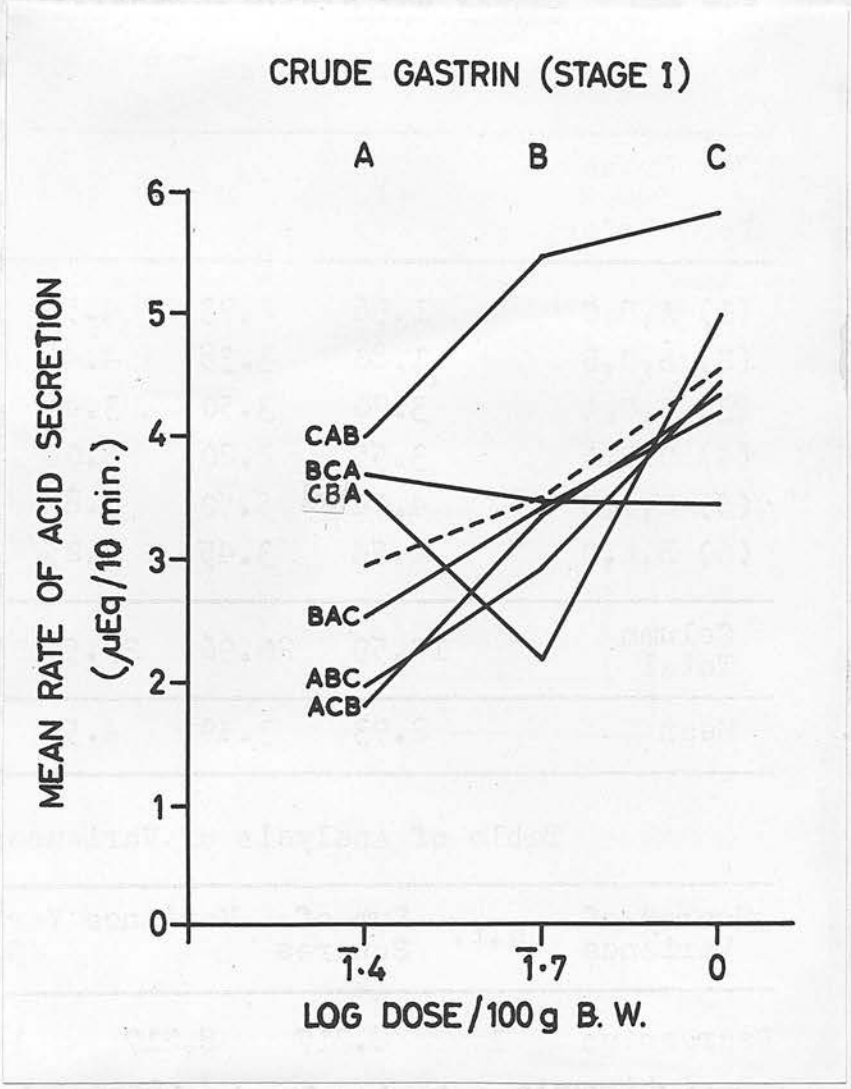

Fig. 6. Response to Graded Doses.

Dose A = 0.25 mg/100 mg body weight of rat.
Dose B = 0.50 " " " " " "
Dose C = 1.00 " " " " " "

Dotted line: mean of the responses.

The letters at the end of each dose-response curve indicate the order of doses given in that rat. This applies also to Figs. 7-12.

results (Fig. 7 and Table 3) showed that the dose-response relationship within the tested range was approximately linear in all 6 rats, and that the group analysed as a whole showed highly significant regression and little evidence of deviation from linearity. It also confirmed considerable variation between animals.

(3) "Purified Gastrin", Batch B, before freeze-drying.

This second batch of material was in the same stage of purity as Batch A, but it was in 0.70% NaH_2PO_4 solution, i.e., the active fraction of the eluate from the calcium phosphate gel, without freeze-drying. Experiments similar to those described above were carried out with this material. The results, as shown in Fig. 8 and Table 4, again gave highly significant regression, though the Error Variance was almost twice and the Variance Ratio for 'regression' only about a fifth of the corresponding values for Batch A.

(4) "Purified Gastrin", Batch B, freeze-dried.

A second series of identical experiments was done with Batch B after freeze-drying. The ensuing data as presented in Fig. 9 and Table 5 showed that, in addition to the highly significant regression as obtained before, both the Error Variance and the Variance Ratio for Regression were again close to those of the freeze-dried Batch A.

TABLE 3

DOSE-RESPONSE RELATIONSHIP WITH GRADED DOSES

"Purified Gastrin", Batch A, freeze-dried.

Rat Number & Dose Order	A	B	C	Block Total
(1) A,C,B	1.60	2.44	3.70	7.75
(2) A,B,C	1.20	2.43	3.22	6.85
(3) B,C,A	1.25	2.04	3.51	6.80
(4) B,A,C	2.00	3.40	4.70	10.10
(5) C,B,A	0.60	2.35	3.70	6.65
(6) C,A,B	1.35	2.80	3.30	7.45
Column Total	8.00	15.46	22.13	45.59
Mean	1.33	2.59	3.69	

Table of Analysis of Variance

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Regression	1	16.638	16.638	218.6	<.001
Deviation from Regression	1	0.018	0.018	0.237	N.S.
Between Columns	2	16.656	8.33	109.5	<.001
Between Blocks	5	2.851	0.57	7.5	<.001
Error	10	0.761	0.076		
Total	17	20.268			

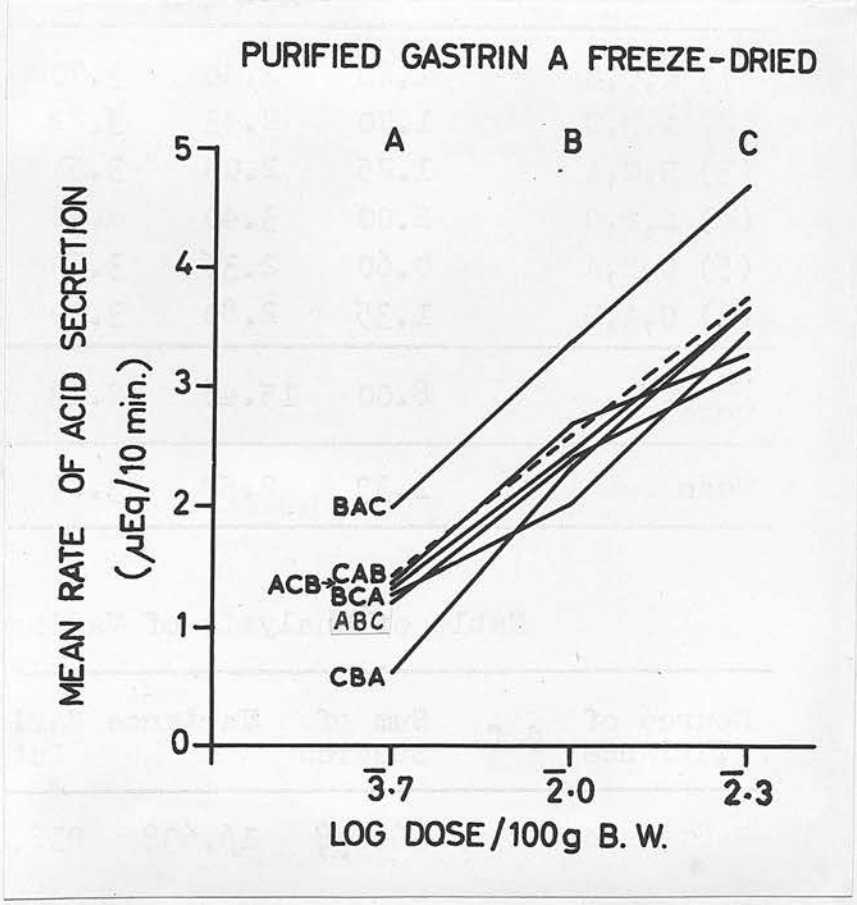

Fig. 7. Response to Graded Doses.

Dose A = 5μg/100 gm body weight of rat.
Dose B = 10 " " " " " "
Dose C = 20 " " " " " "

Dotted line: mean of the responses.

TABLE 4

DOSE RESPONSE RELATIONSHIP WITH GRADED DOSES

"Purified Gastrin", Batch B, before freeze-drying.

Rat Number & Dose Order	A	B	C	Block Total
(1) A,C,B	1.79	2.75	4.43	8.97
(2) C,A,B	1.94	2.65	3.35	7.94
(3) B,C,A	2.23	3.05	3.91	9.19
(4) B,A,C	2.60	2.70	3.26	8.56
(5) A,B,C	1.23	2.20	2.90	6.33
Column Total	9.79	13.85	17.85	40.99
Mean	1.96	2.67	3.57	

Table of Analysis of Variance

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Regression	1	6.496	6.496	46.4	< .001
Deviation from Regression	1	0.03	0.03	0.216	N.S.
Between Columns	2	6.526	3.263	23.3	< .001
Between Blocks	4	1.756	0.439	3.1	.05
Error	8	1.110	0.139		
Total	14	9.392			

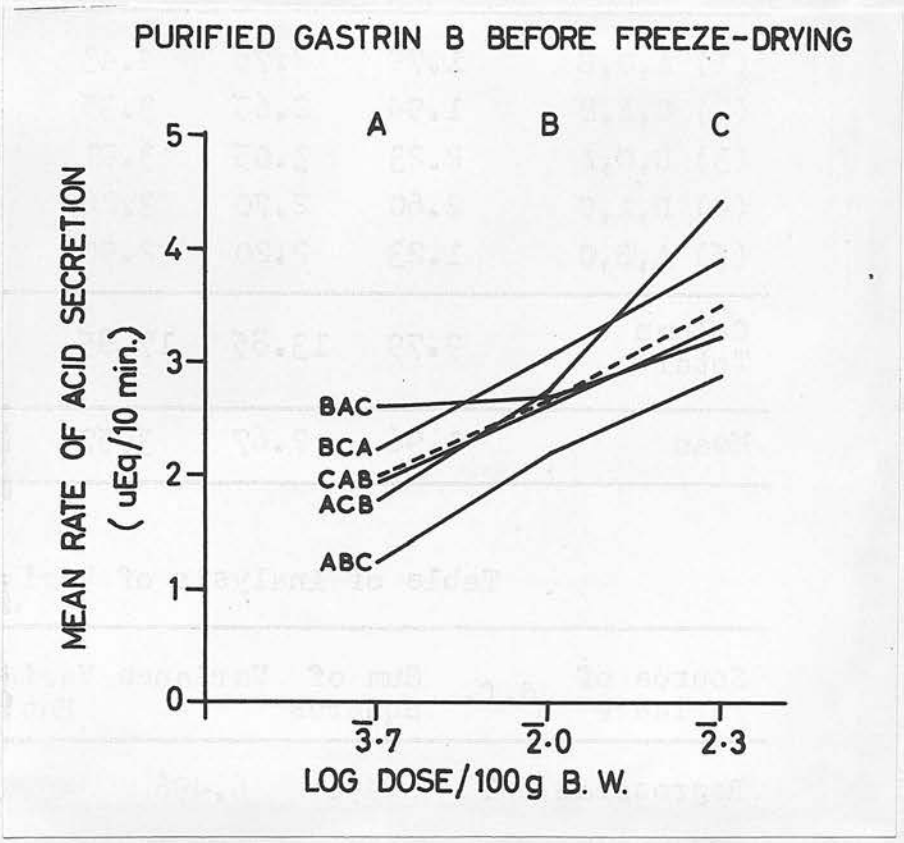

Fig. 8. Response to Graded Doses.

Dose A = 5.5 µg/100 gm body weight of rat.
Dose B = 11.0 " " " " " "
Dose C = 22.0 " " " " " "

Dotted line: mean of the responses.

TABLE 5

DOSE RESPONSE RELATIONSHIP WITH GRADED DOSES

"Purified Gastrin", Batch B, Freeze-dried.

Rat Number & Dose Order	A	B	C	Block Total
(1) A,C,B	1.70	2.90	3.74	8.34
(2) B,A,C	2.93	3.57	4.36	10.86
(3) C,A,B	2.10	3.30	4.51	9.91
(4) B,C,A	1.60	2.56	3.63	7.79
(5) C,B,A	1.85	3.10	4.43	9.38
(6) A,B,C	2.30	3.68	5.11	11.09
Column Total	12.48	19.11	25.78	57.37
Mean	2.08	3.185	4.3	

Table of Analysis of Variance

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Regression	1	14.7408	14.7408	233.2	$<.001$
Deviation from Regression	1	0.0001	0.0001	0.0016	N.S.
Between Columns	2	14.7409	7.37	116	$<.001$
Between Blocks	5	2.936	0.587	92.8	$<.001$
Error	10	0.632	0.0632		
Total	17	18.3086			

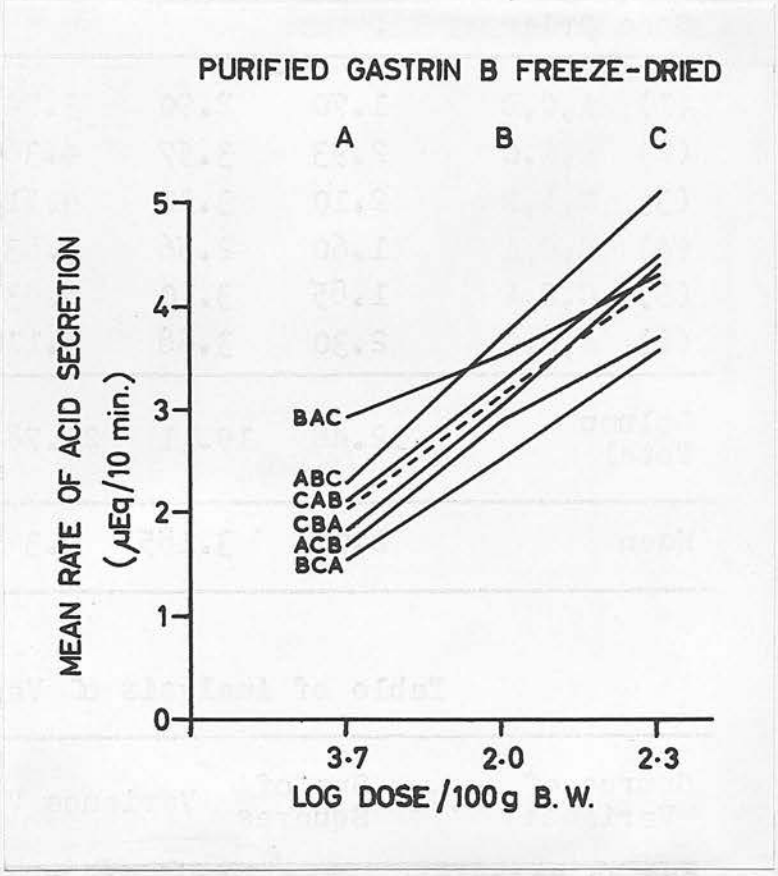

Fig. 9. Response to Graded Doses.

Dose A = 5.5 μg/100 gm body weight of rat.
Dose B = 11.0 " " " " " "
Dose C = 22.0 " " " " " "

Dotted line: mean of the responses.

(5) Hexamethonium Basal Infusion

To investigate the effects of blocking the vagal impulses on gastric secretion, with a view to minimizing possible variations on the 'basal' state during the experiment, Hexamethonium bromide was given as follows: 2 mg intravenously followed by a continuous infusion at the rate of 2 mg/hour throughout the experiment in 6 rats. Batch B "Purified Gastrin" before freeze-drying was given again in graded doses. No appreciable effect was noticed on the basal level of secretion or the magnitude of responses to the injected gastrin. The results are shown in Fig. 10 and Table 6. Regression was highly significant, but the Error Variance was raised.

(6) Bilateral Cervical Vagotomy

A further group of 6 rats was studied in the following manner: both vagi were exposed and dissected out at the neck at the beginning of the operative procedure and left till one hour later when they were cut without further handling to avoid stimulation. This procedure invariably produced marked changes in the respiration, which became much deeper and entirely abdominal in type, and the rat as a whole showed gross dyspnoea. Most of the rats died within 5 hours after vagotomy.

TABLE 6

DOSE-RESPONSE RELATIONSHIP WITH GRADED DOSES

Purified Gastrin, Batch B, before freeze-drying.
Basal infusion of Hexamethonium bromide.

Rat No. & Dose Order	A	B	C	Block Total
(1) B,A,C	2.40	3.62	4.61	10.63
(2) A,C,B	1.80	2.10	2.40	6.30
(3) B,C,A	1.90	3.35	4.35	9.60
(4) C,B,A	2.07	2.92	4.25	9.24
(5) A,B,C	2.76	3.20	3.53	9.49
(6) C,A,B	1.36	2.20	4.00	7.56
Column Total	12.29	17.39	23.14	52.82
Mean	2.05	2.9	3.86	

Table of Analysis of Variance

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Regression	1	9.81	9.81	44.7	< .001
Deviation from Regression	1	0.01	0.01	0.0455	N.S.
Between Columns	2	9.82	4.91	22.4	< .001
Between Blocks	5	4.15	0.803	3.7	.05-.01
Error	10	2.195	0.22		
Total	17				

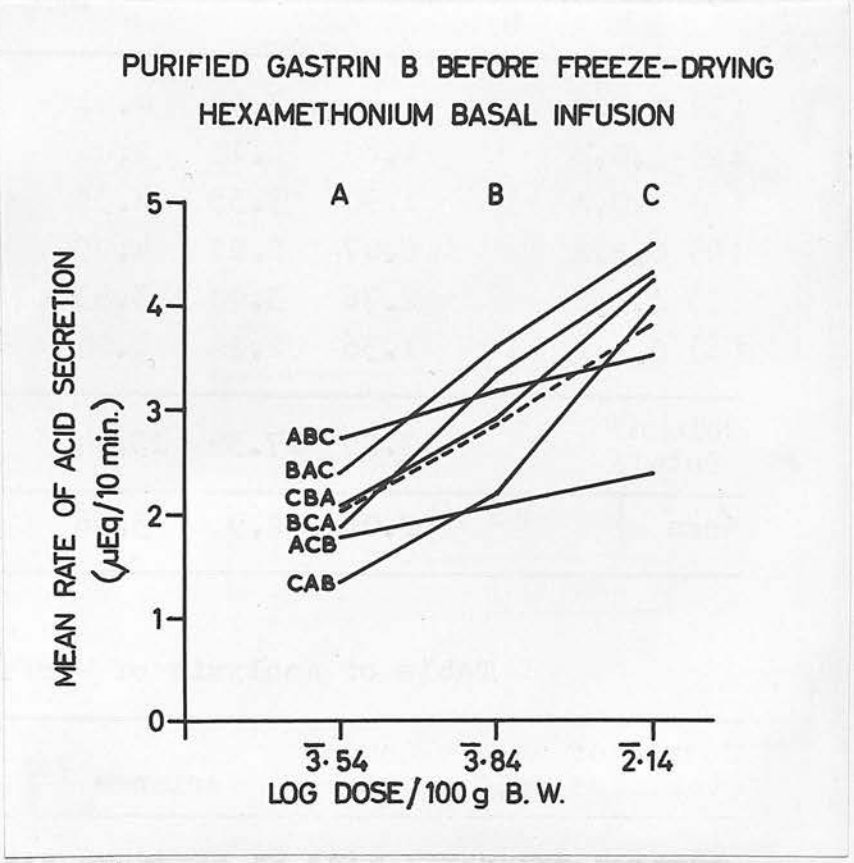

Fig. 10. Response to Graded Doses.

Dose A = 0.0035 ml gastrin solution (≡ 3.5 μg gastrin)/100 gm body weight)
Dose B = 0.0070 ml gastrin solution (≡ 7.0 μg gastrin)/100 gm body weight)
Dose C = 0.0140 ml gastrin solution (≡ 14.0 μg gastrin)/100 gm body weight)

) in 0.7 ml saline given by IV infusion over 15 minutes.

The pattern of response to injected gastrin was also altered. Sensitivity increased considerably so that a third or half the usual dose would produce effects similar to the full dose in other animals. Prompt returns to the baseline kept the duration of responses short. The calculated mean rates of acid secretion showed good correlation with log dose (Fig. 11, Table 7). The results of analysis of the data were similar to those from animals without vagotomy.

(7) Crude Gastrin Extract (Human Material)

Since the subsequent application of this method of bioassay entailed a comparison between the arbitrary standard and crude gastrin extracts of human material prepared by the method of Grossman, Tracy & Gregory (1961) described in Section 3.32, the dose-response relationship of a random sample of these crude extracts was examined. The doses were adjusted to give responses similar in magnitude to those with "Purified Gastrin". above. The results are shown in Fig. 12 and Table 8. It will be noted that the dose-response curves are all approximately linear, and that the regression was highly significant. The Error Variance was similar to that of "Purified Gastrin" Batch B without freeze-drying.

In conclusion, then, the above preliminary work showed that under the specified experimental

TABLE 7

DOSE RESPONSE RELATIONSHIP WITH GRADED DOSES
Purified Gastrin, Batch B, before freeze-drying.
Bilateral Cervical Vagotomy.

Rat No. & Dose Order	A	B	C	Block Total
(1) B,A,C	1.60	2.55	3.20	7.35
(2) B,C,A	1.70	2.43	3.80	7.93
(3) A,B,C	1.40	2.26	3.30	6.96
(4) C,B,A	1.13	1.50	1.84	4.47
(5) A,C,B	1.75	2.57	3.33	7.65
Column Total	7.58	11.31	15.47	34.36
Mean	1.52	2.26	3.1	

Table of Analysis of Variance

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Regression	1	6.225	6.225	77.8	<.001
Deviation from Regression	1	0.006	0.006	0.0741	N.S.
Between Columns	2	6.231	3.12	39	<.001
Between Blocks	4	2.577	0.644	8.05	.01-.001
Error	8	0.648	0.081		
Total	14	9.456			

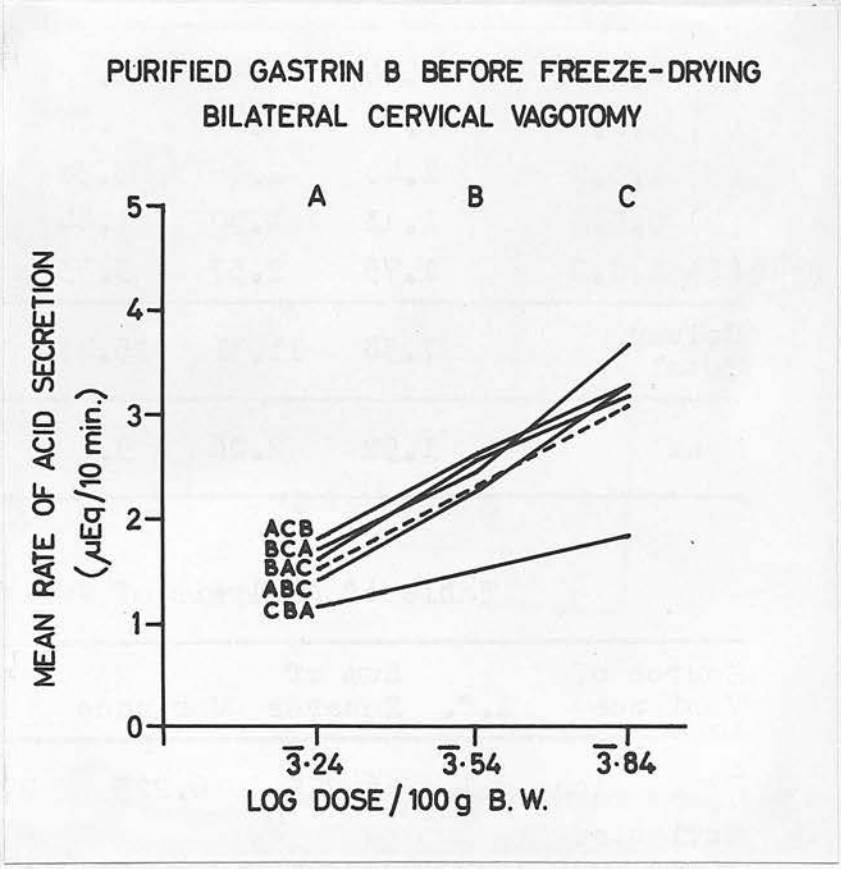

Fig. 11. Responses to Graded Doses.

Dose A = 0.00175 ml gastrin solution (≡ 1.75 μg gastrin)/100 gm body weight)
Dose B = 0.0035 ml gastrin solution (≡ 3.5 μg gastrin)/100 gm body weight)
Dose C = 0.0070 ml gastrin solution (≡ 7.0 μg gastrin)/100 gm body weight)

) in $\frac{1}{3}$ ml. saline given by IV infusion over 15 minutes.

Dotted line : mean of the responses.

TABLE 8

DOSE-RESPONSE RELATIONSHIP WITH GRADED DOSES

Crude Gastrin Extract (by method of Grossman, Tracy & Gregory)

Rat No. & Dose Order	A	B	C	Block Total
(1) A,B,C	1.30	2.24	3.36	6.90
(2) C,B,A	0.50	1.91	3.90	6.31
(3) A,C,B	2.10	3.70	4.50	10.30
(4) B,A,C	2.07	3.10	4.40	9.57
(5) C,A,B	0.70	1.32	2.30	4.32
(6) B,C,A	0.80	1.32	2.25	4.37
Column Total	7.47	13.59	20.71	41.77
Mean	1.245	2.26	3.45	

Table of Analysis of Variance

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Regression	1	14.6081	14.6081	103.0	<.001
Deviation from Regression	1	0.0278	0.0278	0.196	N.S.
Between Columns	2	14.6359	7.318	51.6	<.001
Between Blocks	5	10.690	2.14	15.1	<.001
Error	10	1.4179	0.1418		
Total	17				

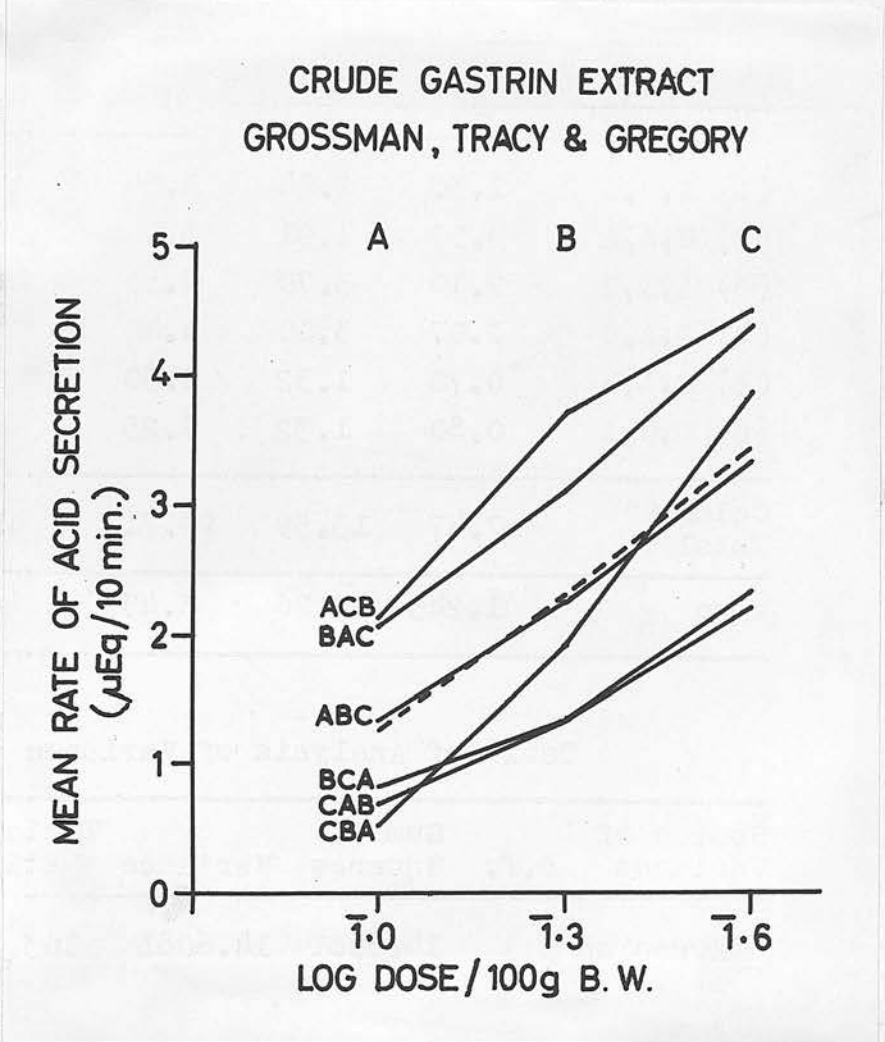

Fig. 12. Responses to Graded Doses.

Extract of human antral mucosa.

Dose A = 0.1 ml/100 gm body weight of rat.
Dose B = 0.2 " " " " " "
Dose C = 0.4 " " " " " "

Dotted line: mean of the responses.

conditions and method of assessment of response, the log dose:response relationship remained linear over the given range for both the crude and the purified gastrin preparations. To compare the accuracy of these estimations, however, the following data are extracted from Tables 2-8:

	Error Variance	Variance Ratio for 'Deviation from Regression'
"Crude Gastrin" (Stage 1)	0.56	0.50
"Purified Gastrin" Batch A	0.076	0.237
"Purified Gastrin" Batch B before freeze-drying	0.139	0.216
"Purified Gastrin" Batch B freeze-dried	0.063	0.0016
Hexamethonium infusion	0.22	0.045
Bilateral vagotomy	0.081	0.074
Crude Gastrin Extract (Human Material)	0.142	0.196

It will be noted that the Variance Ratio for 'Deviation from Regression' and the Error Variance with the freeze-dried "Purified Gastrin", Batch B were the smallest, being about $^{1}/_{300}$ and $^{1}/_{9}$ respectively of the corresponding values for "Crude Gastrin" (Stage 1).

4.5 The Procedure in the Bioassay - The Balanced Incomplete Block Design

In the design of a method of biological assay, it is desirable to arrive at one which would provide information concerning:

(a) the significance of linear regression and of deviation from parallelism of the dose-response curves of the Standard and the Test preparation.

(b) variability due to differences between blocks and between different positions in each block (order of administration of treatments), and the consequent effect on the potency estimate, and

(c) the error of the assay, and hence the fiducial limits of the estimate.

The simplest method answering to these requirements is the 4-point assay with construction of 4 x 4 Latin Square Designs. However, the strict limitation of three doses to each rat excluded this possibility. The difficulty was overcome by application of the Balanced Incomplete Block Design of Youden (1937-8, 1949-41). The statistics of the design and its application to this problem have been worked out by Mr. David Colquhoun with assistance from Dr. D.J. Finney. A simplified account of the mathematics involved is presented by Mr. Colquhoun in the Appendix.

There are many possible balanced incomplete block designs, but the pattern employed in this assay method had 12 treatments (doses) in each square, (i.e. group of 4 rats). An example is shown in Table 9. Each of the 4 rats received 3

TABLE 9

An Example of a Balanced Incomplete Block Design

(a)

Rat No.	Dose Order		
	1st	2nd	3rd
1	HS	LS	HT
2	HT	LT	LS
3	LS	HS	LT
4	LT	HT	HS

(b)

Rat No.	Dose			
	HS	LS	HT	LT
1	x	x	x	
2		x	x	x
3	x	x		x
4	x		x	x

HS = High dose of the standard
LS = Low dose of the standard
HT = High dose of the test preparation (Unknown)
LT = Low dose of the test preparation

treatments, which were so distributed that each column, i.e., the four first, second or third doses in the 4 rats (see Table 9(a)), was formed

by a complete replicate and that no repetition of treatment occurred in any rat. Table 9(b) shows the same block in which the doses have been re-arranged according to their size. It can be seen again that each block consists of 3 complete replicates of 2 graded doses (i.e. a 'high' and a 'low' dose) each of the Standard and the Test preparation. The 'high' doses were double the 'low' ones, and their sizes were adjusted so that the magnitude of the corresponding responses were similar. One hundred and forty-four different blocks of 12 treatments can be designed when taking into account variation in the order of animals used. Statistical analysis of results obtained gave all the necessary information as listed at the beginning of this section.

The smallest 'balanced' block comprised 4 rats, but greater accuracy of estimation could be obtained with more blocks and pooling of the results.

4.6. Fiducial Limits of the Estimate

In order to determine the error of the assay method, the same "purified Gastrin" (Batch B, freeze-dried) in different known concentrations was used as both Standard and Test and assayed against itself. Three blocks of 4 rats each were

used and the results analysed in single blocks, in combination of 2 blocks, and all 3 blocks together (The data for each block are shown in Table 10, and the results of analysis of these data in Table 11). The detailed calculations for a single block and the pooling of all 3 blocks will be given in Appendix IV. All the fiducial limits of the estimate included the true potency. For single blocks of 4 rats, the fiducial limit ranged from about ± 15% to about ±20%. When 2 blocks were combined, it was about ±10% to about ±20%. With 12 rats (3 blocks), it was down to ±12%.

4.7 Discussion

The observation that gastrin extracts were much more effective when given by slow intravenous infusion than when the same amount was given rapidly intravenously in the rat, confirmed the findings of Gregory & Tracy (1961) in dogs and man, and those of Blair et al. (1961) in cats. Sensitivity of the assay was thereby increased, with corresponding reduction in dosage and risk of accumulation of effect, and hence its choice as the method of administration in the assay.

The pattern of response limited the assessment of response to the only suitable measurement of 'mean rate of acid secretion'. A number of others, including the peak secretory rate and the total acid output to a dose with or without a

TABLE 10

Results of 3 Balanced Incomplete Blocks in the Assay of Purified Gastrin Batch B, freeze-dried.

BLOCK NO. 1

Dose Order / Rat No.	1st	2nd	3rd	Block Total
1	LS = 2.53	HS = 4.11	LT = 2.17	8.81
2	LT = 1.96	HT = 3.54	LS = 3.15	8.65
3	HS = 4.79	LT = 2.30	HT = 4.24	11.33
4	HT = 3.67	LS = 3.14	HS = 4.67	11.48
Column Total	12.95	13.09	14.23	40.27

BLOCK NO. 2

5	HS = 3.70	LS = 2.60	LT = 2.7	9.00
6	HT = 3.24	LT = 2.46	LS = 2.77	8.47
7	LS = 1.70	HT = 2.63	HS = 3.34	7.67
8	LT = 1.30	HS = 2.60	HT = 2.0	5.90
Column Total	9.94	10.29	10.81	31.04

BLOCK NO. 3

9	HS = 2.19	LS = 0.98	HT = 1.70	4.87
10	LT = 1.57	HS = 3.13	LS = 1.85	6.53
11	LS = 1.15	HT = 2.27	LT = 0.73	4.15
12	HT = 2.57	LT = 1.68	HS = 3.00	7.25
Column Total	7.48	8.06	7.28	22.82

TABLE 11

Results of analysis of data from Blocks No.1, 2, and 3

In all the blocks, the Standard ≡ Test preparation
= 1100 µg/ml.

Block Number	Estimated Potency of Test Preparation (µg of standard/ml)	Fiducial Limits of Estimate (P = 0.95) (µg of standard/ml)
1	1001	782 - 1207 (78.2%- 120.6%)
2	1186	940 - 1444 (79.3%- 121.7%)
3	1213	1028 - 1405 (84.7%- 115.8%)
1 & 2	1071	870 - 1270 (81.3%- 118.6%)
1 & 3	1090	945 - 1255 (86.6%- 115.1%)
2 & 3	1201	1071 - 1344 (89.1%- 111.9%)
1, 2 & 3	1115.4	978 - 1252 (87.7%- 112.3%)

specified time limit, have been found unsatisfactory.

The study on the effects of purity of the extract and of blocking of vagal influence on the dose-response relationships have led to the conclusion that the freeze-dried purified gastrin extract of Gregory & Tracy would form a suitable arbitrary standard for the assay of gastrin activity in the rat preparation. Hexamethonium infusion and bilateral vagotomy did not improve linearity of regression or reduce the error of the assay.

The balanced incomplete block design of Youden has hitherto been largely applied to agricultural problems, but its use in the present assay has been demanded by the restriction of the number of treatments in each block. The design of the block and the statistical analysis of data, however, have yielded at least as much information as would have been available from other designs with the same amount of data. The fiducial limit of the assay improved with more blocks so that the fiducial range with 12 rats was about half of that with 4 rats. When balancing economy of time with gain in accuracy of the assay, however, it was decided to use single blocks of 4 rats for each assay.

All subsequent assays are based on the assumptions: (1) that the effect produced by the active agent in the Test preparation (human tissue extracts) is identical with that in the Standard, (2) that this agent is the only one in the Test preparation which is capable of stimulating acid secretion, and (3) that all other impurities in the extracts are inert. Whether these assumptions are justified or not remains to be seen.

p. 85.

In the method of G T & G

& the tissue was ground with sand

5. REPRODUCIBILITY OF THE EXTRACTION PROCEDURE OF GROSSMAN, TRACY & GREGORY (1961).

To test this aspect of the method, 4-5 hog stomachs were obtained within 1 hour after death, washed clean of their contents, everted, and fixed in 5% TCA, then brought back to the laboratory. The pyloric mucosa was stripped off, cut up into small pieces (about $\frac{1}{4}'' \times \frac{1}{4}''$), thoroughly mixed, and then redivided into equal portions of 30 gm. each. These were extracted separately at the same time by the method of Grossman, Tracy & Gregory (1961) as described in Section (b) above, until the stage of the aqueous residue after removal of the TCA and acetone. These squeous portions were all kept deep frozen, and one portion was then taken each day and brought to the last stage of extraction in an identical manner. The final solution was made up to 12 ml in each of the portions, and then stored deep frozen till assayed.

Five batches of 3 portions each were thus extracted. Each batch was tested in a group of 3 rats: identical doses were taken from each portion, and each rat received one dose from each portion, administered in random sequence to avoid bias in treatment order. The results were analysed as for a 3 x 3 complete block and shown in Table 12. There was no significant difference between

Table 12

Reproducibility of the Extraction Procedure of Grossman, Tracy & Gregory (1961). Human Antral Extract.

Rat No. \ Dose	1	2	3	Block Total
1	3.37	3.7	3.7	10.77
2	1.8	1.6	1.74	5.14
3	1.95	1.6	1.45	5.00
Column Total	7.12	6.9	6.89	20.91

Source of Variance	d.f.	Sum of Squares	Variance	Variance Ratio	P
Between Columns	2	0.0092	0.0046	0.4	Not Significant
Between Blocks	2	7.1582	3.5791	294.1	$<.001$
Error	4	0.0486	0.0122		
Total	8	7.2160			

the responses to the different portions in the same batch.

It will be noted that the results only

indicated that similar proportions of the original total activity could be recovered from same amounts of tissue which were considered to possess the same amounts of activity at the start. It was assumed that this proportion remained constant throughout the subsequent work. The percentage recovery of activity remained unknown, though it is probably quite high. Quantitative comparison of activity between the extracts, however, is still valid.

PART III

QUANTITATIVE STUDIES OF GASTRIN-LIKE ACTIVITY IN THE HUMAN GASTRO-INTESTINAL TRACT.

6. THE DISTRIBUTION OF GASTRIN-LIKE ACTIVITY ALONG THE GUT

6.1 Introduction

In the study of the topographic distribution of gastrin-like activity in the gastro-intestinal tract, much attention has been paid to the stomach and far less to the rest of the gut.

Distribution in Animals

The evidence for the existence of gastrin in the antral mucosa of the dog, both on physiological and on pharmacological basis, has been reviewed. The presence of antral gastrin in other animals has been supported, so far, mainly by the demonstration of activity in extracts of the antral mucosa. This applies to the hog (Edkins, 1906; Uvnäs, 1943, 1945; Munch-Petersen et al., 1944; Harper, 1946; Jorpes & Mutt, 1952; Gregory & Tracy, 1959, 1960, 1961; Blair et al., 1961; Fletcher et al., 1961), cat (Uvnäs, 1943; Blair et al., 1961), and the cow and sheep (Fletcher, 1961). Edkins (1906) also detected activity in extracts of the cardiac mucosa of hogs; this has been confirmed by Gregory & Tracy (1961). The fundic (or body) mucosa was persistently negative in the hands of many workers.

Keeton & Koch (1915) had noted much activity in the pyloric, fundic, and cardiac mucosa of the hog, far less in the duodenal mucosa, very small amounts in the oesophageal mucosa, and none in the pancreas. Since the presence of histamine could not be excluded from the extracts with certainty, the validity of the positive results is to be questioned. However, the negative result in the pancreas was probably significant.

The gastrin content of the stomach wall at various depths has also been studied. Lim (1922-23) noted that the major part of the activity was found in the superficial portion of the pyloric mucosa. Blair et al. (1961) have confirmed his findings. Baugh et al. (1958) and Baugh (1961), by studying the mode of release of gastrin from pyloric pouches made from the superficial layer of the mucosa only (e.g. down to the muscularis mucosae) came to the conclusion that the source of gastrin was concentrated in the deeper parts of the gastric glands. This is in accordance with the suggestion by Redford et al. (1962) based on indirect evidence.

Further down the gut, Sircus (1953) provided physiological evidence strongly supporting the existence of a hormonal mechanism in the upper small intestine which stimulated acid gastric

secretion. He showed that in a dog distension of a duodeno-jejunal loop, amongst other stimuli, led to acid secretion from a denervated fundic pouch of the stomach, and that this stimulus could be nullified by prior procainization of the mucosa of the loop. It would appear that the known mechanisms of release and mode of action of this intestinal hormonal agent are similar to those of gastrin.

Komarov (1942b) extracted some activity from the upper and lower duodenum, a little activity from the jejunum, and none from the liver of the dog. Uvnäs (1943a) in contrast, could find no extractable activity from the duodenal, ileal, and colonic mucosa of the dog, cat, and pig. Harper (1946) reported activity in the duodenal mucosa of the hog. All of the above authors tested their extracts by intravenous infusion into anaesthetized cats. Gregory & Tracy (1961), however, could find no activity extractable from hog and dog duodenal mucosa, when tested in the conscious dog, though much activity was found in hog antral mucosal extracts prepared by the same method (which yielded material of high purity and therefore minimized possible interference by impurities) and tested in the same way.

Little attention has been paid to the normal

pancreas. Apart from Keeton & Koch (1915), only Hallenbeck et al. (1962) reported negative findings in the pancreas of the dog, hog, and monkey.

Distribution in Man

Extracts of the human gastric mucosa have been studied by several workers. Lim & Ammon (1923-4) extracted postmortem specimens (12-63 hours after death) and found little or no activity, though histamine was also probably present in their extracts. Ivy & Overhelman (1923-4) applied Keeton & Koch's method (1915) of extraction to gastric and duodenal mucosa obtained 1 hour to 4 days after death and found activity in all extracts, the gastric mucosa being approximately equipotent with hog antral mucosa, and the duodenal mucosa about half of that. Uvnäs (1945b) studied 24 postmortem human stomachs (obtained 10-36 hours after death) using the extraction method of Munch-Petersen et al. (1944) and reported considerable activity in 14 and slight activity in 4 extracts of the antral mucosa; extracts of the body mucosa and of the 'Boundary zone' between the body and antral mucosa were all negative. Fifteen postmortem specimens of duodenal mucosa were also extracted, and only slight activity found in 3, Harper et al. (1962), using their own extraction

procedures (Blair et al., 1961), also found activity in the antral mucosa obtained soon after death.

No published information is available for the gastrin-like activity in the rest of the gut.

The pancreas has attracted considerable attention since Zollinger & Ellison (1955) described 2 patients with pancreatic tumours associated with the syndrome which has since been named after them. Marked hypersecretion of hydrochloric acid from the stomach, with its sequel, was a prominent feature of the syndrome, and the causal relationship of the pancreatic tumours was substantiated when Gregory et al. (1960) demonstrated extractable gastrin-like activity in one of them. Ample confirmation was obtained later. Grossman et al. (1961) found activity in the primary pancreatic tumour as well as the metastatic deposits in the liver of another case of the syndrome. These results were confirmed by Code et al. (1962). Hallenbeck et al. (1962) reported activity extractable from Zollinger-Ellison tumours in 8 out of 9 cases; of these, 2 were primary tumours and the rest metastatic nodules. Three other pancreatic tumours without Zollinger-Ellison syndrome were also extracted: 2 produced hypoglycaemia and secretion from a Pavlov pouch (vagally innervated)

but not a Heidenhain pouch (vagally denervated) in a dog, and the third was negative. Normal pancreatic tissues were also negative.

6.2 Materials

Apparently normal tissues, confirmed histologically, were obtained at various distances along the gut from different patients at operation, and were extracted within 30 minutes of their resection by the method of Grossman, Tracy & Gregory (1961) as described in Chapter 2. The materials included:

(a) the pancreas, gastric antrum, duodenum, and a short segment of the adjacent jejunum from one patient in whom Whipple's operation was carried out for a localized leiomyosarcoma in the second part of the duodenum; (Patient A)

(b) 2 other pieces of pancreas from block dissection during gastrectomy for carcinoma of the stomach; (Patients B and C)

(c) 2 lengths of terminal ileum from a patient with endometriosis involving the gut and another patient with total colectomy for polyposis coli; and (Patients D and E)

(d) 2 portions of sigmoid colon removed from 2 patients because of redundancy leading to partial volvulus. (Patients F and G)

TABLE 13

Patient	Tissue	Unit Activity (μg Standard/ Gm Tissue)	Approximate Ratio of Unit Activity	Total Activity (μg of Standard) with Fiducial Range (ρ = .05)
A,B & C	Pancreas (3 specimens)	0	0	0
A	Antral Mucosa	46	13.5	1300 (74-130%)
A	Duodenal Mucosa	33.5	10	2180 (89-127%)
A	Jejunal Mucosa	12	3.5	
D	Ileal Mucosa (1)	3.4	1	
E	" " (2)	4.7	1.5	
F	Colonic Mucosa (1)	about 0.5		
G	" " (2)	about 1.5		

6.3 Results

The findings are shown in Table 13. It will be noted that:

(a) the highest concentration of gastrin-like activity occurred in the antral mucosa,

(b) there is a clear gradient of concentration of activity down the gut, with its maximum in the antral mucosa,

(c) although the unit activity in the duodenal mucosa was less than that in the antral mucosa, the total extractable activity was distinctly greater, and

(d) the normal pancreas persistently yielded no activity.

The histamine content of all these extracts have been estimated in the superfused guinea pig ileum preparation (Adam, Hardwick & Spencer, 1954) and found to be less than 50 ng/ml of the extract. The total volume of the extracts ranged from 5.5 - 13 ml each, and not more than 1 ml was used in each dose during the assay.

6.4 Discussion and Conclusion

In Edkins' (1906) original definition, the source of gastrin was limited to the gastric mucosa. However, the demonstration of gastrin-like activity extractable from the mucosa of the duodenum and upper jejunum confirmed the findings of previous workers and further substantiated the

physiological evidence for the presence of an intestinal phase of gastric secretion. It may seem appropriate, then, to expand the definition to include the agent from the upper small intestine which stimulated gastric secretion.

Since release of the hormonal agent from the antrum as well as the small intestine can be brought about by similar methods and inhibited by similar agents, it is tempting to suggest that the upper small intestine could play an important role in the physiological control of gastric secretion. This does not, however, necessarily mean that the active agents from the two sources are identical in structure, though their modes of release and action may be closely similar. Indeed there are apparent discrepancies. Thus the hog duodenal mucosa yielded no activity when extracted by a method which was highly effective with hog antral mucosa (Gregory & Tracy, 1961, Blair et al., 1961); and the duodenal mucosa of one animal might give active extracts with one method of preparation as tested in one animal and be negative with other methods of extraction and testing.

These observed discrepancies could be accounted for by one or more of the following possibilities:

(a) a difference in behaviour between the conscious and the anaesthetized animal.

(b) a real difference between gastrin of antral and duodenal origin,

(c) the presence of impurities in these extracts which inhibited gastric secretion in the conscious but not the anaesthetized animal, thereby concealing the stimulant effect on gastric secretion.

The answer must await repetition of these tests using the purified agent from the duodenum, whether it be gastrin or a gastrin-like substance.

Experimental evidence suggesting a role of the pancreas in the causation of peptic ulceration started when Elman & Hartman (1931) observed that diversion of the exocrine pancreatic secretion away from the duodenum almost invariably led to duodenal ulceration. Total pancreatectomy, with similar deprivation of the alkaline pancreatic juice, however, rarely did so (Dragstedt et al., 1939). Based on these findings, Poth et al., (1948) suspected that the islet tissues might produce an endocrine secretion causing peptic ulceration. The abundant activity extractable from Zollinger-Ellison tumours supports the above idea in so far as the pathological pancreas is concerned. However, the persistent absence of gastrin-like activity in the normal pancreas, in confirmation of the findings of Hallenbeck et al. (1962), seem difficult to fit in with the postulation. Whether this is a quantitative or really qualitative difference remains to be seen.

Zollinger & McPherson (1958) and Summerskill (1959) had reported instances where the Zollinger-Ellison syndrome occurred in patients in whom the pancreas showed hyperplasia of the islets (most probably the alpha cells) without any detectable neoplasm. Furthermore, Summerskill et al. (1961) noted that histologically normal pancreas could yield activity when the rest of that same gland was studded with multiple adenomata which yielded much higher activity when extracted and tested in the same manner. These findings could of course be interpreted either, (a), that the amount of gastrin-like substance produced (if any) by the normal number of islet cells could not be detected by the methods of extraction and assay used, or (b), that the normal pancreas produced no gastrin but the factors which (probably) led to hyperplasia and neoplasia in these islets also conferred upon them the ability to produce a gastrin-like substance.

Two further points are worthy of note:

(1) There has been, so far, no positive histological identification of the cell type(s) in these Zollinger-Ellison tumours (Zollinger & Craig, 1960; Chvoyka, 1961), nor real proof that they did arise from islet cells, apart from morphological resemblances to the alpha cells in some of the cases.

(2) Morphologically, no single type of cells has been found common to all the known sites of formation of gastrin (or gastrin-like substances) in man, viz., the antral and duodenal mucosa and Zollinger-Ellison tumours. However, the pancreas shares a common embryological origin with the gastro-intestinal mucosa in the endodermal lining of the gut (Ham & Leeson, 1961, and others). If one assumes that gastrin is produced by only one type of cells, and that all gastrin-like substances are in fact gastrin, then it may be that cells from this common embryological origin retain their potential ability to produce gastrin irrespective of the final differentiation.

The solution to this problem most probably lies in a reliable method of identifying the 'gastrin cell'. The immunological approach with the fluorescent antibody technique, as suggested by Monaco et al. (1961) may prove valuable.

Little interpretation can be made of the presence of gastrin-like activity in the lower gut. It is perhaps appropriate to re-emphasize that the antral and upper small intestinal mucosa are, to date, the only sites where the existence of gastrin

(or gastrin-like substances) is supported by physiological evidence. All other active fractions are extraction products. This does not exclude their existence, but their physiological role has not been established. Their positive identification on biochemical grounds in future may add more meaning to the present findings.

7. THE GASTRIN-LIKE ACTIVITY IN THE STOMACHS OF PATIENTS WITH PEPTIC ULCERATION AND GASTRIC CARCINOMA

7.1 Introduction

Little published data is available for the contents of gastrin-like activity in the human gastro-intestinal tract in disease, due perhaps partly to lack of a sensitive and accurate method of assay of gastrin.

Uvnas (1945b) obtained operative specimens of stomach from 4 patients with chronic duodenal ulcers, 5 with chronic benign gastric ulcers, and 2 with gastric carcinomata. The gastric mucosa was extracted, within 1-2 hours after resection, by the method of Munch-Peterson et al. (1944) and assayed in the anaesthetized cat. In 8 out of the 9 cases with peptic ulceration and 1 of the 2 cases of carcinoma, the antral mucosal extracts excited marked acid secretion. The body mucosa was persistently negative.

Ferguson (1950) also studied stomachs removed at operation from patients with peptic ulceration and gastric carcinoma. The pyloric mucosa was extracted with boiling N/10 HCl and the active fraction was precipitated by 10% TCA and stored as dry powder. Activity was assayed in anaesthetized cats with vagotomized isolated stomachs. In this

series, there were 15 patients with inactive duodenal ulcer, i.e., a typical history, a scarred duodenum and usually some past complication but no recent symptoms; 9 had active duodenal ulcer, i.e. persistent pain, high acid secretion, and an active ulcer with crater; 2 had chronic gastric ulcers, 9 had gastric carcinomata, and 2 had apparently normal stomachs. The mean unit activity of the mucosa (mEq HCl secreted in one hour by the cat/gm mucosa extracted) was similar with the inactive D.U's (.029 $\pm$ .005 standard error) and gastric carcinomata (.026 $\pm$ .01). Both the gastric ulcers and the normals fell within this range. The active D.U.'s, however, was about 4 times as large (.125 $\pm$ .021. Studies on 23 dogs gave corresponding values of .022 $\pm$ .004. These findings were taken to suggest that hypersecretion of acid may be due partly to antral hyperactivity in gastrin production.

The results of the above authors can only be taken as roughly quantitative, since the well known variation of responses between animals has not been overcome, and the pooling of data from a group, which was only assumed to be homogeneous, in the calculations is not entirely warranted.

Harper et al. (1962) reported the study on the gastrin-like activity in the antral mucosa of operative specimens from a series of patients with

peptic ulceration and gastric carcinoma. The 'normal' controls were apparently normal stomachs obtained soon after death. The methods for the preparation of the extracts and their assay in the anaesthetized cat have been reviewed (p.48,26). They showed that the amounts of gastrin-like activity extractable from equal portions of hog antral mucosa which have been left at room temperature for periods up to 22 hours were closely similar. This tested the reproducibility of their extraction procedure and the stability of gastrin in tissues.

The results on the human material revealed that the concentration of gastrin in the antral extracts from cases of gastric ulcer is about the same as that from normal controls but only about half of that from cases with D.U. Extremely low unit activity was found in one case of gastric carcinoma and one of intestinal metaplasia of the antral mucosa. These findings agreed in general with those of Ferguson (1950). The body mucosa was also negative.

7.2 Materials

Partial gastrectomy specimens (Bilroth I or Polya operation) from 34 patients were studied, comprising 27 with chronic duodenal ulcers, (D.U.) 3 with benign chronic gastric ulcers, and 4 with

gastric carcinomata. All patients had the following tests done before operation:

(a) The basal secretion - the acid output during a one-hour period at 'basal' state. Two or more values were obtained from each patient and the mean taken.

(b) The 'Maximal Histamine Output' (Card et al., 1955) - Histamine phosphate 0.04 mg/kg body weight was given subcutaneously under anti-histamine cover, and the total acid output during the ensuing hour was determined by titration to Topfer's reagent.

In addition, 22 of the patients with duodenal ulcers had an Insulin Secretion Test done, the total acid output during a two-hour period following an intravenous dose of soluble insulin (0.1 unit/kg body weight) being taken as the response.

In all the above tests, continuous aspiration of gastric juice was practised. All tests were done at least 4 days before operation.

Duodenal Ulcer Cases

This group of patients were selected in the sense that they all had definite indications for surgery, mostly because of complicating stenosis in the duodenum or pylorus, and the remainder because of persistent dyspepsia or high acid secretion. They have been separated into groups, as follows:

(a) Uncomplicated cases (7 patients)

(b) Cases with severe stenosis, evidenced by a fasting aspirate of 100 ml or more, radiological evidence of gross dilation of the stomach, and/or operative confirmation (5 patients)

(c) Cases with severe stenosis and dilated antrum as well as the body of the stomach (2 patients).

(d) Cases with mild to moderate stenosis, not included in the above (10 patients).

(e) Cases with combined gastric and duodenal ulcers (3 patients). All of the gastric ulcers were well healed scars in the antrum.

The reason for this classification will be apparent later.

Gastric Ulcer Cases

Two of these required surgery because of recurrence after medical treatment, and the third was operated on for massive haemorrhage from the ulcer.

Gastric Carcinoma Cases

All were adenocarcinomata. Three were pre-pyloric lesions (two ulcerative and the third proliferative), and the remaining one in the body of the stomach on the greater curvature.

All specimens of stomach were opened at the lesser curvature, washed clean of mucus, and divided at the border between the antrum and the body. This border could roughly be judged by the

naked eye with some accuracy. Occasional strips of mucosa at the supposed border were studied histologically and all found to be within 0.5 cm of the true border. Both antral and body mucosa were then stripped, weighed, and extracted separately. The final volume of extract from each portion was 10-12 ml. Each extract was assayed, and the total gastrin-like activity then worked out. Unit activity per gram of mucosa was also calculated.

7.3 Results

The results are summarized in tables according to their groups (Tables 14 & 15).

In Fig. 13, the total gastrin-like activity extractable per antrum was plotted against the 'Maximal' histamine output. It will be noted that, in general, the contents of gastrin-like activity in uncomplicated D.U.'s are smaller than those with stenosis, that benign gastric ulcers and carcinomata all possess considerable activity similar to or even greater than the average uncomplicated D.U., and that the maximal histamine outputs of patients with severe stenosis were higher than those of the rest. This last finding is in accordance with the results of Hunt & Kay's (1954) analysis using Ihre's (1938) data, and presumably indicate an increased parietal cell mass

TABLE 14 Data on Patients with Duodenal Ulcers

Grouping	Patient Number	Age (Yrs)	Duration of History (Yrs)	Body Weight (Kgm)	Antral Mucosal Weight (Gm)	Basal Secretion		Maximal Histamine Output (m Eq/hr)	Insulin Secretion Test (m Eq/2 hrs)	Unit Activity (μg standard per Gm Tissue)	Total Activity (μg standard) with Range (P = .05)
						m Eq Hcl per Hour	P.S. (ml/Hour)				
Uncomplicated	3	61	8	52.3	33	2.64	39.2	40	73.45	19.9	655 (63-140%)
	9	41	10	70.5	29	4.8	41.6	59.8	52.5	14.2	413 (84-119%)
	11	57	15	77.8	22	7.0	85.4	51.07	79.83	32.4	713 (95-107%)
	14	43	18	64	27	8.37	159.0	51.62	41.6	5.2	160 (90-111%)
	15	33	8	61	18	2.56		35.8		32.4	583 (60-143%)
	27	61	5	67	22	6.5	60.8	33.59	76.9	36.2	796.3 (82-126%)
	29	61	18	59	14	9.7	93.0	44.6	43.4	46.8	655.4 (68-138%)
Severe Stenosis	5	39	9	70.9	33	4.9	59.0	60.6	85.86	27.3	901 (91-110%)
	10	19	4	70	32.5	15.6	118.0	60	90.86	23.4	761 (78-126%)
	13	55	10	60	27	16.8	103.2	69.84	91.6	57.3	1547 (70-143%)
	25*	41	10	77.3	26	7.2	48.0	81.41	99.5	91.1	2470 (55-140%)
	33*	46	10	60	23	13.5	100.8	61	80.92	160.7	3695 (84-124%)
Severe Stenosis Antrum Di-lated	6	36	12	59.5	43.5	4.41		40.56		6	261 (66-150%)
	18	25	1 1/4	58.7	25	1.6	38	34.95	28.46	6.3	158.3 (75-131%)
Moderate Stenosis	4	30	12	77.4	34	6.84		46.5		25.1	1155 (75-140%)
	7	47	22	87	24.5	4.0	34.4	52.54	49.16	95.12	2330 (78-120%)
	12	45	15	77.7	15.5	1.87	25.6	36.4		26.7	413 (83-120%)
	16	51	6		22	9.52	77.2	34.05		127.7	2810 (84-119%)
	17	25	30	77	22	1.39	19.5	72.15	50.27	99.4	1839 (75-135%)
	23	42	8	55	21	5.1	42.9	44.9	42.8	129.3	2716 (85-118%)
	24	62	10	57.5	27.5	3.0	26.7	47.0	10.1	125.3	3445 (70-140%)
	31	44	19		32.5	2.8	26.8	41.7	40.8	16.6	540 (73-126%)
	32	55	5	70	24	5.2	41.3	34.8	46.2	76.3	1832 (81-132%)
	34	53	20	73	14.5	1.1	11.6	38.8	31.5	101.8	1477 (63-166%)
With Gastric Ulcer	26	56	12	72.7	24	2.6	25.1	17	21.9	100	2335 (80-120%)
	28*	15	2	45	25	10.7	81.1	37.55	66.01	65.3	1631.5 (82-125%)
	30	41	20	52	20.5	1.9		38.5		61.7	1265.7 (88-113%)

*Females

P.S. = estimated parietal component of gastric secretion

TABLE 15 Data from Patients with Benign Gastric Ulcer & Gastric Carcinoma

Diagnosis	Patient's Number	Age (yrs)	Duration of History (yrs)	Body Weight (yrs)	Antral Mucosal Weight (gm)	Basal Secretion (m Eq/hour)	Maximal Histamine Output (m Eq/hour)	Unit Activity (µg standard per Gm Tissue)	Total Activity (µg standard) with Fiducial Range (P = .05)	Remarks
Benign Gastric Ulcer	20*	52	8	64	29	3.5	13	87.3	2533 (81-134%)	
	21	53	11	45	22.5	0.8	8.25	28.6	643 (74-127%)	
	22	67	10	60	22	1.0	14	50.9	1120 (71-145%)	
Gastric Carcinoma	1	56	3	50	30	0.5	5	21.6	650 (70-135%)	Pre-pyloric ulcer
	2	39	6	39.3	18	4.0	14	14.2	255 (75-127%)	Pre-pyloric ulcer
	8	47		60	41.5	0	0	25.7	1066 (84-119%)	Presented with spastic diplegia for 7 months. Large pre-pyloric ulcer.
	19	66	½	66.7	8.5	2.1	10.23	56.7	481.5 (60-155%)	Ulcer in middle of body of stomach.

* Male

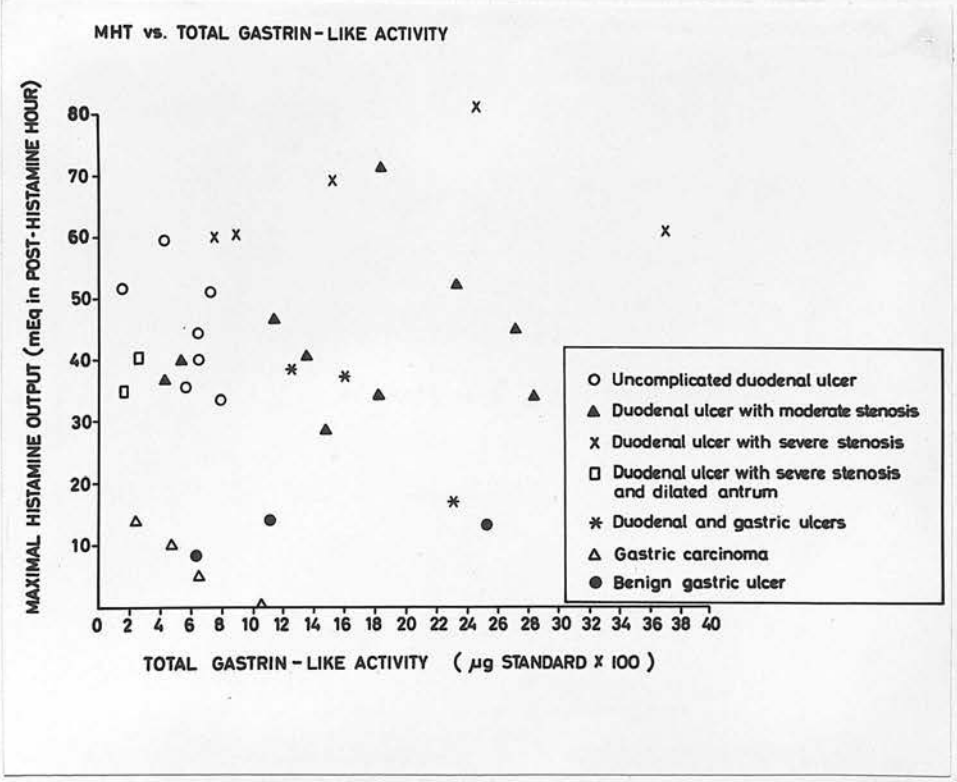

Fig. 13. Total gastrin-like activity plotted against 'Maximal Histamine Output'.

in the cases with severe stenosis. Both of the cases with dilated antra had very low total gastrin-like activity. The cases with combined duodenal and gastric ulcers, however, yielded more activity than the average uncomplicated duodenal ulcers.

No definite correlation exists between the total activity and maximal histamine output in any of the above groups. The same applied to the Basal Secretion plotted against the total gastrin-like activity (Fig. 14).

The total gastrin-like activity was also plotted against the Insulin-stimulated acid secretion (Fig. 15). It may be noted that the uncomplicated D.U.'s fell in line with a correlation coefficient of 0.73, and the slope was relatively steep. Also, the acid output of cases with severe stenosis were distinctly above the less marked ones. This could perhaps be explained by their larger parietal cell mass, as shown previously. In order to correct for that, the insulin-stimulated acid secretion in each case was divided by the maximal histamine output, and the ratio plotted against the total gastrin-like activity. The results as shown in Fig. 16 revealed that the correlation with the uncomplicated D.U.'s was improved ($r = .83$, $P < 0.5$), but all the stenosed cases now fell into the same zone, and

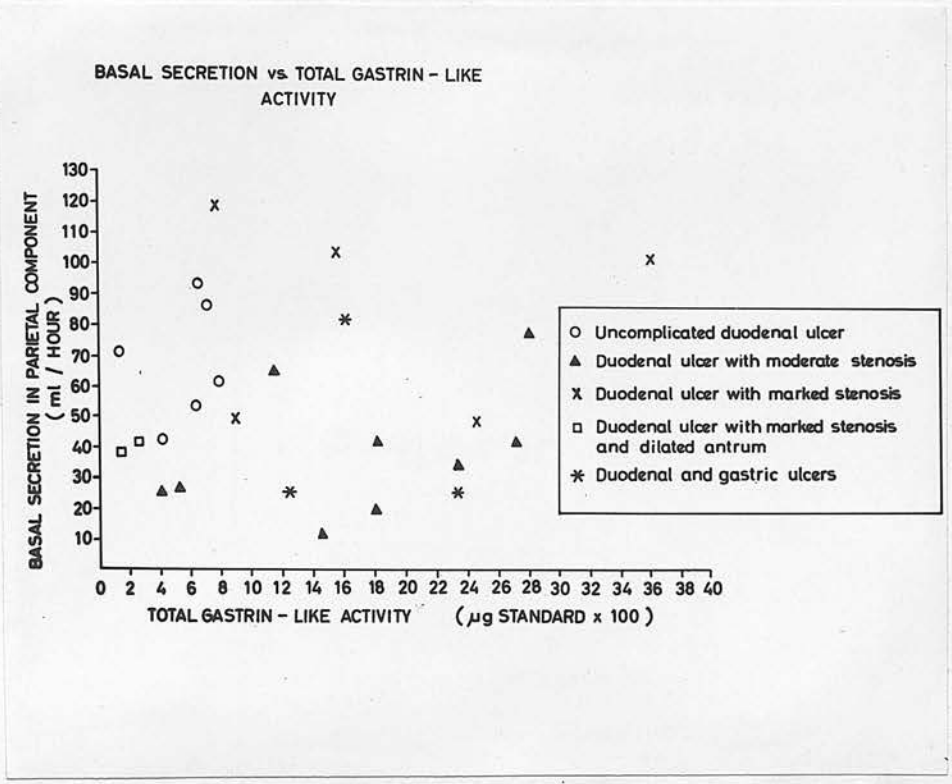

Fig. 14. Total gastrin-like activity plotted against Basal Secretion expressed as its estimated parietal component.

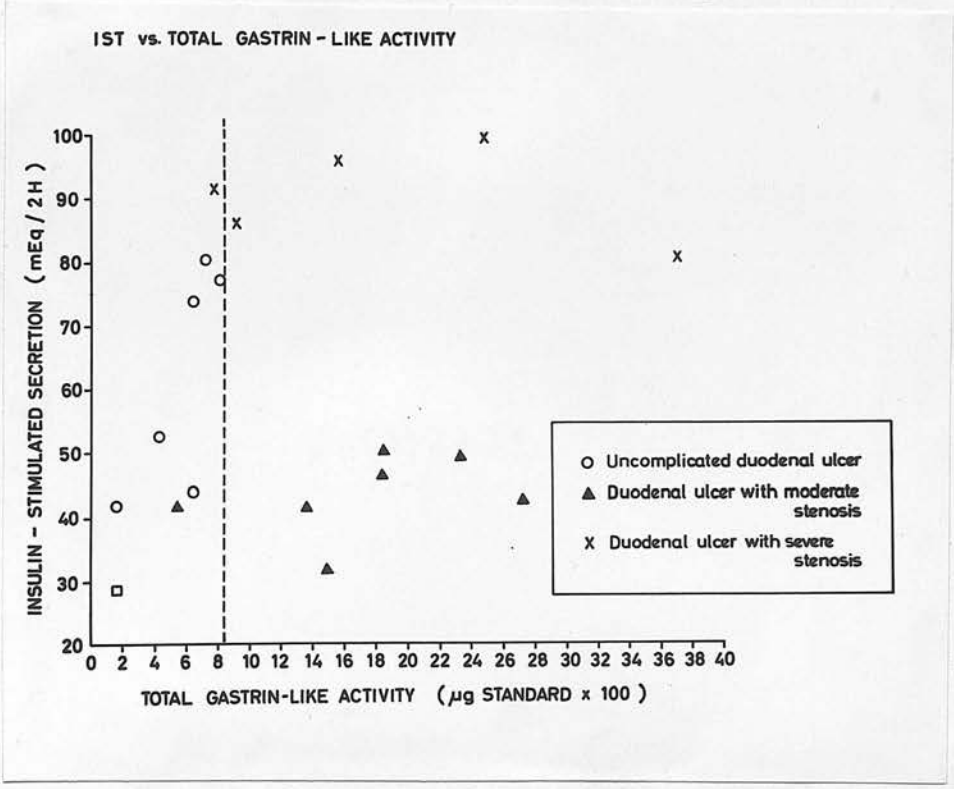

Fig. 15. Total gastrin-like activity plotted against Insulin-stimulated Acid Secretion.

The uncomplicated cases could largely be separated from cases with stenosis by the dotted line.

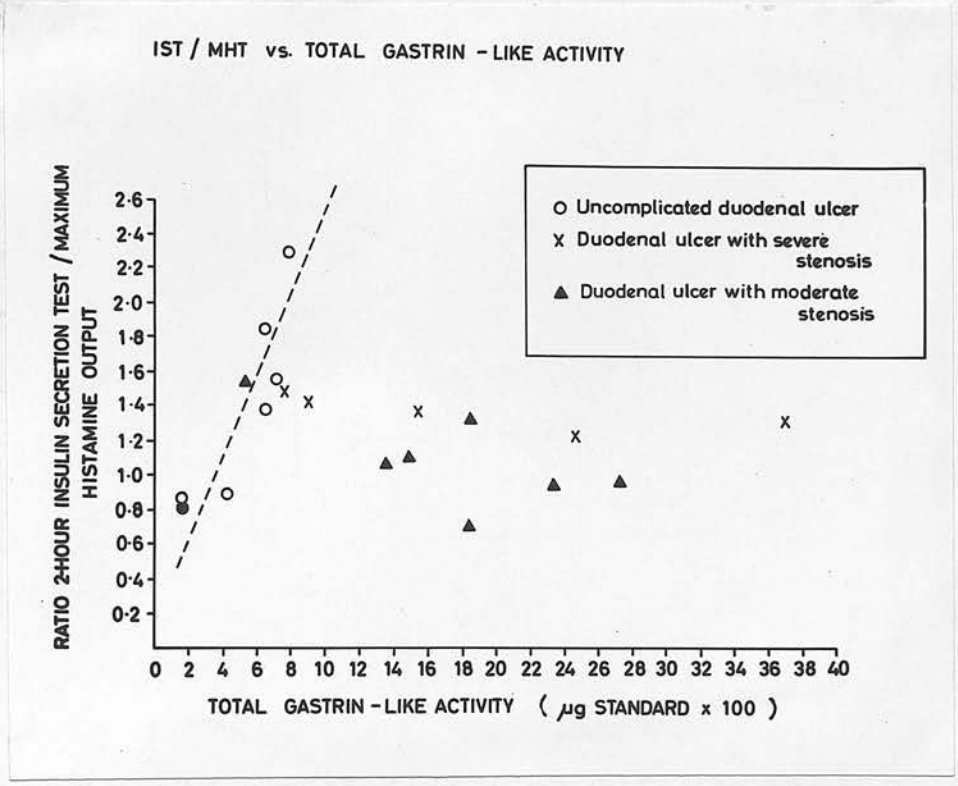

Fig. 16. Total gastrin-like activity plotted against the Ratio 'Insulin-stimulated Acid Secretion/ Maximal Histamine Output'.

Dotted line: the calculated regression line for uncomplicated cases.

$r = 0.83$ $P < .05$

though the correlation was not significant, the trend appeared distinct from the uncomplicated cases. It must be admitted that the number of uncomplicated cases in this study is small and hence sampling error possibly large. But the good correlation with or without correction for parietal cell mass suggests that they do form a separate group.

There is no correlation between total gastrin-like activity or unit activity on the one hand, and age and body weight of patient, duration of history, or size of the antrum on the other.

All body mucosal extracts were inactive.

7.4 DISCUSSION AND CONCLUSION

The relatively small number of cases in this series, aggravated by the need for further division into subgroups, makes interpretation difficult. The situation is made worse by a number of factors as yet unknown, e.g., the effect of anaesthesia and duration of operation on the gastrin content, the rate of turnover of gastrin and the significance of a single estimate of extractable gastrin-like activity. Any comment on the above results must therefore be speculative.

There is now good experimental evidence indicating that vagal excitation stimulates acid gastric secretion by (a), causing release of gastrin, (b), sensitizing the parietal cells to various stimuli including gastrin, and (c), direct stimulation of the parietal cell. If one assumes that, under strong and possibly maximal vagal stimulation by insulin-induced hypoglycaemia, direct stimulation of the parietal cells is responsible for a constant proportion of the acid output, then the remainder of the acid secretory response to insulin would be accounted for by the amount of gastrin liberated. Since, however, there is a positive correlation between the insulin-stimulated acid secretion and the total

gastrin-like activity in the uncomplicated D.U.'s in this study, it would seem reasonable to assume that, in these cases, the amount of gastrin liberated into circulation is proportional to the total gastrin-like activity extractable from the stomach. This is assumed to be true also for D.U.'s with stenosis.

Hunt & Kay (1954) have suggested that the increase in parietal cell mass in D.U.'s with stenosis was a result of repeated distension of the stomach with consequent stimulation of the parietal cells. The data presented in Fig. 15 and 16 is compatible with this view. The cases with stenosis had more gastrin in the antral mucosa (and hence probably in the circulation) than the uncomplicated cases. The observation that cases with mild as well as severe stenosis had similar total gastrin-like activity could be explained by the possibility of operative intervention at different phases of progression of the disease, it being assumed that a considerable time lag exists between the increase in gastrin content (and production) and the associated growth in the parietal cell mass.

The results in Fig. 16 indicate that the insulin-stimulated secretion per secretory unit, represented by the ratio 'insulin-secretion test/ maximal histamine output', remained about the same

despite increase in the total gastrin-like activity beyond a point at about the equivalent of '00 µg of the Standard. This might represent a plateau response to maximal or supramaximal levels of circulating gastrin.

The possible role of gastrin in the aetiology of duodenal ulceration has been suggested by Gillespie & Kay (1961) who showed that antrectomy alone led to healing of the duodenal ulcer in four patients. The Zollinger-Ellison syndrome provides an extreme example of possible effects of excessive gastrin (or gastrin-like substance) in circulation. The idea of gastrin possibly playing a role as a trophic hormone to the parietal cells has been summarized by Card (1962), who cited in support a case of Zollinger-Ellison syndrome of Dr. Bryan Alton when the 'maximal histamine output' of the patient progressively fell after partial resection of the pancreas and left adrenalectomy without any surgery on the stomach. The failure to demonstrate a correlation between the 'maximal histamine output' and gastrin-like activity in this present study does not support this idea but certainly does not exclude it, since apart from all the unknown factors mentioned above, this series could well have included cases with a large parietal cell mass to start with, irrespective of the gastrin content in the antrum.

The whole problem of the clinical significance of gastrin is obviously a dynamic one, a better approach to which would probably be the assay of gastrin in blood or urine, methods for which remain to be devised.

APPENDIX I (a) PHYSIOLOGICAL PROPERTIES OF GASTRIN EXTRACTS

Authors		Komarov (1938 (1942 a.b.)		Munch-Peterson et al (1944)	Uvnäs (1943a, 1945 a.b.)		Harper (1946)	Jorpes, Jalling & Mutt (1952)	Blair et al (1961)	Gregory & Tracy (1961)	Fletcher et al (1961)	
Tissues of Origin	Animal Species	Dog, Hog	Dog	Cat, Dog, Hog	Cat, Dog, Hog	Man	Hog	Hog	Cat, Dog, Hog, Man	Hog	Hog	Ox
	Mucosa Extracted	Pyloric	Duodenal + Fundic − Jejunal −	Pyloric	Pyloric ++ Fundic } + Cardiac } − Duodenal)	Pyloric + Fundic − Duodenal ±	Pyloric ++ Duodenal +	Pyloric	Pyloric	Pyloric ++ Duodenal − Cardiac + Fundic −	Pyloric ++	Pyloric ++ Fundic +
Animal Preparation Tested		Dog, G.F. (c)	Dog, Cat (G.A.)	Cat (G.A.)	Cat (G.A.)		Cat (G.A.)	Dog, P.P. (C) Cat (G.A.)	Cat (G.A.)	Dog, HP + GF (c) Man (c)	Dog, HP + GF (c)	
Route of Administration	Subcutaneous	−								++	++	
	Intramuscular	++	+							++		
	Rapid Intravenous	++	+							++		
	Slow Intravenous			++	++			Dog ±, Cat + (Linde 1950)	++	+		
Effect on Secretion of	Pepsin	±	+	−	−				Slight	−		
	Pancreatic Juice	−	++	−					++	−		
	Bile	−	++	−						−		
Effect on Gastric Tone				−					++	±		
Antagonism by Atropine		−							−	++		
Histamine Content				No Effect on B.P.	Mainly −		No Effect on B.P.	−	0.1 µg/mg	0.01 µg/ml (Stage III Eluate)	< 7 µg/dose (10-100 mg/dose)	
Potency		Purified Gastrin I = 6-10 mg/u Purified Gastrin II = 2.5 mg/u		Crude Extract 6 mg/u Purified Extract 2 - 2.5 mg/u	0.1 - 1 mg/u			0.1-1 mg/u	0.1-1 mg/u	Highly Potent	Max. Rate of Acid Secretion 1.8 m eq. per Hour.	

++ : much activity
\+ : definite activity
− : no activity
Blank : not tested

For Potency,
u = secretory unit
amount of gastrin extract causing secretion of 1 ml highly acid gastric juice/hour.

For animal preparations,
G.F. = Gastric fistula
P.P. = Pavlov pouch
H.P. = Heidenhain pouch
(G.A.) = general anaesthesia
(C) = conscious

APPENDIX I (b) PHYSICO-CHEMICAL PROPERTIES OF GASTRIN EXTRACTS

Authors			Komarov (1938, 1942 a.b.)	Munch-Peterson et al (1944)	Uvnas (1943 a.b.) (1945 a.b.)	Harper (1946)	Jorpes, Jalling & Mutt (1952)	Blair et al (1961)	Gregory & Tracy (1961)	Fletcher et al (1961)
Solubility In	H_2O			+	+(Minimum @ pH 6-8)		+	+	+	+(pH 10.5)
	0.9% Na Cl								+	+
	Dilute Acid e.g. 0.1N Hcl		+	+	+		+		+	
	Organic Solvents	Acetone 80%	+						+	
		Acetone 100%	-		-			-	-	
		Ether	-		-			-	-	
		Benzene			-		-			
		Alcohol	+		-					
		Methanol	+				+			
	Protein Precipitants	Tannic Acid		-	-				-	
		4-10% TCA	-	-	-				-	
		30% Na Cl	-	-	-				-	
Isoelectric Point (pH)					4 - 5.5		7.0		About 5.5	Bovine 3.4 Porcine 5-5.5
Dialysis Through Cellophane Membrane			No		Yes	No	No		Yes (Considerable loss)	No
Destruction By Enzymes	Pepsin			Yes	Yes	Yes				
	Duodenal Juice			"	"					
Inactivation by Ultra Violet Light					in 15 min.					
Stability	Room Temperature				+(24 hrs,pH7)		Some loss of activity after months		+(pH 10,15')	
	Heating in	Acid			+(boiling 30' 0.1N Hcl)N				+(75°C,30' pH3)	
		Alkali			-(boiling /10 NaOH)				+(75°C,30' pH8)	
	Refrigeration				+ 1 year			+ 4 yrs.		+ 8 months or more at -20°C
Remarks									Active fraction probably a peptide. 3-4 other inert substances	Peak absorption at 258-262 mn. Probably nucleotide-like substance

Stability. + Indicates Resistance to
- Indicates Destruction by

APPENDIX II. Preparation of Gastrin (Gregory & Tracy, 1961).

Stage I. (a) Fresh hog stomachs from abbatoir (fixed in 5% aq. TCA)
1 kgm of mucosa stripped in narrow pieces

↓

Add 2 L of 4% TCA in 90% acetone. Stand overnight (room temperature)

↓

Filter through fluted paper (Green 904½)

↓

(b) Filtrate + 10 ml 10N HCl. Extract once with 2 vol. & 3 times with 1 vol. of ether.

↓

Aqueous phase (volume x ml) + 2N NaOH till pH 3. Warm to expel ether.

↓

(c) Heat to 70-80°C. Add 2N NaOH till pH 5-5.5; copious ppt. Leave 10 min.

↓

Add NH_3 solution (0.88 NH_3 solution + equal vol H_2O) till pH 8.5. Leave 5 min. Cool

↓

Centrifuge 5 min. (about 500 G).

Supernatant (golden yellow, clear) + 10N HCl till pH 4.5

Residue + 0.1 NHCl (x ml), warm to 70°C. Partial solution. Leave 5-10 min.

↓

+ $\frac{1}{20}$ vol. of 30% NaCl solution

↓

+ 2N NaOH till pH 5-5.5; leave 10 min.

↓

+ dil. NH_3 solution till pH 8.5; leave 10 min. cool, centrifuge 5 min. (500G)

Supernatant + 10N HCl till pH 4.5 | Residue discarded

(d) Add NaCl crystals to final concentration of 30%, with constant stirring. Flocculent precipitate formed. Stand 60 min. at room temperature.

↓

Filter through coarse paper (Green 904) by suction with aid of 2G acid-washed hyflo-supercel. Wash cake with 30% NaCl, then suck dry.

(e) Resuspend cake in 100 ml 0.1 N HCl, and repeat precipitation at pH 5-5.5 and 8.5 as in (c) above.

↓

Combined supernatant + 10N HCl till pH 7. Cool to 10°C. (Vol. y cc.)

↓

(f) Add TCA crystals (to give final concentration of 4% in solution), dissolved in 20 ml distilled water, drop by drop (over 15 minutes) with constant stirring.

Heavy white precipitate formed. Stand in refrigerator for 45 min.

↓

Centrifuge 10 min. (500 G)

↓

(g) Supernatant discarded. Dry inside of cup.

Residue + y cc distilled water. Warm gently. Green solution.

Cool to 10°C and repeat TCA precipitation as in (f) above.

↓

(h) Residue + 100 ml acetone slowly. Green viscid solution formed first, then heavy flocculent precipitate.

↓

+ 10N HCl slowly (2 ml/100 ml acetone). Shake well.

↓

+ ether till cup full (about 100 ml). Shake well. Stand 5 min. Centrifuge.

↓

Supernatant discarded. Gummy precipitation resuspended finely in acetone. Centrifuge.

↓

Supernatant removed by suction. Precipitate shaken up with ether. Centrifuge.

↓

Ether sucked off. Residue left in cup overnight. Dry powder collected next morning and weighed and stored in the dark.

Usual yield: 600-700 mg/kgm mucosa.

APPENDIX II. Preparation of Gastrin (Gregory & Tracy, 1961) Continued.

Stage II.

600 mg of Stage I powder dissolved in 30 ml distilled water (may warm gently to aid solution but cool before proceeding).

↓

+ 2N NaOH slowly till pH 5-5.5; flocculent precipitate formed.

↓

+ few drops of piperidine till pH 10-10.5; perfectly clear green solution.

↓

+ 120 ml acetone slowly, with stirring. Curdy precipitate with cloudy supernatant.

↓

+ Dilute acetic acid slowly till pH 8.5

↓

Pour into dry centrifuge, shake vigorously, stand a few minutes, centrifuge briefly

Clear supernatant

↓

+ 600 ml ether, shake, stand

Aqueous phase

Ethereal Phase

+ 20 ml dist. H_2O, shake, stand

↓

Aqueous phase

Residue + 30 ml dist. H_2O

↓

+ 1-2 drops piperidine. Clear solution

↓

Pour into original beaker.
+ 0.1 ml 30% NaCl soln.

↓

+ 120 ml acetone slowly with stirring

↓

+ dil. acetic acid till pH 8.5

↓

Shake in centrifuge cup. Stand a few min. Centrifuge 5 min.

Clear Supernatant

Residue discarded

+ 600 ml ether, shake, stand

Aqueous phase

Ethereal phase + 20 ml. dist H_2O. Shake, stand a few min.

↓

Aqueous phase

Re-extract twice with 2-3 volumes of ether.

Stand overnight in wide dish in dark cupboard at room temperature.

Solution clear, faintly pigmented, with pH of about 7.

Stage III.

Calcium phosphate gel was prepared by slowly mixing equal volumes of 0.5 molar $CaCl_2$ and 0.5 molar Na_2HPO_4, boiling the mixture for an hour, and washing the precipitate with distilled H_2O till the pH was <10. Before use, the pH of the gel was adjusted to 7 by dilute acetic acid. A column was made with the gel, measuring 3-4 cm long and 15-20 mm diameter.

Stage II solution run on to column at 25 ml/hr.

↓

Column washed with 50 ml dist. water at 40 ml/hr.

↓

Elution of column with Na_2HPO_4 (0.07% anhydrous salt) at 10 ml/hour. Most of the activity usually eluted in the first 20 ml.

APPENDIX III. Preparation of Crude Gastrin Extract
(Grossman, Tracy & Gregory, 1961)

Mucosa (or pancreatic tissue) cleaned, weighed, and cut in small pieces (¼ in. x ¼ in.)

↓

Immerse in 10 vol. of 4% TCA in 90% acetone. Stand overnight at room temperature, with occasional stirring. Drain.

Supernatant (dark cloudy solution)

Tissue re-extracted twice with 5 vol. of 4% TCA in 90% acetone.

Supernatant

Residue discarded

Add 10N HCl (5 ml/litre of extract)

Extract once with 2 vol. and 3 times with 1 vol. of ether

↓

+ 2N NaOH till pH 3. Warm in bath at 70°C to expel ether. Measure volume of extract.

↓

Cool to 10°C. Carry out twice TCA precipitation as in Stage I procedure (f) & (g) in Appendix I.

↓

Precipitate + convenient volume of 0.15N HCl (about 1 ml/2-4 gm wet weight of mucosa)

↓

Extract once with 2 vol. and 3 times with 1 vol. of ether.

↓

+ 2N NaOH till pH 3. Warm to expel ether.

↓

Heat to 70-80°C. Add 2N NaOH till pH 5.5; dense ppt. Leave 10 min.

↓

+ NH_3 solution (0.88 NH_3 solution diluted 10 times) till pH 8.5; leave 10 min. Cool. Centrifuge 10 min. (500 G).

↓

Clear supernatant. Volume measured. Stored in deep freeze till assayed.

APPENDIX IV (a) (1)

Analysis of Variance for the Balanced Incomplete Block

Rat Number	Dose Order 1st	2nd	3rd	Block Total
1	LS_1	HS_1	LT_1	B_1
2	LT_2	HT_2	LS_2	B_2
3	HS_3	LT_3	HT_3	B_3
4	HT_4	LS_4	HS_4	B_4
Column Total	Ca	Cb	Cc	G

Rat Number	Dose HS	LS	HT	LT	Block Total
1	HS_1	LS_1		LT_1	B_1
2		LS_2	HT_2	LT_2	B_2
3	HS_3		HT_3	LT_3	B_3
4	HS_4	LS_4	HT_4		B_4
Column Total	V_{HS}	V_{LS}	V_{HT}	V_{LT}	G

t = number of different treatments (e.g. HS, LS, HT, LT)

k = number of doses per block

r = number of replicates (i.e. number of times each treatment appears in the 4 rats or their multiples.

b = number of blocks (i.e. number of rats)

$N = tr = bk$

G = grand total of all treatments within a balanced group.

$\lambda = \frac{r(k-1)}{t-1}$

V_i = total for i^{th} treatment within a balanced group (e.g. $V_{LS} = LS_1 + LS_2 + LS_4$)

$\frac{G^2}{N}$ = correction factor.

(A) For Columns (i.e. for effect of dose order)

$$\text{Sum of squares (s.s.)} = \frac{\Sigma C}{k} - \frac{G^2}{N}$$

(B) <u>For Blocks</u> (ignoring treatments, i.e., Interblock Variation)

$$\text{Sum of Squares} = \frac{\Sigma B}{b} - \frac{G^2}{N}$$

T_i = sum of block totals containing i^{th} treatment (e.g. $T_{LS} = B_1 + B_2 + B_4$)

<u>Interblock Treatment Component</u>

$$\text{s.s.} = \frac{\Sigma(T - \bar{T})^2}{k(r - \lambda)}$$

$$= \frac{1}{k(r-\lambda)}\left[\Sigma T^2 - (\Sigma T)^2/t\right]$$

$$= \frac{1}{k(r-\lambda)}\left[\Sigma T^2 - (kG)^2/t\right]$$

<u>Partition of s.s.</u>

$$\text{s.s.} = \frac{\Sigma aT}{4k(r-\lambda)}$$

$$\text{Regression s.s.} = \frac{(L_1)^2}{4k(r-\lambda)} = \frac{(-T_{LS} + T_{HS} - T_{LT} + T_{HT})^2}{4k(r-\lambda)}$$

$$\text{Parallelism s.s.} = \frac{(L_I')^2}{4k(r-\lambda)} = \frac{(T_{LS} - T_{HS} - T_{LT} + T_{HT})^2}{4k(r-\lambda)}$$

$$\text{Preparation s.s.} = \frac{(L_P)^2}{4k(r-\lambda)} = \frac{(-T_{LS} - T_{HS} + T_{LT} + T_{HT})^2}{4k(r-\lambda)}$$

(C) For Treatments (eliminating blocks, i.e. error depending on variation within rats only).

For each treatment, $Q_i = kV_i - \sum_r B_i$

where $\sum_r B_i$ = sum of block totals over blocks containing i-th treatment

(e.g. $\sum_r B_{LS} = B_1 + B_2 + B_4$)

$$\text{Sum of squares} = \frac{t-1}{Nk(k-1)} \sum Q^2$$

Partition of the s.s. :

$$\text{Regression s.s.} = \frac{(t-1)(L_I)^2}{4Nk(k-1)} \quad \text{where } L_I = -Q_{LS} + Q_{HS} - Q_{LT} + Q_{HT}$$

$$\text{Parallelism s.s} = \frac{(t-1)(L_I')^2}{4Nk(k-1)} \quad " \quad L_I' = Q_{LS} - Q_{HS} - Q_{LT} + Q_{HT}$$

$$\text{Preparation S.s.} = \frac{(t-1)(L_P)^2}{4Nk(k-1)} \quad " \quad L_P = -Q_{LS} - Q_{HS} + Q_{LT} + Q_{HT}$$

(D) Total sum of squares $= \sum (y - \bar{y})^2$

$$= \sum y^2 - \frac{G^2}{N}$$

Potency Ratio

The standard was expressed in actual weight of material in the dose (viz. HS = 11 µg). The test preparation was expressed in the volume used in the dose (viz. HT = 0.0075 ml)

D = dose interval

$$\text{Potency ratio (R)} = \frac{Z_S}{Z_T} \text{ antilog} \left(\frac{2L_P}{L_I} \log \sqrt{D} \right) \quad \ldots (1)$$

$$= \frac{Z_S}{Z_T} \text{ antilog} \left(\frac{L_P}{L_I} \log D \right) \text{ in } \mu g/ml.$$

Fiducial Limits

In general, Feiller's Theorem (Feiller, 1944; Finney, 1952) gives the fiducial limits (F.L.) of a ratio, say, $m = \frac{a}{b}$, as

$$\text{F.L. of } m = \frac{m}{1-g} - \frac{g V_{12}}{(1-g)V_{22}} \pm \frac{ts}{b(1-g)} \sqrt{V_{11} - 2mV_{12} + m^2 V_{22} - g\left(V_{11} - \frac{(V_{12})^2}{V_{22}}\right)}$$

where $\text{Var}(a) = v_{11}s^2$, $\text{Var}(b) = v_{22}s^2$, and $\text{Cov}(a,b) = v_{12}s^2$.

If $v_{12}s^2 = 0$, then

$$\text{F.L. of } m = \frac{m}{1-g} \pm \frac{ts}{b(1-g)} \sqrt{V_{11} - gV_{11} + m^2 V_{22}} \quad \ldots (2)$$

Also, if a is represented by $(\bar{y}_T - \bar{y}_S)$, then

$$m = \frac{(\bar{y}_T - \bar{y}_S)}{b} = M - (\bar{x}_S - \bar{x}_T), \text{ where } M = \log R \quad \ldots (3)$$

Substituting equation (3) in (2):

$$\text{F.L. of } M - (\bar{x}_S - \bar{x}_T) = \frac{M - (\bar{x}_S - \bar{x}_T)}{1-g} \pm \frac{ts}{b(1-g)} \sqrt{(1-g)V_{11} + (M - \bar{x}_S + \bar{x}_T)^2 V_{22}} \quad \ldots (4)$$

For this assay, $v_{11} = \frac{k(t-1)}{N(k-1)}$, $v_{22} = \frac{1}{4} \times \frac{k(t-1)}{N(k-1)}$

$$g = \frac{t^2 s^2 v_{22}}{b^2}$$

$$b = \frac{\Sigma (x-\bar{x})(y-\bar{y})}{\Sigma (x - \bar{x})^2}$$

Since $\Sigma (x-\bar{x})(y-\bar{y}) = \Sigma y(x-\bar{x}) = \Sigma y_H - \Sigma y_L = n(\bar{y}_H - \bar{y}_L)$

and, in this assay, $\Sigma (x - \bar{x})^2 = N$, and $n = \frac{N}{2}$

$$\therefore \quad b = \frac{\frac{1}{2}N\,(\bar{y}_H - \bar{y}_L)}{N} = \frac{\bar{y}_{HS} + \bar{y}_{HT} - \bar{y}_{LS} - \bar{y}_{LT}}{4}$$

Substituting $\bar{y}_i = \frac{t-1}{N(k-1)}\,Q_i + \frac{G}{N}$,

$$b = \frac{t-1}{N(k-1)} \times \frac{Q_{HS} + Q_{HT} - Q_{LS} - Q_{LT}}{4} = \frac{(t-1) \times L_I}{N(k-1) \times 4} \quad \ldots (5)$$

$$(\bar{y}_T - \bar{y}_S)\ \text{Corr.} = \frac{t-1}{N(k-1)} \times \left(\frac{Q_{HT} + Q_{LT}}{t/2} - \frac{Q_{HS} + Q_{LS}}{t/2} \right)$$

$$= \frac{(t-1)}{N(k-1)} \times \frac{2L_P}{t}$$

$$\therefore \quad M - (\bar{x}_S - \bar{x}_T) = \frac{\bar{y}_T - \bar{y}_S}{b} = \frac{2L_P}{L_I} \times \frac{4}{t} = \frac{2L_P}{L_I}$$

since $t = 4$. $\ldots (6)$

Substituting equation (6) to (1):

$$R = \frac{Z_S}{Z_T}\ \text{antilog}\ \left\{ (M - (\bar{x}_S - \bar{x}_T)) \times \log \sqrt{D} \right\}$$

$$= \text{antilog}\ \left[\log \frac{Z_S}{Z_T} + \left\{ (M - (\bar{x}_S - \bar{x}_T)) \times \tfrac{1}{2} \log D \right\} \right]$$

$$\text{F.L. of R} = \text{antilog}\ \left\{ \log \frac{Z_S}{Z_T} + (\text{R.H.S. of eq. (4)}) \times \tfrac{1}{2} \log D \right\}.$$

APPENDIX IV (b) Analysis of Single Block of 4 Rats (Block No. 1)

LS	2.53	HS	4.11	LT	2.17		8.81
LT	1.96	HT	3.54	LS	3.15		8.65
HS	4.79	LT	2.3	HT	4.24		11.33
HT	3.67	LS	3.14	HS	4.67		11.48
	12.95		13.09		14.23		40.27

$V_{LS} = 8.82$ $\quad t = 4$

$V_{HS} = 13.57$ $\quad k = 3$

$V_{LT} = 6.43$ $\quad r = 3$

$V_{HT} = 11.45$ $\quad b = 4$

$G = 40.27$ $\quad N = tr = bk = 12$

$\frac{G^2}{N} = 135.1394$ $\quad \lambda = \frac{r(k-1)}{t-1} = 2$

Between Columns s.s.

$$= \frac{12.95^2 + 13.09^2 + 14.23^2}{4} - 135.1394 = 0.24647$$

Between Blocks s.s.

$$= \frac{8.81^2 + 8.65^2 + 11.33^2 + 11.48^2}{3} - 135.1394$$

$$= 2.39323$$

Between Treatments (Intrablock

$Q_{LS} = (3 \times 8.82) - (8.81 + 8.65 + 11.48) = -2.48$

$Q_{HS} = (3 \times 13.57) - (8.81 + 11.33) + 11.48) = 9.09$

$Q_{LT} = (3 \times 6.43) - (8.81 + 8.65 + 11.33) = -9.50$

$Q_{HT} = (3 \times 11.45) - (8.65 + 11.33 + 11.48) = 2.89$

$$\text{s.s.} = \frac{t-1}{Nk(k-1)} \Sigma Q^2 = \frac{\Sigma Q^2}{24} = 7.8075$$

Partition:

1) Regression: $L_1 = 23.96$ s.s. $\frac{(L_1)^2}{96} = 5.9800$

2) Preparation: $L_p = -13.22$ s.s. $\frac{(L_p)^2}{96} = 1.8205$

3) Derivation from parallelism:

$L_1' = 0.82$ s.s. $\frac{(L_1')^2}{96} = 0.007$

Check : $5.98 + 1.8205 + 0.007 = 7.8075$

Total s.s. $= \Sigma y^2 - \frac{G^2}{N} = 10.5073$

Potency Ratio:

HS = 11 µg HT = 0.0075 ml) of same solution
LS = 5.5µg LT = 0.00375 ml) (1100 µg/ml)
D = 2

$$R = \frac{Z_S}{Z_T} \text{ antilog } \left(\frac{L_P}{L_1} \log_{10} D\right) = \frac{11}{.0075} \text{ antilog}\left(\frac{-13.22}{23.96} \times .3010\right)$$

$$= 1000.56 \text{ µg/ml.}$$

Corrected Means		Cf. Uncorrected Means
$HS = \frac{t-1}{N(k-1)} Q_{HS} + \frac{G}{N}$	$= 4.492$	$\frac{13.51}{3} = 4.523$
HT	$= 3.717$	$\frac{11.45}{3} = 3.816$
LS	$= 3.046$	$\frac{8.82}{3} = 2.940$
LT	$= 2.169$	$\frac{6.43}{3} = 2.143$

	d.f.	s.s.	Variance	F	P
Interblock Total (Bet. rats)	3	2.3932	0.797	12.8	0.05 - 0.01
Regression	1	5.98	5.98	96	.001
Deviation from parallelism	1	0.007	0.007	1	N.S.
Preparation (Bet. S & T)	1	1.82	1.82	2.93	0.01 - 0.001
Treatments (intrablock)	3	7.807	2.60		0.1 - 0.05
Order	2	0.25	0.125	Pooled = 0.062 with 5 d.f.	
Intrablock error	3	0.06	0.02		
Total about mean	11	10.507			

Interblock error not available with 1 block (no d.f. available).

Fiducial Limits:

$v_{11} = \frac{k(t-1)}{N(k-1)} = \frac{3}{8}$ $\quad v_{22} = \frac{1}{4}\frac{k(t-1)}{N(k-1)} = \frac{3}{32}$ $\quad b = \frac{L_1}{32} = 0.7487$

$s^2 = 0.062$ with 5 d.f. $\quad t(P = 0.95,\ 5\ \text{d.f.}) = 2.571$

$$\therefore g = \frac{t^2 s^2 v_{22}}{b^2} = \frac{(2.571)^2 \times 0.062 \times 3}{(0.7487)^2 \times 32} = 0.0706$$

$\therefore 1 - g = 0.9294$

$$M - (\bar{x}_S - \bar{x}_T) = \frac{2L_P}{L_1} = \frac{-2 \times 13.22}{23.96} = -1.1035$$

$$s = \sqrt{s^2} = 0.2490$$

$$\log \frac{Z_{LS}}{Z_{LT}} = \log \frac{11}{.0075} = \log 1467 = 3.1662$$

$$\text{F.L. of } (\bar{y}_T - \bar{y}_S)/b = \frac{-1.1035}{0.9294} \pm \frac{0.2490 \times 2.571}{0.7487 \times 0.9294} \times \sqrt{(0.9294 \times \tfrac{3}{8}) + (1.1035)^2 \times \tfrac{3}{32}}$$

$$= -1.8131,\ -0.5615$$

$\therefore$ Upper Fiducial limit of R = antilog(3.1662 -(0.5615x0.1505))

= 1207

Lower Fiducial limit of R = antilog(3.1662 -(1.8131x0.1505))

= 782.2

Result: Potency estimated as 1000.6 µg/ml

Fiducial limits of estimate (P = 0.95)

= 782.2 - 1207 µg/ml

=-21.8% to +20.6% of estimate

Actual potency = 1100 µg/ml

Fiducial limits in terms of actual potency

= -28.9% to +9.7%

APPENDIX IV (c) Analysis of a Block with 12 Rats
(Blocks No. 1 + 2 + 3)

Gastrin Assay (1), (2) & (3) analysed as Single 12 Block Assay

$t = 4$ $N = 36$ $V_{LS} = 19.865$
$k = 3$ $\lambda = 6$ $V_{HS} = 31.53$
$r = 9$ $G = 94.13$ $V_{LT} = 16.87$
$b = 12$ $\frac{G^2}{N} = 246.1238$ $V_{HT} = 25.865$

Between Columns s.s. $= \frac{30.37^2 + 31.44^2 + 32.32^2}{12} - 246.1238 = 0.1589$

Between Blocks s.s. $= (137.53263 + 82.1266 + 45.46575) - 246.1238 = 19.002$

Interblock Treatment Component:

$T_{LS} = 69.65$ $T_{LT} = 70.115$
$T_{HS} = 72.855$ $T_{HT} = 69.77$

Check : $\Sigma T = kG = 282.39$

$$s.s. = \frac{1}{k(r-\lambda)}\left[\Sigma T^2 - \frac{(\Sigma T)^2}{t}\right] = 0.7679$$

Partition of Interblock Treatment Component:

1) Regression: $L_1 = 2.86$ s.s. = 0.2272
2) Parallelism: $L_1' = -3.55$ s.s. = 0.3500
3) Preparation: $L_p = -2.62$ s.s. = 0.1906

Check: 0.19067 + 0.35007 + 0.2272 = 0.7679

Intrablock Treatment:

$Q_{LS} = -10.055$ $Q_{LT} = -19.505$
$Q_{HS} = 21.735$ $Q_{HT} = 7.825$

$$s.s. = \frac{\Sigma Q^2}{72} = 14.09984$$

Partition of Intrablock Treatment s.s.:

1) Regression: $L_1 = 59.12$ s.s. = 12.1360

2) Parallelism: $L_1' = -4.46$ s.s. = 0.0690

3) Preparation: $L_p = -23.36$ s.s. = 1.8948

Check : 12.13602 + 0.069068 + 1.89475 = 14.0998

Total s.s. = (145.6467 + 85.3746 + 49.7794) - 246.1238

= 34.6769

$$\text{Potency Ratio} = \frac{11}{.0075} \text{ antilog}\left(\frac{-23.36}{59.12} \times .301\right)$$

= 1115.4 µg/ml

Fiducial Limits:

$b = \frac{59.12}{32x3} = 0.6158$ $v_{11} = \frac{1}{8}$ $v_{22} = \frac{1}{32}$

$s^2 = 0.07504$ with 21 d.f. $t(P = .95, 21 \text{ d.f.}) = 2.08$

$g = \frac{s^2 t^2 v_{22}}{b^2} = 0.0268946$ $\therefore 1-g = 0.973105$

$M - (\bar{x}_s - \bar{x}_T) = \frac{2L_p}{L_1'} = -0.79025$ $s = 0.27295.$

$\log \frac{Z_{LS}}{Z_{LT}} = 3.1662$

$\therefore$ F.L. of $M - (\bar{x}_s - \bar{x}_T)$

$$= \frac{-0.79025}{0.9731} \pm \frac{0.27295 \times 2.08}{0.6158 \times 0.9731} \sqrt{\frac{0.9731}{8} + (0.79025)^2 \times \frac{1}{32}}$$

$= - 0.8121 \pm 0.35723$

$= - 0.45485 - 1.1693$

Upper F.L. of R = antilog(3.1662 - (0.4549 x .1505))

= 1252 µg/ml.

Lower " " = antilog(3.1662 - (1.1693 x 0.1505))

= 977.9 µg/ml.

	d.f.	Sum of Squares	Variance	Variance Ratio	P
Regression	1	0.2272	0.2272	< 1	N.S.
Parallelism	1	0.35007	0.35007	< 1	N.S.
Preparation	1	0.19067	0.19067	< 1	N.S.
Interblock Treatment	3	0.7679	0.2559	< 1	N.S.
Interblock error	8	18.2332	2.27915		
Interblock Total	11	19.00115	1.72737	23.02	< 0.001
Regression	1	12.1360	12.1360	161.7	< 0.001
Parallelism	1	0.0690	0.0690	0.9204	N.S.
Preparation	1	1.89475	1.8947	25.2	< 0.001
Intrablock Treatment	3	14.0998	4.6999	63.0229	< 0.001
Order	2	0.1589	0.0794	) Pooled:) 0.07504	
Intrablock Error	19	1.4169	0.07457	) with 21 d.f.	
Intrablock Total	24				
Total about mean	35	34.67685			

Results: Estimated Potency =1115.4 µg/ml (1.4% high)

Fiducial Limits(P=.95) = 977.9 - 1252 µg/ml,

= -12.3% to + 12.3% of

Estimated potency.

Corrected means:	HS = 3.52	LS = 2.195
vs. Uncorrected	3.503	2.207
Corrected means:	HT = 2.94	LT = 1.80
vs. Uncorrected	2.874	1.874

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