STUDIES ON THE REGULATION OF BILIARY AND PANCREATIC SECRETION IN MAN

BY

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A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE

UNIVERSITY OF LONDON

OCTOBER 1982

ROYAL POSTGRADUATE MEDICAL SCHOOL

LONDON

ABSTRACT

The mechanisms which regulate the delivery of bile and pancreatic juice into the duodenum are incompletely understood in man. The aims of this work were to evaluate the efficiency of the duodenal perfusion technique for measurement of biliary and pancreatic secretion, and to use this technique to study the secretion of bile and pancreatic juice into the duodenum under various experimental circumstances. The effects on pancreatico-biliary secretion of luminal bile salts and amino acids, of partial diversion of duodenal contents, and of several polypeptides known to be released from the upper gastrointestinal tract and associated organs were studied, and the possibility of a periodicity of the biliary and pancreatic secretion into the duodenum.

The validity of the duodenal perfusion technique was confirmed. The "steady state" conditions of the concentration of duodenal recovery markers were shown to be dependent on the efficiency of gastric and duodenal aspiration and high perfusion flow rate. Duodenal perfusion of the bile salt sodium taurocholate (STC) suppressed biliary excretion of bilirubin and the exogenously infused hepatophile indocyanine green in subjects with gall bladders, while in cholecystectomised subjects such effects were not found. The inhibitory effect of STC was overcome by luminal perfusion of amino acids. Pancreatic enzyme secretion was not suppressed by STC nor was there any effect of the bile salt on basal bicarbonate secretion. STC suppressed plasma levels of motilin but had no effects on pancreatic polypeptide (PP), gastrin or gastric inhibitory polypeptide in plasma. Bovine pancreatic polypeptide (BPP), simulating postprandial plasma levels of PP, suppressed biliary and pancreatic enzyme secretion in subjects with gall bladders, while in cholecystectomised subjects PP suppressed pancreatic secretion only. Partial jejunal diversion of bile-pancreatic juice had no effect on trypsin secretion but reduced the secretion of bile salts, bilirubin and bicarbonate into the duodenum.

Power spectral analysis of the secretory data revealed a periodicity of biliary and pancreatic secretion with an average period around one hour in basal and caerulein/secretin stimulated studies and with subsidiary periods of around 40 min and 90-110 min in the stimulated studies. The fluctuations of the secretion were reduced by exogenous hormonal stimulation, as estimated by reduction in peak power density as well as total power density in stimulated studies compared with basal studies.

PREFACE

This thesis is introduced by a short historical review and a summary of the recent literature on the regulation of the hepato-biliary and pancreatic secretion (*Chapter I*). This is followed by six chapters, each containing its own introduction, description of materials and methods and experimental designs, results, discussion and a brief summary. Cross references are as necessary. The first of these chapters (*Chapter II*) deals with the duodenal perfusion technique as it was used to estimate biliary and pancreatic secretion in man under various experimental conditions. Recovery and steady state conditions of the reference markers in the duodenal lumen were evaluated and the effects of various factors on the steady state. This is followed by several physicochemical studies on the hepatophile dye indocyanine green (*Chapter III*), which was used as a biliary marker in many of the following studies. The luminal effects of amino acids and bile salts on basal and exogenously stimulated biliary and pancreatic secretion are described in *Chapter IV*, whereas *Chapter V* describes the effects of bovine pancreatic polypeptide on the biliary and pancreatic secretion. In *Chapter VI* the effects of partial jejunal diversion of duodenal fluid on the biliary and pancreatic secretion were studied. A further analysis of the secretory patterns of these studies, using the technique of power spectral analysis, is described in the final chapter (*Chapter VII*). Thereafter is an *Appendix*, containing detailed tables and methodological descriptions, which were not easily included in the main text. At the end are the *References* and a *List of Publications* from this work.

The main bulk of the experimental work presented in this thesis is perfusion studies carried out in man. All protocols of these studies were accepted and signed by (1) the Supervisor, (2) the Professor of Medicine and (3) the Professor of Endocrinology, before submission to the Royal Postgraduate Medical School Research Ethics Committee, where they were approved. I wish to thank my Supervisor, the Professor of Medicine and the Professor of Endocrinology for their support, and Mrs. Jean De Luca for her most excellent secretarial help, not only on this thesis, but on all previous publications leading up to this work. I also wish to thank Mr. Doig Simmonds and his staff of the Medical Art Studio for teaching me for many years how to draw my pictures myself, and the librarians Miss Eileen M. Read and Miss Lindsey Curtis and their colleagues of the Wellcome Medical Library for providing me for years with working facilities and other library services.

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" IV - Buffers of constant and low ionic strength for spectrophotometric pK determinations

" V - Calculations of bisquare mean and variance

" VI - Cubic spline interpolation

" VII - Equations for power spectral analysis

LIST OF ABBREVIATIONS

α	:	slope constant
AR	:	analytical reagent
Asp	:	specific area
BDH	:	British Drug Houses
BPP	:	bovine pancreatic polypeptide
BSP	:	bromsulphthalein
CCK	:	cholecystokinin
CCK-8	:	cholecystokinin octapeptide
CCK-33	:	cholecystokinin triacontatriapeptide
CCK-39	:	cholecystokinin triacontanonapeptide
CHR	:	Crick Harper Raper
cpm	:	counts per minute
CU	:	clinical unit
CV	:	coefficient of variation
dc	:	direct current
dcGMP	:	dibutyryl cyclic guanosine monophosphate
DMF	:	dimethylformamide
dpm	:	disintegrations per minute
ε	:	extinction coefficient
Ea	:	activation energy
EAA	:	essential amino acid
ECDF-plot	:	empirical cumulative distribution function plot
EEDQ	:	N-ethoxycarbonyl-2-ethoxy-1,2-dihydro-quinoline ethyl-1,2-dihydro-2-ethoxy-1-quinoline-carboxylate
FFT	:	Fast Fourier transformation
GIH	:	gastrointestinal hormone
GIP	:	gastric inhibitory polypeptide
h	:	hour
HCl	:	hydrochloric acid
^{99m}Tc-HIDA	:	^{99m} technetium-labelled dimethyl iminodiacetic acid
HPP	:	human pancreatic polypeptide
ICG	:	indocyanine green (Cardio Green®)
IU	:	international unit
i.v.	:	intravenous
k	:	rate constant
kg	:	kilogram
KIU	:	kilo international unit
min	:	minute
n	:	total number of (subjects, samples, *etc.*)

β-NAD	:	β-nicotinamide adenine dinucleotide
ng	:	nanogram
PEG 4000	:	polyethylene glycol mol. wt. 4000
^{3}H PEG 4000	:	^{3}H-labelled polyethylene glycol mol. wt. 4000
^{14}C PEG 4000	:	^{14}C-labelled polyethylene glycol mol. wt. 4000
^{99m}Tc-PIPIDA	:	^{99m} technetium-labelled paraisopropyl iminodiacetic acid
PP	:	pancreatic polypeptide
PR	:	phenol red
PSD	:	power spectral density
R_f	:	chromatographic mobility relative to solvent front
SD	:	standard deviation
SEM	:	standard error of the mean
STC	:	sodium taurocholate
$t_{\frac{1}{2}}$	:	half-life
TLC	:	thin layer chromatography
To	:	centre value of periodicity
yr	:	year
VIP	:	vasoactive intestinal peptide
wk	:	week
Wsp	:	specific width
s	:	second

CHAPTER I

GENERAL INTRODUCTION

1.1 HISTORICAL

1.1.1 Anatomy

According to Sigerist (349), detailed descriptions of the surface anatomy of the liver, the gall bladder, the hepatic and bile ducts in animals can be found in Sumerian scripts (religious hepatoscopy). The Sumerian believed that the liver was the seat of the soul (349), and there they agreed almost with the Great Lakes' Ojibway Indians of North America, who placed the soul in the gall bladder (76).

It is well known that bile played a major role in the ancient Greek theory of medicine, but the Hellenians are not known for anatomical dissection, and probably they limited it only to animals. There is a story that Aristotle (384-322 B.C.) noticed when working on sheep that the sheep of Naxos had large gall bladders while the sheep of Chalcis had none (238).

The Greek physician Claudius Galen (129-199 or 201 *A.D.*) from Pergamus in Asia Minor, made his name in Rome, and not least for his extensive dissections on animals. Necropsy had become illegal at that time, and Galen had to limit his dissections to animals. He chose particularly the Barbary ape (*Macacus inuus*) as a dissection animal, as he thought it structurally closest to man of all animals available to him. According to Galen's doctrine, the liver had five lobes, there were two common bile ducts, and the spleen, where the "black bile was formed", emptied straight into the stomach (351). Nonetheless, Galen noticed during dissections that the gall bladder was "sometimes very full, sometimes empty and sometimes in an intermediate state", and he argued that its content entered and left the gall bladder at different times through one and the same canal (47).

Many errors on human anatomy attributed to Galen are certainly due to false analogy drawn from his animal works, and Galen's influence, with all its truth and errors prevailed for centuries, or until Andreas Vesalius (1514-1564) and his followers published their revolutionary works on human studies (280).

Information on the anatomy of the pancreas from ancient times is scarce. Hypotheses that the pancreas acted as "a packing for the

stomach and neighbouring organs" or that it was "the gall-bladder of the spleen" are known (352). Precise anatomical discoveries date only from the seventeenth century (62, 133). The discovery of the pancreatic duct is usually attributed to the Bavarian anatomist Johann George Wirsung (1600-1643) in 1642, although, according to Foster (127), Wirsung's pupil, Johann Maurice Hofmann claimed to have actually done that work for him. However, the Bolongian anatomist, Mondino de'Luzzi (*ca.* 1270-1326) described the duct in his work "*Anothomia*" in 1316 (351).

The English anatomist and pathologist Francis Glisson (1597-1677) is said to have first described the sphincter musculature at the terminal end of the common bile duct (152). More than 200 years later this sphincter was rediscovered independently by Simon P. Gage in 1878 and Rugero Oddi in 1887, and after whom the sphincter was called. In 1720 Abraham Vater of Wittenberg (1684-1751) described the ampulla of the distal end of the biliary tract, which bears his name (133).

1.1.2 Physiology

1.1.2.1 "the period of *atra bilis*" - "bile as a mere excrement" - "bile as a digestive ferment". The ancient Greek theory of the balance of the four bodily fluids - water, blood, yellow bile and black bile - is well known. In various forms it dominated medical thinking in the Western world until the Renaissance, "the period of *atra bilis*" (232). In contrast, in ancient India and China people believed that the saliva and the semen were the most important fluids of the body (276). However, according to some reviewers (162) Aristotle and later Galen abandoned the idea of the importance of bile for vital functions and considered it "as a mere excrement, and of no other use than by its acrimony to promote the excretion of the guts". Other texts state that Galen was a great believer in the dangerous nature of the black bile (? melena), and which he considered the cause of all cancer (232).

Revolutionary ideas on the digestive role of bile are associated with the name of the Dutch anatomist Ysbrand van Diemerbroeck (1609-1674) (162), who suggested that bile, by mixing with the "alimentary mass", caused the mass to "ferment" (change from one substance to another). Diemerbroeck claimed that bile "dissolves and separates the thinner parts of the chyle from the thicker ones, and attenuates them so much that they may easily be taken up by the narrow orifices of the lacteal vessels" (these had recently been discovered by Gaspare Aselii, 1581-1625).

Diemerbroeck noticed the difference between hepatic and gall bladder bile. He thought that the gall bladder emptied by the effect of the intra-abdominal pressure, indeed the pressure of the full stomach, which he considered a "timely agent for evacuating the gall bladder when its contents would be most needed" (47).

Another Dutchman, Regnier de Graaf (1641-1673) was probably the first to perform experimental studies of the secretion of pancreatic juice and bile in dogs after having prepared the animals with permanent biliary and pancreatic fistulas (127). This enabled him to collect the juice from the living animal, and he noticed that the volume of pancreatic juice secreted varied greatly. Once de Graaf had the opportunity to examine the pancreatic juice of a sailor who had died suddenly, and he found the human juice identical in its properties to that of the dog (127). De Graaf was aware of the accessory pancreatic duct (the duct of Santorini, G.D. Santorini, 1681-1737), and he devised a clamp made of two ivory plates, which he applied to the duodenum in the region of the accessory duct in order to occlude it. He also cannulated the parotid duct which had been discovered by Niels Stensen (1638-1686), and he collected from the same animal simultaneously pancreatic and parotid juice and observed that the two juices differed in their characteristics.

Although the Italian microscopist Marcello Malpighi (1628-1694) and Johann Wirsung may have collected bile and pancreatic juice before (127, 133), they did not carry out experiments on the secretion, and neither they nor de Graaf seem to have done any noteworthy chemical analysis on the fluid they collected. De Graaf's only comments, as far as this was concerned, were that the fluid was acid (?) and salty (?), and this he based only on tasting the fluid (127). His writings about the physiological functions of the pancreatic juice and bile were no more than echoes of his teacher Franciscus Sylvius of Leyden (127), *i.e.* that the function of the pancreatic juice was to cause "effervescence" when mixed with the alkaline bile; the "effervescence" attenuated the viscid mucus, which is found in the intestine, and also helped to separate the useful parts of the food from the useless. De Graaf published his studies in 1664 in his "*Disputatio medica de natura et usu succi pancreatici*". It is sometimes quoted that during the next 200 years no such work was carried out on pancreatic secretion, or until Claude Bernard performed his studies in the mid-nineteenth century (127).

However, de Graaf's work fell into disrepute later in the seventeenth century. This is partly blamed on Johann Conrad von Brunner of Schaffhausen (1653-1727), who claimed that after almost a total removal of the pancreas from dogs, he was able to keep them alive and well for

a considerable time. He therefore concluded that the pancreatic fluid and the pancreas were of no such importance as de Graaf had claimed. Von Brunner's work had a considerable influence, and old ideas about the stomach as the chief place of digestion became popular again (127).

However, knowledge about the function of bile and pancreatic secretion continued to be gathered. The British physician John Arbuthnot (1667-1735) realised that bile was a substance which "mixed the oily and watery parts of the aliment together" (162) and Albrecht von Haller (1708-1777) wrote in 1736 "that those animals which never eat fat are not so much in need of acrid bile as those which take fat with their food" (47).

William Beaumont (1785-1853) found that human bile assisted with the digestion of oily food, and Sir Benjamin Collins Brodie (1783-1852) tied the common bile duct and noticed that the lymph of the thoracic duct became thin and depleted of fat (162). John Eberle claimed in 1834 that the pancreatic juice emulsified fat, and in 1844 Gabriel Gustav Valentin (1810-1883) showed that pancreatic juice acted upon starch. Johannes Evangelista Purkinje (1787-1869) observed the proteolytic power of pancreatic extracts in 1836, and Lucien Corvisart (1834-1882) carried out series of experiments during the years 1857-1863 where he observed that proteins were converted by the pancreatic juice into what he called "the ordinary digestive products", and this occurred at body temperature and in alkaline, neutral or acid media (133). Friedrich Heinrich Bidder (1810-1894) and Carl Schmidt (1822-1894) in 1852 demonstrated, by using artificial bile fistulas in dogs and total diversion of bile from the intestine, that the animals treated in this way lost weight and produced steatorrhoea in spite of ravenous appetite (162). In fact they recovered from the faeces more than half of the fat the animals had ingested. In contrast, absorption of proteins and carbohydrates seemed to be normal (352).

The most famous work on the digestive role of the pancreatic juice during the nineteenth century was probably that conducted by the French physiologist Claude Bernard (1813-1878) (352). Claude Bernard became interested in pancreatic juice when carrying out a post-mortem examination on a rabbit, which had had a fatty meal just before death. He observed that the lacteals in the mesentery were filled with a white fluid, but only below the point of entry of the pancreatic duct. He therefore concluded that the pancreatic juice played a part in the absorption of neutral fats. This was followed by a series of publications during the years 1849-1856, where Bernard showed that pancreatic juice could split fat into fatty acids and glycerol, starch into sugar, and protein into proteids. It was left to his pupil Willie Kühne (1837-1900)

to study the proteolytic enzymes of the pancreatic juice further and in 1876 he probably discovered trypsin. In the year 1900 Eduard F.W. Pflüger (1829-1910) introduced the theory of hydrolysis of triglycerides, but it was not until 1945 that A.C. Frazer and H.J. Sammons finally proved that monoglycerides were the important end products of pancreatic lipolysis (162).

The present view of the role of bile salts as detergents is much based on the work of B. Moore (162) who demonstrated, at the turn of this century, the great dispersity of fatty acids and lecithin in bile, and that fatty acids and lecithin could be made to form a water-clear solution in bile. In 1913 J.W. McBain (162) put forward the idea that soap solutions contained highly charged colloid particles, which he called "micelles". This theory was extended further by G.S. Hartley and others (162) who showed that fatty acids and monoglycerides were rapidly taken up by the intestinal mucosa when placed in such an artificial "micelle" solution. The role, which bile salts play in forming water soluble micelles with fatty acids and monoglycerides in the intestinal lumen, was accepted, and the importance of these micelles for fat absorption (162).

1.1.2.2 The enterohepatic circulation of bile salts. Giovanni Alfonso Borelli (1608-1679), Professor of Mathematics in Naples, had already in the mid-seventeenth century suggested, based on calculations, that there was a "particular circulation of the bile through the abdomen" (162). Edward Barry (1696-1776) in Dublin, wrote in 1759, that such a circulation of "the most concocted humour in the body" could be economic, as the "active parts of the bile, which remain dissolved in the intestines" might thereby be passed to the liver and "by these means supply it with active, genuine materials, fit for the more easy preparation, and secretion of new bile" (162). It remained to Edward Felix Hoppe-Seyler (1825-1895) to postulate in 1863 that there were the bile salts which circulated in man, and in 1870 Moritz Schiff (1823-1896) produced choleresis in dogs with fistulas by refeeding them with bile salts. A. Weiss seems to have finally settled the matter in 1884, that there was an enterohepatic circulation of bile salts when he fed dogs whose bile contains only taurine conjugates (162) with glycocholic acid, and demonstrated that the ensuing bile secretion contained glycine conjugates.

Before the end of the nineteenth century, H. von Tappeiner had observed that bile salts were only absorbed by the ileum (213), but it was not until 1961 that Lack and Weiner (212) showed, using everted

gut sacs *in vitro* from rats, that active absorption of bile salts occurred only in the distal third of the ileum, absorption elsewhere being passive.

The quantitative significance of bile salts passively absorbed by the intestine is still obscure (162), but it seems to depend on the type and conjugation of the bile salts (205). Krag and Phillips have demonstrated in man that while bile salt absorption in the distal ileum was confirmed to be active (occurred against electrochemical gradient, was saturable and subject to competition), absorption in the proximal ileum and the jejunum was only passive (passive diffusion). The passive permeability of the jejunum to bile acids was found to be twice as much as in the ileum (205).

1.1.2.3 Neural *versus* hormonal regulation of biliary and pancreatic secretion. One cannot see that much attention was paid to the regulation of the secretory functions of bile and pancreatic juice until the nineteenth century. According to Victor Mutt (269), two Frenchmen, Leuret and Lassaigne, described in 1825 an experiment which they carried out on dogs. This showed that instillation of dilute acetic acid into the upper intestine resulted in dilatation of the duodenal orifices of the pancreas and bile ducts and an increase in flow of pancreatic juice and bile into the intestine. They suggested that since the chyme in the duodenum is normally acidic, it could physiologically produce the same effect as the acetic acid under the experimental conditions. This is the first description known of the effect now believed to be due to the hormonal action of secretin and cholecystokinin on the biliary tract and the pancreas. However, until the end of the nineteenth century research workers were preoccupied with explaining the regulation of biliary and pancreatic secretion by the nervous system.

Rudolf P.H. Heidenhain (1834-1897) (381) in 1875, was probably the first to demonstrate that neural (vagal) stimulation increased the secretion of the pancreas. He and his pupils (including Ivan Petrovitch Pavlov, 1849-1936) also claimed that the vagus could inhibit the secretion, and the vagus nerve was therefore considered to have both stimulatory and inhibitory nerves. Pavlov was also the first to observe the blocking effect of atropine on vagally-induced pancreatic secretion in 1878 (381).

According to Gregory (146), I.L. Dolinsky rediscovered in 1894 the effect of acid in the duodenum on the pancreatic secretion. He was working in Pavlov's laboratory, and an extensive work was started there to explain the effect by neural reflexes (146, 269). In 1902 William M. Bayliss (1860-1924) and Ernst H. Starling (1866-1927) demonstrated that extracts of the mucosa of the upper intestine contained a material

which if injected into the bloodstream caused the pancreas to secrete, and also stimulated the flow of hepatic bile. They named the substance of the extract responsible for this action secretin, and introduced the name "hormone" for substances acting on a similar principle. Endocrinology in the modern sense was founded,[1] and Bayliss and Starling postulated that pancreatic secretion should be explained by hormonal regulation (146, 269). This swept away in many countries Pavlov's ideas of the vago-vagal reflexes, which were subsequently considered to be only of minor importance as a regulatory mechanism for pancreatic function monitored from the gastrointestinal tract.

In 1905 John S. Edkins (1863-1940) reported the gastric acid-stimulating effect of a "hormone" he called gastrin, but his results were not accepted until some 30 years later. In 1913 S. Okada, working in Starling's laboratory, described the contraction of the gall bladder during intestinal acidification (270), but as early as 1879 Rutherford had described the secretion of bile into the duodenum in dogs following acidification of the intestine (318). In 1923 Boyden (46) was able to demonstrate a gall bladder contraction after ingestion of egg yolk and cream. A.C. Ivy *et al.* (182) suggested that this contractile effect was due to a hormone, which they called cholecystokinin, and in 1928 they supported this hormonal theory by extract injections and cross-circulation experiments in dogs (182). This was not only a remarkable support for the endocrinological control of the gall bladder, but also did much to settle the longstanding dispute as to whether the gall bladder was contractile in man or not. Still in the 1920's it had been claimed that the gall bladder emptied by siphonage or due to an increase in intra-abdominal pressure, or that it did not empty through the biliary tract at all but that all material entering the gall bladder was removed by absorption through the gall bladder wall (238).

The 1940's and 50's are often considered a stagnant period in this research of the biochemistry and physiology of the gastrointestinal tract. Extracts used were crude, and assay techniques to measure their effect were primitive. In the 1960's secretin, gastrin and cholecystokinin were isolated and structurally identified and the radioimmunoassay subsequently developed. For a period of nearly two decades the field was dominated by research on peptide chemistry and studies on the possible physiological role of the newly-discovered "candidate peptide hormones". Recently, Morton I. Grossman

[1]The idea of a material secreted into the blood by the glands of the body, and acting upon organs elsewhere is, however, much older and was already argued by the Parisian doctor Théophile de Bordeau (1722-1776) (133).

(1919-1981) and co-workers re-examined the evidence for neural regulation and this promptly resulted in publications (353-355) showing strong evidence for a neural mechanism underlying the pancreatic enzyme responses to intestinal stimuli, and that "hormones", like secretin and cholecystokinin, might be there of limited importance (see further *Summary of the Recent Literature*).

1.1.3 Chemistry

1.1.3.1 Bile pigments. The Jewish physician Moses Maimonides (1135-1204 A.D.) wrote that the pigment in bile stones probably came from the blood. In 1847 Rudolf Virchow (1821-1902) supported this idea by experiments, where he isolated bilirubin crystals from ecchymotic areas of the skin and other tissues (18). Ivan R. Tarchanoff (1848-1909) injected blood into dogs with biliary fistulas and observed an increase in bile pigment secretion. F.C. Mann finally confirmed that bile pigment formation occurred mainly as a breakdown of haemoglobin in the cells of the reticulo-endothelial system (18).

In 1913 van den Bergh and Snapper (18) introduced the diazo reaction for the estimation of bilirubin in body fluids and this method in various modifications has since been the most popular method for estimation of bilirubin in biological fluids. This reaction was first described by Paul Ehrlich (1854-1915) in 1883, and is based on the coupling of bilirubin with diazotized sulfanilic acid, which results in splitting of the bilirubin molecule with the formation of two relatively stable dipyrrole azopigment molecules, which have a purple/red colour.

Hans Fischer (1881-1945) demonstrated in the late 1930's that the breakdown product of haemoglobin was bilirubin IXα, and in 1942 he and H. Plieninger succeeded in synthesizing the pigment and in identifying its structure.

In 1956-1957 Billing, Talefant and Schmid published separately (18) their results on glucuronic conjugation of bilirubin in the liver, and that bilirubin was virtually secreted into bile only as a di- or monoglucuronide.

In 1912 G. Fromholdt (137) noticed that after tube feeding of a solution of crystalline bilirubin in subjects with common bile duct obstruction, no significant increase of urobilin was found in the faeces of these subjects. He thought that the tube-fed bilirubin

had been absorbed. This observation got support from several following workers but was rejected by others (137). Lester and Schmid showed later that ^{14}C-labelled conjugated and unconjugated bilirubin was absorbed from the intestine in rat and reappeared in the bile (224). The absorption of conjugated bilirubin was slower and less in quantity than the absorption of unconjugated bilirubin. A possible hydrolysis of conjugated bilirubin to free bilirubin in the intestine was suggested. Gilbertsen *et al.* (137) observed that unconjugated bilirubin was absorbed in man, and shortly thereafter Lester and Schmid (223) found that while unconjugated bilirubin was freely absorbed in man, less than 5% of the tube-fed conjugated pigment was absorbed, suggesting that the "conjugate barrier" was more effective in the human than in the rat.

1.1.3.2 Bile salts. Louise-Jacques Thénard (1777-1857), Jöns Jacob von Berzelius (1779-1848) and Leopold Gmelin (1788-1853) found that by treating bile with lead acetate they could separate two distinct substances from bile, which they called "bile resin" and "picromel", corresponding to what are now known to be glycine and taurine conjugates of cholic acid (211). In 1848 Adolf Strecker (1822-1871) claimed that there were two acids in bile, a nitrogen-containing acid (now glycine conjugated) and both nitrogen- and sulphur-containing acid (now taurine conjugated) (249).

Bile acids were first crystallised in 1844 by Ernst Alexander Platner (a mixture of taurocholic and glycocholic acids). Hans Fischer isolated lithocholic acid in 1911 from gall stones while carrying out his classical work on bilirubin. Chenodeoxycholic acid was isolated separately by Henrik Wieland (1877-1957) and Adolf Windaus (1876-1959) in 1924 (249). Much of the work on the structure of bile acids stemmed from these two latter workers during the second and third decades of this century. They showed that cholic acid, from where all primary bile acids in man can be derivated, can be obtained either from cholesterol or cholanic acid. *In vivo* and *in vitro* studies on the conversion of cholesterol into bile acids is still an active field in research (271).

1.1.3.3 Pancreatic enzymes. In 1833 Anselme Payen (1795-1871) and Jean-François Persoz (1805-1868) discovered amylase (diastase) in malt extracts (121). Claude Bernard discovered lipase in pancreas in 1849, and Willie Kühne discovered trypsin in 1877. To the last of

these four we owe the word "enzyme". Emil Fischer (1852-1919) carried out extensive work to show the specific action of enzymes, and he introduced the "key-lock" concept to describe the interaction between enzymes and their substrates (97).

It was not until 1926 that the first enzyme was crystallised (urease by James B. Sumner, 1887-1955), and this was followed by purification and crystallisation of several digestive enzymes by John H. Northrop and co-workers in the late 20's and early 30's (121). A long dispute about the chemical nature of enzymes was thereby settled and it was finally proven that enzymes are protein. The fate of pancreatic enzymes in the intestinal lumen remains somewhat obscure, since it has recently been reported that pancreatic enzymes are absorbed to some extent and might re-circulate in the blood (158).

1.1.3.4 Foreign compounds. M.J.B. Orfila in 1813-1815 was probably the first to demonstrate the elimination of foreign compounds from the body by bile (359). Claude Bernard found that copper sulphate, potassium iodide, turpentine spirit and fructose were rapidly passed into bile when injected into the blood, and Chrzonszczewsky demonstrated in 1866 the biliary excretion of the dyes aniline red and indigo carmine, and that these dyes could be used to visualize the bile duct (359).

1.1.4 On the collection of gastric and duodenal fluid for therapeutic or experimental purposes: the development of biliary and pancreatic function tests

Lazaro Spallanzani (1729-1799) collected gastric juice from animals and himself in the interest of studying the acidity of the stomach (127). He devised metal buckets and perforated wooden tubes to collect the juice. John Hunter (1728-1793) invented flexible tubes, which he passed into the stomach for artificial feeding (133). Alexander Monro *secundus* (1733-1817) in Edinburgh was probably the first (in 1767) to use gastric tubes to remove poisons from the stomach (62). This technique, "the stomach pump", was later modified and re-introduced and much practiced by the American surgeon Philip Syng Physick (1768-1837) (62), and the two English physicians Edward Jukes and Francis Bush (133). Carl Anton Ewald (1845-1915) devised new types of gastric tubes and used them extensively to study acidity of the stomach, "the Ewald's meal test" (133).

John Conrad Hemmeter (1864-?) and Max Einhorn (1862-?) (133) are considered the pioneers of duodenal intubation and they exercised the technique already before the turn of this century (48). In 1914 Martin E. Rehfuss (1887-?) described the technique of fractional analysis of the gastric content, and the aspiration technique was adapted to collection of duodenal juice (the duodenal Rehfuss tube) (62). In the 1920's duodenal drainage was a widely popular practice applied with magnesium sulphate for the therapeutical purpose of "non-surgical drainage of the gall bladder", a procedure introduced by S.J. Meltzer and B.B. Lyon (the Meltzer-Lyon test) (152)[2]. Later it fell into disrepute, but as a research tool and as a technique for evaluation of pancreatic function, the duodenal aspiration technique has survived in ever-modified forms.

Chiray, Salmon and Mercier (68) were probably the first to carry out pancreatic function test with secretin in 1926. In 1936 Ågren and Lagerlöf (9) published their large series of studies on pancreatic secretion, using a much purer preparation of secretin than had been used before. They realised that gastric aspiration was essential for a reliable pancreatic test to be carried out, and they introduced therefore a double-lumen tube for simultaneous aspiration of the stomach and the duodenum, an idea which had already in 1923 been described by Lim, Matheson and Schlapp (229). A mercury balloon was attached to the tube to facilitate passage into the intestine (9) and the tube material became more flexible and with a more favourable internal-to-wall ratio when polyvinyl and polyethylene materials became easily available in the 1950's. During that time a revolutionary progress was made when non-absorbable markers were introduced to the aspiration technique (45). It changed from being a simple aspiration technique without correction for losses of unaspirated fluid, to being an intestinal perfusion technique, which under "steady state" conditions allowed total volume of fluid in the intestinal segment under observation to be estimated (138).

The pancreatic function test developed as a diagnostic and research tool with ever-purer preparations of secretin becoming available, and finally secretin was synthesized (268). A combined pancreozymin-secretin test was introduced (372) and bolus injections were replaced by continuous i.v. infusions (21).

[2]As an ingredient of Karlsbad Sprüdelwasser magnesium sulphate had been used as a cathartics since the 14th century (48).

1.2 THE DUODENAL PERFUSION/ASPIRATION TECHNIQUE WITH GASTRIC ASPIRATION AND INERT RECOVERY/DILUTION MARKERS

1.2.1 Polyethylene glycol used as a reference substance (dilution marker) in intestinal perfusion studies

In 1947 Shaffer & Critchfield (345) showed that the polymer polyethylene glycol (PEG) of molecular weight 1000-6000 was not absorbed to any significant extent from the human intestine. Further studies by Sperber (364) and Hydén (173) supported these results and showed that polyethylene glycols were non-toxic and not destroyed or chemically changed in the intestinal lumen. In 1957 Borgström and co-workers (45) introduced polyethylene glycol (molecular weight 4000, PEG 4000) into medical research as a reference substance in digestive studies. Since then PEG 4000 has been used extensively as a non-absorbable reference marker for estimating recovery of intestinal aspiration and calculation of the volume of fluid in the intestine (29, 123, 138).

Polyethylene glycols are easily water-soluble polymers of the general formula:

$$HOCH_2\ (OCH_2)n\ OH$$

At the molecular weight of 1000 and above they are in a solid state (Carbowax®). Like most polymers they are polydisperse so that PEG average molecular weight 4000 is a mixture of oligomers ranging in molecular weight from 3000 to 3700.

Initially PEG 4000 was measured by gravimetric or colourimetric methods (345). Later Hydén (172) invented a turbidimetric method for analysis of PEG 4000 in biological fluids. The method of Hydén is based on the formation of an unstable oil emulsion in water in the presence of trichloroacetic acid and barium ions. Although this method, with slight modifications (246), has been the method of choice for measuring PEG 4000 during the last 25 years, it is cumbersome and laborious. The sensitivity of the method has also been questioned (260, 405) and doubts have been raised about its reliability when used on re-thawed samples which have been stored frozen for a while (37). PEG 4000 has been radiolabelled with ^{14}C (260), and more recently with ^{3}H (206, 382). This has allowed the compound to be determined quickly by scintillation counting which has made the measurement at least easier and probably more reliable (405). Studies comparing the results of ^{3}H-labelled PEG 4000, ^{14}C-labelled PEG 4000 and PEG 4000 as recovery markers have, however, shown good correlation (163, 206, 260).

1.2.2 Phenol red used as a recovery marker for gastric aspiration

Of the many reference substances which have been used in gastro-intestinal perfusion studies, phenol red (phenolsulfonephthalein) is probably the oldest, first applied by Gorham and Briggs in 1923 (141) as a dilution marker in gastric aspiration studies. Phenol red is non-toxic, water-soluble and distributes homogenously within the water-soluble components, and is physiologically inert. Although phenol red is only of molecular weight 376, the molecule is probably too large to be absorbed to any considerable extent by the normal gastric mucosa (42, 98). Phenol red can be measured colourimetrically after alkalinisation of the solution (pH 10.5) by strong NaOH. Chromogens in the intestinal fluid (bile pigments) may interfere with the absorbance of phenol red, and in order to solve this problem the absorbance of the sample can be read at two different wavelengths and the interference corrected for (23) (see also *Section 2.2.7*).

1.2.3 Criteria for reference markers in intestinal perfusion studies

Fordtran (124) has described the following criteria for reference markers in intestinal perfusion studies:

(1) the reference substance has to be strictly non-absorbable;
(2) it should not be subject to degradation in the intestinal organ under observation;
(3) it should not be trapped (sequestered) by mucous or other intestinal content;
(4) it should not influence intestinal motility;
(5) it should be measurable with accuracy;
(6) it should have the same solubility characteristics as the test substance; and
(7) it should not influence digestion of the test substance.

To this list has to be added that the reference substance should add minimally to the effective osmotic pressure of the intestinal fluid. Although PEG 4000 has been found to fulfill adequately most of the criteria listed above, recent publications show that the marker may increase the osmotic pressure of the intestinal fluid and interfere with the absorption of water and electrolytes (10, 82).

1.2.4 The assumption of a "steady state"

The duodenal perfusion technique is based on the assumption that a reference marker, infused at a constant rate into the proximal part of the duodenum, is at any time uniformly mixed with the duodenal content, before it is aspirated further apart in the duodenum. Under such circumstances the concentration of the reference substance in the aspirate represents the concentration of the substance in the whole duodenal test segment. Based on this, the total volume of fluid in the test segment can be calculated at any point in time as the product of the marker's infusion rate and the ratio of the marker's concentration infused and aspirated (138). Changes in volume of the duodenal fluid will then be reflected in changes in concentration of the marker in the test segment and the aspirate.

Although perfusion studies carried out in the jejunum and the ileum may have shown that these assumptions are valid, and a satisfactory steady state of complete mixing is likely to exist there, this is not necessarily so in the duodenum.

1.2.5 Factors influencing the "steady state"

"Steady state" conditions in the intestine, reflected by steady concentrations of the reference substance in the intestinal fluid, have been found to depend on the following (79, 123, 399):

(1) the distance between the port, where the perfusion fluid is infused into the intestine and the port(s) where the fluid is aspirated (the perfusion/mixing segment);
(2) the flow rate of the perfusate;
(3) the length of the equilibration period; and
(4) the prevention of endogenous fluid from suddenly entering the test segment.

The accuracy of the perfusion technique has been shown to increase by lengthening the mixing segment above 10 cm (399) and by increasing the equilibration period above one hour (79, 123, 399). A further lengthening of the mixing segment and a further increase of the equilibration period would probably improve the "steady state" further, but a compromise has to be made. The total duration of the study has to be kept within the limits that most volunteers can tolerate. And the longer the mixing segment is the greater distance has

the bile to pass before it is aspirated from the intestine for analysis. This can be of disadvantage, as pancreatic enzymes may be absorbed or destroyed in the duodenum and bicarbonate neutralised (138). Bilirubin and bile salts may also be absorbed to a certain extent in the duodenum (see *Section 1.1.3*). The inevitable delay caused by the passage of the bile through the duodenum to the aspiration ports is obviously directly related to the length of the mixing segment. This delay is influenced by the duodenal motility and the perfusion flow rate.

The flow rate of the perfusate has to be kept within physiological limits, and a flow rate above 10 ml min^{-1} can hardly be considered to be physiological. Perfusion of fluid at a high flow rate may distend the intestinal organ and interfere with peristalsis. High perfusate flow rate is also likely to wash away intestinal content, including digestive products and bacteria, and interfere in that way with normal absorption. However, this is likely to affect more studies on digestion and absorption of food than studies on biliary and pancreatic secretion described here.

The efficiency of intestinal aspiration depends on the diameter of the aspiration tube and the accompanying air ventile, and the efficiency of the pump mechanism used. High aspiration efficiency is reported to give more accurate results (225), although this may be of disadvantage in studies where aspirated fluid, not required for analysis, is discarded and not infused into the jejunum to conserve the bile salt pool, and this may obviously interfere with the enterohepatic circulation of bile salts. Finally, it has been claimed that the sensitivity of the perfusion technique can be increased by shortening the collection intervals (225).

1.2.6 Total volume of fluid/flow rate/transit time

Although the perfusion technique has been found to be reliable for estimation of fluid volume in the intestinal lumen (79, 142, 183, 399), this does not apply to the estimation of flow rate in the test segment (the amount of fluid passing the sampling point in the intestine per unit time), or the transit time through the test segment (the average time required for the fluid to travel from a proximal perfusion point of the intestine to a distal sampling point) (142). The technique, however, was not used for these purposes here, and at least theoretically it has been questioned whether it can be used in that way (78).

1.2.7 Advantages and limitations of the perfusion technique

The duodenal perfusion technique is the only technique available which simultaneously allows the biliary and pancreatic secretions into the duodenum to be monitored in man. One of its limitations is that it requires introduction of tubes into the duodenum and the stomach. Tubes may irritate the intestinal mucosa and possibly stimulate vagal reflexes and cause hormone release, although I am not aware of any studies which do show such changes due to the presence of a tube in the duodenum or stomach.

The duodenal perfusion technique has been criticised for not being reliable for estimating pancreatic secretion in man, *i.e.* for the purpose used here. Worning (414) reported that in his hands this technique had failed to provide sufficiently exact calculations of pancreatic secretion. This worker carried out his studies in man with a perfusion flow rate of 0.6-2.5 ml min^{-1} (414) and with an average aspiration recovery of 20%. The aspiration recovery fluctuated from zero to 100%, and it reached zero or close to zero in one or more of his studies at every 20 min collection interval. The concentration of the recovery marker (PEG 4000) in the perfusion fluid is not mentioned in Worning's paper, so that the amount of the marker perfused into the duodenum cannot be estimated. The bore of the duodenal aspiration tube was 2.1 mm and, according to the methodological description, no air ventile was used. Even more, gastric aspiration was either not done or with an unknown efficiency, which means that neither the duodenal reflux into the stomach nor the contamination by the gastric fluid was known. All this may explain why the aspiration recovery in these studies was so poor and no "steady state" conditions were established and the results on pancreatic secretion were not valid as rightly concluded by the author. However, contrary to some authors (138), no information should be extrapolated from this work on the validity of studies carried out by a standard duodenal perfusion technique.

1.3 SUMMARY OF THE RECENT LITERATURE ON THE REGULATION OF HEPATOBILIARY AND PANCREATIC SECRETION

1.3.0 Introduction

During the last decade the production of literature in gastrointestinal physiology and related disciplines has been plethoric. Probably this is due to the rapid development of the immunoassay and peptide chemistry in general, which has resulted in identification and isolation of numerous peptides with biological activities which have been tested for possible physiological functions in the gastrointestinal tract and associated organs.

This summary concentrates mainly on studies in man, although animal work will frequently be mentioned, as much of the recent work has been carried out on animals. It will centre around factors which possibly regulate the discharge of bile and pancreatic juice into the duodenum under physiological circumstances. It will stress the most recent findings which are often contradictory, rather than repeat the generally accepted views found in textbooks. Studies on anatomy and histology are just mentioned when necessary to explain the functional control of the biliary and pancreatic secretion. Absorption from the gall bladder, and the secretory processes at receptor/cellular levels will be mentioned, but are also covered in recent reviews by Diamond (93) (gall bladder absorption) and Gardner (132) (pancreas).

1.3.1 Hepatic secretion

1.3.1.0 Introduction. Hepatic secretion is an excretory route for bile salts, cholesterol, phospholipids, bilirubin, hormones, metabolites, certain inorganic ions, foreign compounds and among these anionic dyes. Although secreted continuously in all species, bile is delivered intermittently into the intestine in those species which have a gall bladder. The hepatic formation and secretion of bile is poorly understood, and research in this field is hampered by the lack of a suitable technique for studying canalicular and ductular bile formation (126).

1.3.1.1 The bile salt dependent secretion. It has been a generally accepted view (51, 126, 136, 188) that the hepatic secretion of bile is regulated by the amount of bile salts entering the liver after

absorption from the intestine (the enterohepatic circulation). Entering the liver by the hepatic portal circulation, bile salts pass into the hepatic sinusoids, where they come in contact with the basolateral membrane of the hepatocytes and gain access to the cytosol by an uptake mechanism which is poorly understood. The uptake seems not to depend on the portal pressure, since bile salts can be taken up by the hepatocytes against a pressure gradient which exceeds the sinusoidal pressure (110).

Bile salts at physiological pH are organic anions, ($pKa \leq 6$), and the active cellular uptake of these anions builds up an electrochemical gradient across the hepatocyte membrane. This gradient is associated with movement of water and inorganic cations into the hepatocyte by electro-osmosis. The active uptake mechanism (occurs against electrochemical gradient and is dependent on cellular energy) for bile salts may be sodium-dependent (sodium coupled carrier mechanism) and regulated by Na^+ K^+ ATPase (51). The process may not be exclusive for bile salt anions, but capable of an active uptake of other anions as well (51).

Within the hepatocyte bile salts are bound to proteins and conjugated with taurine and glycine. Conjugation of bile salts is probably necessary for their secretion into bile (51, 188).

The mechanism by which bile salts leave the hepatocytes and enter the biliary canaliculi is even less understood than the hepatic uptake of bile salts from the blood. The hepatic cytoplasm is negatively charged (-30 to -40 meV) (41,51), which favours extrusion of bile salts out of the cell into the canaliculi. In the canaliculi the concentration of bile salts is likely to reach the critical micelle level and micelles are formed (51, 136). The formation of micelles greatly reduces the osmotic effect of bile salts in the bile, and this may, by passive binding of bile salts and other organic ions to micelles, create a sink which reduces the luminal conc. of these anions in the non-micellar phase of bile, decreasing its availability for back diffusion across the canalicular membrane. The theory does not account for the secretion of cations, and most authors consider an active transport necessary to account for hepatic secretion of bile.

The close correlation between bile salt secretion and the biliary flow (as estimated by mannitol or erythritol clearance) is well established, but the relationship is now thought to be curvilinear, *i.e.* the intercept with the *Y*-axis (the flow rate) becomes much close. to the origin at low bile salt secretory rates than previously considered (41) As an explanation for this it is suggested that at low secretory rates bile salts are osmotically more effective than at higher rates where,

due to micelle formation, the osmotic effect is reduced (51). The previously estimated "bile salt-independent" bile flow up to 40% or more in man (188), based on a rectilinear relationship between bile flow and bile salt secretion, is therefore likely to have been an overestimation (41, 51).

The secretion of bile salts is considered necessary for the secretion of lecithin, and the correlation between bile salts and lecithin secretion has been found to be curvilinear, the lecithin output reaching a plateau at high levels of bile salt secretion (136). Biliary secretion of cholesterol seems to be associated with bile salt secretion in a similar way as does lecithin (53, 136, 335). It has been suggested that both lecithin and cholesterol may require bile salt micelles in the vicinity of the canalicular membrane for their secretion (136).

1.3.1.2 The bile salt independent secretion. A fraction of bile may be secreted independently of the secretion of bile salts, although this fraction is likely to have been overestimated (126) as discussed before. Moreover, the "bile salt independent" secretion seems to vary both between species and within the same experimental animal (196), and may after all be influenced by bile salts (51).

The mechanism of bile salt independent flow is considered to be secretion of water and bicarbonate into the canaliculi and the biliary ductuli in response to an electrochemical gradient possibly regulated by the "sodium pump" (41). The "sodium pump" is likely to be influenced by secretin, CCK, gastrin and other hormones, *e.g.* thyroxin, insulin and cortisol (101, 188). Further modifications of bile may occur by reabsorption through the ductular epithelium and, recently paracellular pathways, *i.e.* passage through the intercellular spaces and associated "tight" junctional complexes, have been shown to be a possible route for the secretion of water and electrolytes in bile (220).

1.3.1.3 The concepts of "bile salt dependent" and "bile salt independent" secretion challenged. The view that canalicular formation of bile depends on active transport of bile salts and other organic anions, followed subsequently by passive transfer of water and cations, has been challenged by many workers. Klaassen (196) has found in dogs and rats that great variations in hepatic bile salt secretion had little effect on bile flow and that "bile salt independent" fraction, estimated as the positive

intercept at zero bile salt secretion, could be altered by various experimental procedures.
He pointed out that correlation between bile flow and bile salt secretion has to be interpreted with caution, because a plot of flow *versus* bile salt secretion will inevitably show some degree of correlation. This is inevitable because if secretion (flow x concentration) is plotted against flow, the two variables (flow *versus* flow x concentration) would be correlated with each other, because flow is a common factor on both axes.

Recently Oshio and Phillips (283) have reported that canalicular bile flow is facilitated by contractions ("peristalsis") of the bile canaliculi, a conclusion they based on studies on cultured hepatocytes.

1.3.1.4 The secretion of bilirubin. Bilirubin is partly unconjugated and partly conjugated in plasma and bound to albumin. It is taken up by the hepatocytes (membrane-associated carrier-mediated transport) (31) and bound to the transfer protein ligandin. About 98-99% of bilirubin is conjugated with glucuronic acid and secreted into bile as a diglucuronide or, to a minor extent, as a monoglucuronide (336).

The transport of conjugated bilirubin into the canaliculi is the rate-limiting step for bilirubin secretion (136). The canalicular transport system can be saturated, and bilirubin competes there with other anionic dyes, like bromsulphthalein and indocyanine green, for secretion. The carrier-mediated transport for bilirubin is probably different from the carrier transport for bile salts, although interaction seems possible, as bile salts generally increase the maximum transport capacity (Tm) for bilirubin and bromsulphthalein (136, 140).

It is not clear whether bile salts interfere with bilirubin secretion to such an extent in man that bile salt pool depletion affects bilirubin secretion. Mok *et al.* (264) have reported that in cholecystectomised subjects the hourly output of bilirubin is relatively constant over 24 h, although the bile salt output varies with ingestion of meals. Shull *et al.* (348) have found, however, in gallstone patients that bilirubin secretion was linearly related to bile salt secretion. In fact, they found that two-thirds of the bilirubin secretion seemed to be bile salt dependent. In patients with sickle cell anaemia and a greatly increased bilirubin secretion, 90% of the bilirubin output was independent of bile salt secretion (348).

1.3.1.5 The secretion of various anionic dyes. Bilirubin and ICG seem to compete for the same hepatic uptake mechanism (2,31,294), while bile salts are transported by a separate pathway (136). However, ICG administrated at high doses (exceeding the maximal hepatic transport capacity) results in a decrease in bile salt independent bile flow (170), and a similar cholestatic effect has been observed with rose bengal (92). Infusions with BSP have shown a choleretic effect (136). Studies in rats show that biliary excretion of ICG is increased by i.v. injection of bile salts (taurocholate, dehydrocholate) (396).

1.3.1.6 Neural regulation. The liver is richly innervated by the autonomic nervous system (219). However, to what extent neural regulation affects hepatic secretion is uncertain. By present techniques it is not possible to differentiate between direct and indirect effects of the vagus on the hepatic secretion. For example, the choleretic effect observed after vagal stimulation (188) may be mediated by gastrin, released by the vagus (117). Section of the vagus nerve has been reported to increase bile flow, indicating that the vagus may carry inhibitory nerve components (219). Truncal vagotomy has been found to reduce insulin-induced choleresis (101, 188).

Stimulation of the splanchnic nerves reduces bile flow in dogs, and a section of the nerve increases the flow, which might be due to an alteration in hepatic blood flow, an effect which at present cannot be separated from the possible direct effect of the sympathetic nerves on the hepatocytes (219).

1.3.1.7 The effect of the enterohepatic circulation on hepatic secretion. Klapdor and co-workers (197) found in cholecystectomised piglets that CCK increased bile flow and secretion of bile salts, and this seemed to be due to a decrease in transit time of the bile salts through the intestine. Hardison and co-workers (155) found in man that sorbitol shortened the intestinal transit time and increased the biliary secretion, and atropine lengthened the transit time and reduced bile salt secretion. In these studies no effect was observed on the secretion of cholesterol and bilirubin.

1.3.2 Biliary secretion

1.3.2.0 Introduction. At least until recently the general view has been (152, 230, 320) that in the fasting state the gall bladder acted as a constant low pressure area (a gall bladder sink) into which the major fraction of all fasting hepatic bile was diverted. The pressure within the biliary tract was thought to be mainly regulated during the fasting state by the hepatic secretory pressure and the resistance to bile flow generated by the sphincter of Oddi, which was considered to remain closed or almost closed during fasting. Inside the gall bladder bile is concentrated by a continuous active absorption of electrolytes followed by absorption of water (93). The passage of food into the duodenum caused the stimulus for the synchronised contraction of the gall bladder and the reciprocal relaxation of the sphincter of Oddi seen after a meal (237, 415), and this caused the concentrated gall bladder bile to flow into the duodenum. CCK was considered the main mediator for this (230), and as soon as the CCK was cleared from the blood, the gall bladder relaxed, the tonus of the sphincter of Oddi increased, and the bile, continuously secreted by the liver, started to run into the gall bladder again.

This generalised view of the regulation of the flow of bile into the duodenum has been challenged and modified by many workers and some of these newer concepts will be summarised here.

1.3.2.1 Studies based on parenteral administration of regulatory factors. (i) Cholecystokinin: (a) the effect on the gall bladder. The earlier studies on the effect of CCK on the gall bladder used impure preparations of the peptides (152, 230) which make these studies now obsolete, at least as far as the doses used are concerned. Sturdevant *et al.* (371) used the synthetic octapeptide of CCK (CCK-8) from Squibb, and a cholecystogram to estimate the gall bladder size under various doses of parenterally administered CCK. They observed a dose-response relationship between the bolus doses of CCK-8 and the gall bladder size up to 20 ng kg^{-1} of the peptide. At higher doses, 40 ng kg^{-1} and 80 ng kg^{-1}, no further dose-response relationship was found. An excessive initial contraction of the infundibulum of the gall bladder was suggested to be the possible explanation for this.

Rock *et al.* (313, 314) studying the gall bladder motor function by scanning (^{99m}Tc-PIPIDA), showed that the gall bladder emptied

during infusion of CCK-8. Maton *et al.* (248), using a similar scanning technique, but a continuous i.v. infusion of CCK-8, observed a dose-related reduction in gall bladder size over a dose range producing plasma CCK concentrations similar to that observed after a meal (<3 pmol/l to 26.9 ± 2.9 pmol/l, SEM).

Spellman *et al.* (363) used ^{99m}Tc-HIDA to estimate gall bladder emptying following administration of the impure Boots "cholecystokinin". They estimated that the threshold dose of this preparation for gall bladder contraction was only 0.010 CHR units $kg^{-1}min^{-1}$, and they found a linear relationship over a wide dose range of infused CCK.

Lilja *et al.* (227, 228) used ultrasound scanning to estimate gall bladder contraction in man. They found that gall bladder contraction correlated well with an increase in plasma CCK during the infusion of 99% pure CCK. Gall bladder relaxation correlated with the fall in plasma CCK levels after the cessation of the CCK infusion. Yau *et al.* (416) compared the effect of CCK-33 (10% pure), CCK-8 (synthetic, Squibb), caerulein and pentagastrin on the guinea pig gall bladder strip *in vitro*. They found that the efficacies (the maximal responses) of these peptides were similar but that they had a different molar potency. CCK-8 was ten times more potent than CCK-33, and caerulein was two times more potent on molar basis. Pentagastrin was 1800 times less potent.

However, there are reports where no correlation was found in man after a meal between gall bladder emptying and rise in serum CCK, as estimated by a bioassay and cholecystogram (278).

It has been stated repeatedly since Jung and Greengard (190) in 1933 observed that parenteral atropine did not inhibit the action of CCK on the gall bladder muscle *in vitro*, that the effect of CCK on the gall bladder muscle was "direct", and that neural action was therefore not involved (136, 230, 366). This was thought to be in contrast to the action of CCK on the intestine, where atropine was found to prevent the action of CCK, and acetylcholine was thought to be the mediator (270). Several recent studies, however, have shown that the mechanism of the action of CCK on the gall bladder is not likely to be as simple as was previously stated.

Peikin and Ryan (297) studied the effect of atropine and CCK on circular muscle strip from the guinea pig's gall bladder. The dose-response curve to CCK-8 was not altered by atropine, but was shifted in parallel to the right by dcGMP, a specific and competitive

inhibitor of the action of CCK. However, the contractile response caused by CCK-8 persisted for at least 90 min after the muscle strip had been washed repeatedly and resuspended in CCK-free medium. This persistently stimulated state of smooth muscle contraction was abolished by atropine or dcGMP. However, while atropine reduced tension below the initial resting level, dcGMP reduced tension to the resting state only. From these findings the authors concluded that the resting gall bladder muscle tension might be dependent on an intrinsic cholinergic stimulation, and that the persistently stimulated state of the smooth muscle contraction, caused by the CCK-8, might be under cholinergic control, since it could be reversed by atropine.

Behar and Bianchi (27) studied cats *in vivo* and found the effect of synthetic CCK-8 or CCK-33 on the cat's gall bladder was partially blocked by tetrodotoxin, hexamethonium alone, or a combination of hexamethonium and atropine. The gall bladder response to CCK-33 or CCK-8 was not blocked by either atropine alone or adrenergic antagonists. The fact that the action of CCK-8 or CCK-33 on the gall bladder was only partially, but not completely blocked by tetrodotoxin, suggested that CCK acted on two excitatory receptors in the gall bladder, one placed direct on the gall bladder muscle and which was not blocked by any neural antagonist, and a second receptor for CCK at the post-ganglionic cholinergic intramural neurons. These results, however, contrast with earlier observations by Yau *et al.* (416) who failed to alter the dose-response curves obtained by CCK-33, CCK-8, caerulein or pentagastrin on the guinea pig gall bladder muscle *in vitro*, either by anticholinergic or antiadrenergic agents or tetrodotoxin. As pointed out by Behar and Bianchi, no attempt was made in these *in vitro* studies to determine whether the intrinsic cholinergic innervation of the gall bladder muscle was functionally intact, as it might have been damaged by hypoxia.

Poitras *et al.* (301) have demonstrated that dcGMP is a competitive inhibitor of CCK-8 on the gall bladder *in vitro*, and that its inhibitory effect appears to be restricted to peptides which have the common COOH-terminal pentapeptide sequence to that of CCK.

(b) The effect on the sphincter of Oddi. The sphincter of Oddi is composed of intrinsic muscular fibres which differ from the musculature of the duodenum (50), both in embryological development and structure. There are also muscular fibres around the distal end of the pancreatic duct.

The old dispute about whether or not the sphincter of Oddi acted as a physiological entity (152, 320) seems to have been settled. Ono *et al.* (282) observed a good correlation between spike burst activity of the sphincter of Oddi and the increase in the intracholedochal pressure and the interruption of bile flow into the duodenum in rabbits. Electrical stimulation of the duodenum increased the duodenal motility but did not affect the sphincter, the choledochal pressure or the discharge of the bile into the duodenum.

Ono and co-workers (281) have also shown by using similar technique in man, that bile flow was interrupted at the terminal end of the common bile duct when electrical activity of the muscle of Oddi was observed, and the flow of bile into the duodenum was observed to occur only in the absence of electrical activity of the sphincter. Finally, these workers stated that the electrical activity of the muscle layer of the duodenal wall did not necessarily produce suppression of the bile flow.

More recent results by Honda *et al.* (168) from the opossum show that the sphincter of Oddi exhibits rhythmic peristaltic contractions which propagate towards the duodenum, and which are associated with simultaneous spike burst activity. The spike activity demonstrated a cyclic pattern with a mean cycle length of approximately 90 min.

In man, Scott *et al.* (344), Geenen *et al.* (135), Carr-Locke *et al.* (59) and Miranda *et al.* (261) have provided results which support the view that the sphincter of Oddi has a rhythmic activity independent of the motility of the duodenum.

Sandblom *et al.* (323) were probably the first to report that CCK had an inhibitory (relaxing) effect on the sphincter of Oddi, but increased the tone of the duodenum. The effect of the CCK on the sphincter was considered independent of its action on the musculature of the duodenum. These findings have been supported later by numerous studies (320).

Lin (230) found CCK to lower the choledochal resistance in dogs in a dose-responsive fashion. The threshold doses for the sphincter were lower than for the gall bladder and the latency period was shorter. However, in the opossum Becker *et al.* (26) found CCK-33, CCK-8 and pentagastrin to increase the contractile activity of the biliary sphincter at a wide range of doses and at what they considered a physiological range. While CCK stimulated the contraction of the gall bladder tonically the bilia y sphincter was stimulated in a phasic manner. The phasic contractions and relaxations of the

sphincter were considered to facilitate the expulsion of bile into the duodenum. Similar results have been reported in the rabbit by Sarles (326).

These apparently contradictory results may be due to species differences. Behar and Bianchi (27) studied the effect of CCK-33 and CCK-8 in cats. They found that CCK caused a dose-dependent relaxation of the sphincter and that the half-maximum response of the sphincter of Oddi was obtained by doses definitely lower than that required for a half-maximal response of the gall bladder. The relaxing effect of CCK-8 and CCK-33 on the sphincter was blocked by tetrodotoxin, but not by cholinergic or adrenergic antagonists. It was suggested that tetrodotoxin blocked an unmasked second CCK receptor that mediated a sphincter of Oddi contraction, when stimulated by CCK, and which supported the idea of a direct action of CCK on the circular muscle. These workers concluded that the sphincter of Oddi had two receptors, one inhibitory and most likely present at the intramural, non-cholinergic, non-adrenergic post-ganglionic neurones, the other excitatory, and present in the circular muscle of the sphincter.

It can be concluded from this literature that CCK administered parenterally, causes contraction of the gall bladder and relaxation of the sphincter of Oddi in a dose-responsive manner. The action of CCK on the gall bladder and the sphincter of Oddi is probably partly direct on the smooth muscle and partly through intramural neurones.

(ii) Caerulein. Caerulein, the decapeptide isolated from the skin of the Australian frog *Hyla caerulea*, shares the same carboxyl terminal pentapeptide sequence as CCK. It was introduced into medical research by Erspamer in the 1960's and found to have a similar biological activity to CCK (33).

Bertaccini *et al.* (33) found in man that the threshold dose to contract the gall bladder was 0.5 - 1.0 ng kg^{-1} for caerulein, and the effect was satisfactorily dose-dependent up to 20 ng kg^{-1}. The responses at doses of 3 - 4 ng kg^{-1} were of similar magnitude as those produced after a test meal of milk or egg yolk. The spasmogenic action of caerulein on the gall bladder was found to be atropine resistant. Carratù *et al.* (60), using caerulein infusion, found the gall bladder response to be largely dose-dependent, and that a dose of 0.25 ng $kg^{-1}h^{-1}$ stimulated the gall bladder response seen after a fatty meal. They found the effect of caerulein was not affected by atropine, tetrodotoxin, splanchnectomy or adrenalectomy.

Bertaccini *et al.* (33) found that caerulein relaxed the sphincter of Oddi and that the threshold doses for relaxation of tone were 5-20 ng kg^{-1}. Sarles (326) found caerulein to be eight times more effective on molar basis on the sphincter in rabbits than CCK-33 or CCK-8.

Caerulein has similar biological activity on the biliary tract and the gall bladder as CCK, but it is more potent on molar basis.

(iii) Secretin. *In vitro*, Toouli *et al.* (383) claimed that secretin (Boots) caused contraction of the canine and human gall bladder and the choledochal sphincter, and similar findings with synthetic secretin were reported on human gall bladder strips by Cameron (58). More recent studies *in vitro* using purer preparations of secretin, however, have not supported this (320). In man, i.v. injection of secretin contracts the sphincter of Oddi, but the doses required to cause this effect are pharmacological (at least 1 CU kg^{-1} body weight) (230). However, in the conscious dog Lin and Spray (230) found the choledochal resistance to be decreased by i.v. GIH secretin or synthetic secretin in the dose range of 0.125 to 1.0 CU $kg^{-1}h^{-1}$. Sarles *et al.* (326) have reported no effect of secretin on the spike activity of the sphincter of Oddi in rabbits *in vivo*.

Within expected physiological ranges, pure secretin is likely to have no or only weak effects on the biliary tract and the gall bladder.

(iv) Glucagon. Chernish *et al.* (63) found that the gall bladder shadow increased in size after i.v. injection of glucagon. That this was due to a direct effect on the gall bladder muscle and a relaxation of the gall bladder, however, was not supported by studies *in vitro*. The gall bladder dilatation might have occurred due to an increase in hepatic secretion of bile diverted into the gall bladder, as Dyck and Janowitz (107) found that i.v. infusion of glucagon stimulated the bile flow in man.

According to Lin (230) i.v. glucagon decreases choledochal resistance but the decrease has been found to be preceded by an increase in resistance, indicating a biphasic action of glucagon on the biliary musculature. Lin (230) reported that glucagon relaxes the isolated choledochal sphincter *in vitro*, and the effect is considered to be direct on the smooth muscle, as it was not blocked by propranolol, atropine or phenoxybenzamine. Sarles *et al.* (326) have reported that glucagon decreased the spike activity of the sphincter of Oddi in the rabbit *in vivo*.

The doses of glucagon required to relax the extrahepatic biliary system greatly exceed the doses required for glycogenolytic actions, so the action of glucagon on the biliary system is likely to be pharmacological.

(v) Motilin. Adrian and co-workers (8) found that i.v. infusion of motilin increased gall bladder pressure in conscious pigs in a dose-dependent manner. Takahashi *et al.* (376) found that i.v. motilin contracted the gall bladder in dogs during the inter-digestive state but not during the digestive period. However, the action of motilin differed from the action of CCK, since the effect of motilin was not dose-related and was abolished after a meal. Wilson *et al.* (404) found motilin to have no contractile effect on a rabbit gall bladder strip over a wide dose range of motilin, and suggested that the increase in gall bladder pressure claimed by earlier workers to be due to motilin (8), was caused by something other than gall bladder contraction.

(vi) Histamine. Schoetz *et al.* (337) used conscious baboons with indwelling gall bladder catheters and demonstrated a parabolic dose-response relationship between i.v. injection of histamine and increases in gall bladder pressure. The H_1 antihistamine, diphenhydramine, significantly depressed this effect, but the H_2 antagonist, metiamide, caused a significant enhancement of the gall bladder response to histamine. They concluded that the histamine-induced gall bladder contraction was mediated via H_1 histamine receptor, and that H_2 receptors mediated gall bladder relaxation.

Wise *et al.* (408) reported that histamine might have a physiological role in regulating gall bladder filling and emptying. They found that histamine and 2-pyridylethylamine, a specific H_1-receptor agonist, increased the gall bladder tension in guinea pigs *in vitro* in a dose-responsive fashion, whereas isothiourea, a highly specific H_2-receptor agonist, reduced the tension. The response of histamine was enhanced by the H_2-antagonist, cimetidine, but depressed by the H_1-antagonist diphenhydramine. These workers suggested that histamine might have a "counter-regulatory role" in regulating gall bladder filling and emptying.

Waldman *et al.* (397) found in the guinea pig that the H_2-antagonist, metiamide, augmented the contractile response to histamine, suggesting that the gall bladder contained a contractile H_1-receptor

and an inhibitory H_2-receptor. They also suggested that histamine might modify the response to hormones like CCK.

Toouli *et al.* (386) studied the phasic contractile activity of the sphincter of Oddi in the opossum, using the infused catheter system. They found that i.v. histamine invariably depressed the phasic contractions of the sphincter. The inhibitory effect of histamine was duplicated by the selective H_1-agonist, 2-pyridyl-ethylamine, and abolished by the H_1-blockade with pyrilamine, or neural blockade with tetrodotoxin. After tetrodotoxin, histamine and 2-pyridylethylamine increased the frequency and amplitudes of the sphincter of Oddi contractions. This excitatory effect was blocked by pyrilamine, the selective H_1-antagonist. These authors concluded that the depressant effect of histamine on the sphincter of Oddi was mediated by H_1-stimulation of non-cholinergic, non-adrenergic inhibitory nerves, over-riding an H_1-stimulatory effect on the sphincter of Oddi smooth muscle.

In conclusion, there seem to be H_1-receptors and H_2-receptors on the gall bladder, which when occupied cause contraction and relaxation of the gall bladder respectively. In the sphincter of Oddi there are H_1-receptors and H_2-receptors as well which cause relaxation and contraction of the sphincter of Oddi respectively. The physiological role of histamine receptors on the biliary tract and the gall bladder still remains speculative.

(vii) α-Endorphin. Behar and Biancani (28) found in cats, using end-opening catheters introduced into the common bile duct, that the sphincter of Oddi exhibited high tonic pressures with superimposed phasic contractions. α-Endorphin injections caused a dose-dependent decrease in the tonic pressures and in the magnitude of the phasic contractions, and this was suggested to be due to stimulation of non-cholinergic, non-adrenergic inhibitory neurons. It also caused contractions which appeared to be mediated by mechanisms similar to those involved with enkephalin-induced contractions, *i.e.* by stimulating cholinergic excitatory neurons and antagonising non-adrenergic, non-cholinergic inhibitory neurons, or by stimulating non-adrenergic, non-cholinergic excitatory neurons (28).

Matsumura (250) found that taurocholate (1 mmol/l or 5 mmol/l) released β-endorphin from human duodenal mucosa *in vitro*.

1.3.2.2 Studies based on enteral administration of regulatory factors.

(i) Mixed meals. Rock *et al.* (313, 314) found that liquid meals and solid meals cause gall bladder emptying in man, as estimated by gall bladder scintigraphy. Atropine reduced this effect of the meal, but somatostatin totally inhibited it, suggesting that both cholinergic and hormonal mechanisms were involved in the gall bladder response to meals. Rock *et al.* (313, 314) also showed that gall bladder emptying was likely to be affected by the physical and chemical composition of the ingested meal.

Honda *et al.* (168) observed that feeding abolished the inter-digestive cyclic spike-burst activity of the sphincter of Oddi in the opossum.

Malagelada *et al.* (244) compared the biliary responses to a solid meal and a homogenised meal of identical composition. Nutrients ingested with a solid meal were emptied from the stomach at a slower rate than the homogenised meal, and this resulted in a more prolonged biliary response, since stimulation of these organs continued for as long as the meal was entering the duodenum. However, early biliary outputs were less after solid meals than homogenised meals.

The sustained bile salt output seen in the postprandial period after the initial peak output immediately after meals is considered to be due to the enhancing effect of the meal on the enterohepatic circulation of bile salts and increased bile salt flux through the liver. Moreover, a smaller fraction of bile is considered to be stored in the gall bladder due to CCK-induced contraction.

(ii) Hydrochloric acid. In 1928 Ivy and Oldberg (182) showed by carotid-to-carotid cross circulation studies in dogs that a dilute HCl placed in the duodenum caused a gall bladder contraction. This happened not only in the dog receiving the HCl perfusion but also in the cross-connected dog, suggesting a humoral mediated mechanism. This effect has since been reported in numerous studies (152, 269). HCl has also been found to relax the choledochus (152).

(iii) Amino acids. Malagelada *et al.* (239) reported that a mixture of EAA infused into the duodenum resulted in a sharp peak output of bilirubin within 20 min, but this effect of the amino acids was inhibited by simultaneous perfusion of the duodenum with the bile salt sodium taurocholate (see further *Chapter IV*). Ertan *et al.* (111) perfused the proximal jejunum in man with a similar mixture of EAA and

found it to cause a sharp increase in bile salt secretion, similar to an i.v. infusion of CCK of 0.125 CHR units per kg per min, which they considered to be a maximal tolerated dose of CCK.

Ashkin *et al.* (19) compared the effect of EAA perfusion with an i.v. infusion of CCK on the biliary output and found that although the total output and the mean output were of similar magnitude for both types of stimuli, the secretion due to EAA oscillated greatly, while the biliary output during i.v. infusion of CCK was more constant. It was assumed that luminal bile salts interfered with the release of CCK induced by EAA and this caused the unstable biliary output during EAA perfusion.

Holtermuller *et al.* (167) found that *l*-phenylalanine stimulated bilirubin secretion in man but *d*-phenylalanine was ineffective. Earlier, Meyer *et al.* (253) had observed similar biological ineffectiveness of *d*-aminoacids on the pancreas in dogs.

The effect of amino acids on the sphincter of Oddi have been reported by Lin and Spray (230) who found that duodenal perfusion with *l*-tryptophan and *l*-phenylalanine decreased in a dose-dependent manner the resistance to bile flow through the choledochus in dogs.

(iv) Fatty acids. Boyden in 1923 (46) was probably the first to describe the contractile effect of fat placed in the duodenum on the gall bladder (studies in cats). He also observed gall bladder contraction after pure protein, carbohydrate and mixed meals. Ivy and Oldberg, in their experiment in 1928 (182), found that olive oil, egg yolk and cream caused gall bladder contraction, but only if this material had been "pre-digested" (lipolysed) with "pancreatin". Numerous studies have since shown similar effects of fat on the gall bladder (152, 320).

Recent studies have aimed at studying the effect of the concentration and the chain length of fatty acids in the duodenum on the biliary secretion. Malagelada *et al.* (243) found in man an inverse correlation between fatty acids absorbed and gall bladder responses to fatty acids. That is to say, when they raised the bile acid concentration above the critical micellar point in the duodenum, fatty acid absorption rate was accelerated but the magnitude of the gall bladder responses was decreased. Higher bile acid concentration exerted the opposite effect; it slowed fatty acid absorption and increased the gall bladder response. The gall bladder response was augmented by increasing fatty acid chain length and fatty acid load

until the "maximal secretory capacity" was obtained. Ladas *et al.* have also (215) observed that medium-chain triglycerides were a weaker stimulus to gall bladder contraction than long-chain triglycerides in man.

(v) Carbohydrates. In contrast to the earlier studies of Boyden (46), most studies have found intraduodenal carbohydrates to be ineffective in causing gall bladder contraction (152).

(vi) Divalent ions. Holtermuller *et al.* (166) found that duodenal perfusion of a solution of calcium salts stimulated bilirubin output in a dose-related fashion, indicating gall bladder contraction. The calcium solution gave similar concentration of calcium ions in the duodenum as seen after a calcium-rich meal. I.v. infusion of calcium had no effect on biliary secretion.

Inoue *et al.* (178) found that after oral administration of magnesium sulphate, plasma levels of CCK were increased and the size of the gall bladder reduced. The cholecystokinetic effect of the magnesium sulphate was well demonstrated by Meltzer, and this effect of the salt was used by him for therapeutic purposes to drain the biliary tree (the Meltzer-Lyon test) (152). Boyden *et al.* (49) thoroughly examined the effect of the salt and found that it also caused relaxation of the sphincter of Oddi. They suggested a hormonal mechanism for this action (CCK).

1.3.2.3 The concepts of colonic and ileal phases of biliary secretion. Several studies have suggested that humoral factors were released from the lower intestine, which antagonised the effect of CCK on the biliary system. Harper *et al.* (158, 160) described a polypeptide material which they called pancreatone, obtained from the ileal and colonic mucosa, which in anaesthetised cats inhibited the action of CCK on the gall bladder. The action resembled the inhibitory effects of an intraileal and intracolonic infusion of oleic acid in cats with vagal and splanchnic nerves cut (159). Owyang *et al.* (289) have shown in man that colonic perfusion of oleic acid, essential amino acids or glucose reduced cholecystokinin-stimulated biliary secretion. It has also been pointed out by Sarles *et al.* (325) that gall bladder contraction induced by various types of fat stops at similar times in the same individual, independent of the intensity of the contraction. These workers suggested a delayed humoral mechanism which inhibited the action of CCK.

1.3.2.4 The concept of basal biliary secretion. By basal biliary secretion is meant the secretion of bile into the duodenum during fasting when the individual is at rest. Brunner *et al.* (53) found that during the night there was a considerable biliary secretion into the duodenum, and van Berge Henegouwen and Hofmann (29) reported that there was a continuous secretion of bile into the duodenum, and that even more than half of the bile secreted at night bypassed the gall bladder. A similar view was held by von Bergmann (30), who found that a significant portion of the bile salt pool was not in the gall bladder at the end of an overnight fasting. This view, however, has not been appreciated by Malagelada (245) and Ryan (320) who thought that the major portion of bile was stored in the gall bladder during the night.

1.3.2.5 Sham feeding. Using scintigraphy with ^{99m}Tc-PIPIDA, Rock *et al.* (313) found that gall bladder emptying was significantly stimulated by sham feeding. This effect was inhibited by somatostatin and atropine, suggesting that both cholinergic and hormonal mechanisms were involved in the response of the gall bladder to sham feeding. That cholinergic regulation was involved was further supported by the finding that bethanechol stimulated gall bladder emptying, although to a lesser extent than seen after a meal or sham feeding. Vagotomy or atropine decreased gall bladder emptying induced by sham feeding, but did not abolish it altogether. Read *et al.* (308) found that sham feeding had no effect on bile acid secretion in man.

1.3.2.6 The release of CCK into the blood. It is possible that CCK is the only humoral agent which, under physiological conditions, has motor effects on the gall bladder and the biliary system (245). Although it has been stated frequently that CCK is released into the blood by various individual nutrients such as amino acids and fat (19, 111, 139, 230, 239, 243) these assumptions were based on the observations that biliary output was increased after introduction of these nutrients in the upper intestine. The actual measurements of CCK in plasma were not carried out.

The immunoassay for CCK is still in its infancy. The problems of how to measure the various forms of CCK and the problem of cross-reactivity of the antibodies with other peptides than CCK, have been difficult to solve. Plasma levels of CCK vary greatly between different

centres and have to be interpreted with caution. The most recent publications with the immunoassay of CCK, however, do support that there is an increase in plasma CCK after ingestion of a mixed meal (55, 57, 256, 398), fat (32, 55, 247, 309), amino acids (32, 55, 256, 309), glucose (32, 55) and HCl (55, 309). The biological activity of CCK measured in plasma on the gall bladder and the biliary tract is as yet unknown.

1.3.2.7 The role of the parasympathetic nerve system. Parkin and Johnston (292) found that the gall bladder was dilated in subjects who had undergone truncal vagotomy, or a selective vagotomy. In subjects who had undergone highly selective vagotomy, the gall bladder shadow however was not enlarged. In cats, Liedberg *et al.* (226) found that vagal section decreased the resting pressure of the gall bladder, but it did not alter the pressure response to cholecystokinin injection. Malagelada *et al.* (241), however, have reported that truncal vagotomy increased the sensitivity of the extrahepatic biliary tract to CCK, while the sensitivity of the pancreas to CCK after truncal vagotomy was decreased.

Debas and Yamagishi (87) provided evidence for a cholinergic pylorocholecystic reflex for gall bladder contraction in dogs. They showed that a graded distention of the antrum caused a graded gall bladder contraction, even when gastrin release was suppressed by acidification of the stomach. The gall bladder contraction, induced by the gastric distention, was abolished by parenteral administration of atropine or transthoracic truncal vagotomy. This suggests that the antrum plays a role in the integration of biliary and pancreatic secretion by a vagally-mediated neural mechanism.

The effect of the parasympathetic system on the sphincter of Oddi remains most controversial. Vagotomy has been reported to decrease the tonus of the sphincter but these findings have been challenged by others (320). Electrical stimulation of the vagus has been reported to be ineffective in increasing the tone of the common bile duct or to decrease bile flow in a large series of dogs (169).

1.3.2.8 The role of the sympathetic system. Most studies seem to indicate that sympathetic drugs and stimulation through the sympathetic nervous system increases the tone of the sphincter of Oddi (320). This probably occurs by stimulation of α-adrenergic receptors, whereas β-adrenoreceptors cause relaxation. Similarly, the smooth muscle of

the gall bladder contains both excitatory (α) and inhibitory (β) adrenergic receptors (320).

1.3.2.9 Interactions between "hormones" and between "nerves" and hormones. Chowdhury *et al.* (69) working on isometric gall bladder strips of the cat, found that background doses of secretin shifted the dose-response curve for CCK-8 to the left and increased the slopes significantly with an increase in the respective maximal response. The combined action of CCK-8 and secretin was supra-additive (potentiation), suggesting that CCK-8 and secretin acted on different receptors. Prior addition of submaximal doses of gastrin shifted the dose-response curve for CCK-8 to the right, but neither the slope nor the maximum response was significantly changed. This suggested that CCK-8 and gastrin competed for a common receptor on the gall bladder.

Csendes *et al.* (80) and Lin and Spray (230) have reported in dogs that secretin given intravenously in high doses (1-4 CU $kg^{-1}h^{-1}$) augmented the stimulating effect of CCK on the hepatobiliary system.

Al-Hassani *et al.* (11) found *in vitro*, using perfused whole guinea-pig gall bladder, that the effect of electrical stimulation of the intrinsic cholinergic neurons was augmented when caerulein, bombesin or pentagastrin were added to the bath. On their own, caerulein, bombesin and pentagastrin had little or no effect on the gall bladder muscle tension. However, in the most extreme cases, sub-threshold intrinsic nerve stimulation became highly effective in the presence of sub-threshold doses of these peptides.

In a different publication Al-Hassani and Davison (12) found that the addition of tetrodotoxin to the bath, containing isolated guinea-pig gall bladder, caused a significant loss of gall bladder distensibility, but addition of either atropine or guanethidine was without an effect. This indicated that there was no intrinsic cholinergic or adrenergic tone in the isolated preparation but that non-adrenergic, non-cholinergic inhibitory nerves were active.

Davison *et al.* (83) found in the guinea pig *in vivo* that secretin and pentagastrin, in doses which were not effective on the gallbladder when administered alone, significantly increased the gall bladder contractions evoked by vagal stimulation.

Davison *et al.* (84) found evidence for non-adrenergic, non-cholinergic inhibitory nerves in the gall bladder wall of the guinea pig causing relaxation of the gall bladder. These nerves could be activated by electrical stimulation of the cervical vagus, and were

apparently activated by pre-ganglionic fibres in the vagus nerve, since the transmission was blocked by hexamethonium.

1.3.2.10 Some factors which may inhibit the secretion of bile into the duodenum. For a long time it has been known to some workers that although oral administration of egg yolk leads to a stronger contraction of the gall bladder than a similar quantity of trialaidine or butter, and that long-chain fatty acids cause a stronger gall bladder contraction than short-chain fatty acids, gall bladder contraction appears to cease at similar time in the same individual, notwithstanding the intensity of the contraction (325). This happens even although fat is still being delivered by the stomach to the duodenum. This has been interpreted so that fat in the intestine released a factor which inhibited the action of luminal fat on the gall bladder. This was further supported by cross-circulation studies, where blood of a dog, receiving intragastric injection of egg yolk, inhibited the gall bladder contraction of a connected dog (325).

More recent studies have shown that i.v. injection of an ileal extract inhibited gall bladder contraction, suggesting that the inhibitory factor was released from the ileal mucosa (91). Owyang *et al.* (289) found that colonic perfusion of oleic acid, EAA and glucose inhibited bilirubin secretion in man.

Rutishauser (319) studied intraluminal pressure of the guinea-pig gall bladder *in vitro* and found that various conjugated and unconjugated bile salts in the intraluminal fluid of the gall bladder had an inhibitory effect on the motor activity of the gall bladder, even at very low concentrations (6.0×10^{-6} mol/l). She found that this inhibition affected the contractile action of CCK on the gall bladder. It is of particular interest that of the bile salts she tested, sodium taurocholate had a stimulating effect on the gall bladder motility.

Poncelet and Thompson (303) demonstrated in cats that prolonged spasm of the sphincter of Oddi occurred when infected bile was infused into the common bile duct. They suggested that this effect was caused by secondary bile salts produced by bacterial decomposition of primary bile salts of the infected bile. This suggestion was supported by the finding that when deoxycholate was infused into the common bile duct of cats, spasm was induced.

Al-Hassani and Davison (12) observed in anaesthetised guinea pigs that there was an immediate and sustained rise in the intravesicular pressure following bilateral abdominal vagotomy, suggesting that

spontaneous or reflexly-induced activity in the pre-ganglionic vagal fibres occurred to inhibitory neurons. Boyden (48, 49) reported that while magnesium salts placed in the duodenum in man caused evacuation of the gall bladder, sodium chloride, sodium bicarbonate, sodium phosphate and sodium citrate induced temporary relaxation and filling of the gall bladder. He also noticed in cats (35) that when the gall bladder was emptying after an egg yolk, faradic stimulation of the pylorus or the duodenum temporarily inhibited the emptying of the gall bladder, while under fasting conditions this electrical stimulation caused gall bladder contractions.

Pallin and Skoglund (291) found in cats *in vivo* that vagal stimulation facilitated the effect on the gall bladder tone set up by cholecystokinin and, moreover, that stimulation of the splanchnic nerve inhibited the effect of cholecystokinin, just as it inhibited the effect of vagal stimulation.

1.3.2.11 The duodenal motility and biliary secretion. Keane *et al.* (192, 193) have demonstrated in dogs cyclic changes of biliary output into the duodenum and that these changes were associated with the intestinal motor activity. In man Peeters *et al.* (296) found that the bile acid output into the duodenum during the interdigestive state fluctuated and reached a maximum during the activity front (phase III) of the interdigestive migrating motor complex. However, Itoh and Takahashi (180, 376) found the contraction of the gall bladder in the dog to be closely associated with the initial period of the phase II of the interdigestive migrating contractions of the duodenum.

1.3.3 Pancreatic secretion

1.3.3.0 Introduction. To describe the regulation of the pancreatic secretion in separate phases (cephalic phase, gastric phase, *etc.*) is arbitrary. The response of the pancreas is an integrated response to numerous stimuli which may be evoked simultaneously from the entire gastro-intestinal tract as well as from more separate regions. The "phases" therefore do obviously overlap. However, good physiological studies, taking these complexities into account, are lacking.

1.3.3.1 Basal secretion. By basal secretion of the pancreas is meant the pancreatic secretion of water, electrolytes and enzymes during the fasting state when the individual is at rest. In healthy

subjects this secretion is low. According to Meyer (254), basal bicarbonate secretion is only 2%, and the secretion of enzymes only 15% of the maximal stimulated secretion, respectively.

The secretion of enzymes at cellular level is described as exocytosis of zymogen granules, when there is a meeting and fusion of the plasma membrane with that of the zymogen granule (158). In the basal state this occurs in a random fashion, but during stimulation a synchronous movement of the granules towards the apical membrane occurs, which increases the probability of meeting and fusion and consequently the release of the enzymes (158).

The detailed mechanism underlying the regulation of the secretion in the basal state is uncertain (254, 357). There is some evidence that basal secretion is under neurogenic control. Konturek (200) and Debas (85) reported that truncal vagotomy reduced basal pancreatic secretion in dogs, and Niebel *et al.* (277) found that basal bicarbonate secretion was partly dependent on basal cholinergic activity. Singer *et al.* (355) found that i.v. infusion of a low dose of atropine reduced basal bicarbonate and protein output.

However, at cellular level *in vitro*, atropine was not found to inhibit basal secretion, and extensive washing of pancreatic tissue slices did not reduce the secretion, suggesting that cholinergic mechanism and hormones were not involved in the secretion at that level (207).

Keane *et al.* (193) and Itoh *et al.* (181) have described the fluctuating secretory output of the pancreas into the duodenum in dogs in the basal state. The fluctuations are probably related to the rhythmic activity of the sphincter of Oddi and the phasic movements of the upper gastrointestinal tract.

Rozé *et al.* (316) have reported that synthetic β-endorphin, injected intracerebroventricularly in rats, strongly inhibited in a dose-dependent way basal pancreatic secretion. The effect was reversed with naloxone, suggesting opiate receptors mediating this central effect.

1.3.3.2 Cephalic phase. Stimulation of exocrine pancreatic secretion by the cephalic afferent loci (by sight, smell, taste and by the ingestion of food) was suggested by Pavlov (295) (the psychic phase) and by Kuvshinskii before him (295) and they were able to support their hypotheses by experiments in dogs. Pavlov was well aware that the "psychic" stimulus on the pancreas might

be mediated by gastric acid entering the duodenum, gastric acid being already at that time a well-known stimulus for pancreatic secretion. Pavlov provided a dog with oesophagectomy and both gastric and pancreatic fistulae and submitted the dog to a sham feeding. Under these experimental circumstances he observed an increase in flow of pancreatic juice. Although he admitted that gastric juice might still have escaped into the duodenum in these experiments, he made the interesting observation that the latent period of gastric secretion to sham feeding was never less than 4½ min, while the pancreatic juice began to flow 1 to 1½ min after the beginning of the sham feeding (295). He interpreted these results as evidence for that pancreatic secretion stimulated by sham feeding was not mediated by gastric acid secretion.

The longstanding dispute on the mechanism and physiological importance of the cephalic phase is not yet settled. Preshaw *et al.* (306) carried out studies in dogs, and found that acidification of the antral pouch abolished pancreatic secretion in response to sham feeding, presumably by preventing gastrin release. Partial denervation of the pancreas, while preparing the antral pouch might, however, have destroyed direct vagal stimulation. Konturek (203) repeated these studies in dogs and found that removal of the antral mucosa completely abolished gastrin response to sham feeding but pancreatic response to sham feeding was still preserved, although reduced. Subsequently truncal vagotomy almost completely abolished the pancreatic response, supporting the hypothesis of Pavlov of direct vagal activation of the pancreas.

Sarles *et al.* (324) carried out sham feeding studies in man using a balloon-occluding tube to prevent gastric fluid from entering the duodenum. They observed an increase in fluid and bicarbonate secretion, but particularly in enzyme secretion. Novis *et al.* (279), criticising the use of an intestinal balloon, and still claiming a possible acid leakage into the duodenum in these studies, went around the problem by studying the effect of sham feeding on achlorhydric patients, and observed an increase in enzyme secretion, but not in basal volume or bicarbonate secretion. Defillipi *et al.* (89) using gastric and duodenal aspiration, were unable to demonstrate pancreatic secretory responses to sham feeding, except with a background stimulation with secretin. They found that sham feeding was a powerful stimulant to pancreatic enzyme secretion, and that atropine did not affect the response. Nevertheless, there are studies where sham

feeding has not been found to have any effect on pancreatic and biliary secretion (308).

Although the mechanism of sham feeding may be partly a direct vagal stimulation on the pancreas, this effect may still be mediated by gastrin. Feldman *et al.* (117) observed that vagus releases gastrin, and that in fact during sham feeding gastrin is released (118). Valenzuela *et al.* (392) have reported that gastrin in low doses (submaximal for acid secretion) stimulated pancreatic secretory output.

1.3.3.3 Gastric phase. The gastric phase of pancreatic secretion, denied by Pavlov (295), has been demonstrated in dogs by Vagne (391), in cats by Blair (40) and in man by White (401) by distending the stomach with a balloon. This resulted in a modest increase in volume of pancreatic juice high in enzyme concentration but low in bicarbonate secretion. Therefore it resembled the secretion stimulated during the cephalic phase, and the mechanism is probably through vago-vagal reflexes, where acethylcholine and gastrin are the mediators. Humoral effects of gastrin (71) and other hormones, known to be released by gastric distention, such as PP (71, 340) and motilin (71), however, have not been excluded.

Studies aimed at separating the effects of gastrin and vagus have shown that acidification of the antrum (and thereby inhibition of gastrin release) and removal of the antral mucosa, diminish the stimulating effect on the pancreas, but truncal vagotomy abolishes it (40, 391, 401). Removal of the antral mucosa abolishes the increase in plasma gastrin levels observed during antral distention. Debas and Yamagishi (86) studied in dogs, provided with gastric and pancreatic fistulas, the effect of graded antral distention, both when gastrin release was promoted (alkaline distention), and when gastrin release was blocked (acid distention). Graded alkaline antral distention caused a graded rise in protein-rich pancreatic secretion and a graded increase in the concentration of gastrin in serum. A similar pancreatic response was observed with graded acid antral distention, although this time no rise in serum gastrin was observed. Atropine completely inhibited both types of response, and truncal vagotomy reduced pancreatic response to alkaline distention and completely abolished the response to the distention of the antrum with acid. All this supports the view that the mechanism of the gastric phase, as far as gastric distention is concerned, is mediated through vago-vagal reflexes.

Food in the stomach stimulates gastric acid secretion by an effect mediated by gastrin, acetylcholine and histamine (361). For the effect of gastric acid on the pancreas, see the *Duodenal phase (Section 1.3.3.4.i.a)*.

1.3.3.4 Duodenal phase. (i) The effect of individual luminal factors. (a) Acid. Infusion of dilute hydrochloric acid into the duodenum is a well-established stimulus for water and bicarbonate secretion from the pancreas (43, 109, 113, 157, 288, 411). The hydrochloric acid is assumed to release secretin from the duodenal mucosa, and this peptide has been considered the necessary mediator for the bicarbonate and water secretion (43, 54, 65, 109, 113, 203). However, there is no agreement as to how important this acid-induced secretin mechanism is under physiological circumstances. Repeatedly it has been reported that although ingestion of a meal led to a marked increase in bicarbonate secretion, pH of the proximal duodenum did not fall to a significant extent, and there was no detectable release of secretin into the blood postprandially (43, 54, 113, 311). This has been blamed on lack of sensitivity of the immunoassay for the secretin and recently works have been reported which showed a significant fall in duodenal pH (<4.5) after a meal which, although transient, was followed by intermittent spikes in plasma secretin and a secretion of bicarbonate into the duodenum (65, 299, 332).

The studies of Schaffalitzky de Muckadell and co-workers probably come closest to demonstrating the relationship between plasma secretin duodenal acidity and bicarbonate secretion in man. These workers observed in the fasting state and after a meal, a rapid, transient fall in intraduodenal pH to 2-3, followed by short-lived increments (spikes) in plasma concentration of secretin (332). By injecting weak solutions of HCl into the duodenum, and subsequently neutralising the acid by intraduodenal injection of bicarbonate, these workers were able to simulate the transient falls in duodenal pH, which they had observed postprandially, and measure transient increments in plasma secretin levels as seen after a meal (333). In a separate series of studies, where these workers injected small boluses of secretin parenterally, they were able to elicit small transient increments in plasma secretin, similar to that which they had observed after a meal or acid injections into the duodenum, and this resulted in a stimulation of bicarbonate secretion (334).

(b) Protein. After a protein-rich meal much of the small intestinal mucosa is exposed to the digestive products of protein of various molecular sizes, ranging from large polypeptides, through oligopeptides, to free amino acids. The intraluminal peptide-bound amino acids are present in several times greater concentration than the free amino acids (1). According to Adibi (1), total concentration of free intrajejunal amino acids after a protein-rich meal rose from 3.2 mmol/l to 29.3 mmol/l, but individual amino acids rose no higher than to 5.6 ± 0.9 mmol/l (SEM). In experimental studies, however, the effect of free amino acids has usually been studied at considerably higher concentration levels. Go *et al.* (139) perfused the duodenum with a mixture of essential amino acids of 78 mmol/l, and found the pancreatic response to start within 20 min of perfusion and to be mainly enzymatic. In magnitude it was similar to a submaximal stimulation of CCK. In fact they found that only *l*-phenylalanine, *l*-valine, *l*-methionine and *l*-tryptophan induced cholecystokininetic effects. Konturek *et al.* (204) reported that essential amino acids placed either in the duodenum or administered parenterally, stimulated the pancreas, *l*-tryptophan and *l*-phenylalanine being the most potent. These amino acids were of similar potency when administered intraduodenally and intravenously. Nevertheless, when administered intravenously they did not affect secretin-induced pancreatic secretion, whereas intraduodenal administration augmented the secretin response. Stabile (365) reported, however, that amino acids placed in the duodenum stimulated pancreatic secretion but not when they were given intravenously, and this was also the experience of Go (139).

Perfusion of the jejunum with amino acids is not without an effect, according to DiMagno (95), but simultaneous perfusion of the duodenum and the jejunum with amino acids caused less trypsin output than at any one of these sites. Fink and Meyer (119) have suggested that there is a synergism between the intestinal acid stimulus and protein digest stimulus on pancreatic secretion.

Undigested proteins probably do not stimulate pancreatic secretion (119, 412).

(c) Fat. Luminal fat stimulates pancreas, but only when lipolysed. Corn oil administered directly, or after emulsification with bile salt, does not stimulate pancreatic secretion until lipase has been added to the emulsion. The stimulating effect of fatty acids

has been related to the chain length of the acids, the degree of saturation, concentration, and the total load and concentration of the bile salts relative to the fatty acids (243). According to Stabile and Edelman (108, 365) fat introduced intravenously has no effect on the pancreas, but this has been denied by others (204). There are recent reports (43, 114) that sodium oleate placed into the duodenum stimulates pancreatic bicarbonate secretion, and this has been reported to occur with (114) or without (43) detectable changes in peripheral or portal blood levels of secretin.

Malagelada *et al.* (243) observed a complicated relationship between luminal bile salts and fatty acids in man. By raising the luminal concentration of sodium taurocholate, pancreatic response to stimulation with 18-carbon fatty acids was decreased, while a further increase in concentration (above 10 mmol/l) of the bile salt caused an increase in pancreatic secretion stimulated by the fatty acid. The authors suggested that by raising the concentration of the bile salt, the absorption of fatty acids was decreased, and this increased the intestinal mucosal area exposed to the fatty acids and thereby the assumed CCK release.

(d) Mixed meal. There are realtively few studies on the overall pancreatic response to a food in man. Standard meals are reported to elicit three-quarters of the maximal enzyme response to a combined stimulus by exogenous secretin and CCK (412). The pattern of response depends on the physical and chemical state of the meal. A solid meal is delivered more slowly from the stomach than a liquid or homogenised meal, and therefore the pancreatic secretory response to a solid meal is not so acute, but it lasts longer (244). Digestion seems, indeed, to occur during a longer time after a meal than previously was considered, and to extend throughout most of the length of the small intestine. Breakdown products of protein have been found in the small intestine for 4 h after a meal and have been detected in the ileum. Recent studies by Itoh *et al.* (179) in dogs show the post-prandial period to last for 16 h, and during that time pancreatic enzyme output peaked by 2-3 h after feeding but bicarbonate secretion did not reach a maximum until 8-9 h later. How relevant these studies are to man is not known, but Moore *et al.* (265) have reported a time lag between enzyme and bicarbonate secretion in man after ingestion of a meal. Pancreatic enzyme secretion peaked immediately after the meal, while bicarbonate secretion did not reach a maximum until 1 h

later. This time was suggested necessary to decrease the duodenal pH by the meal to the threshold necessary for secretin release.

(e) Carbohydrates. Chey (66) reported in dogs that i.v. 2-deoxy-D-glucose potentiated the pancreatic bicarbonate secretion stimulated by duodenal acidification. The effect was not mediated by a release of secretin. Stabile (365) reported that intraduodenal glucose stimulated pancreatic secretion, while parenteral administration had no effect. Byrnes (54) found no effect of oral glucose on secretin release. Nakajima and Magee (274) have reported that i.v. glucose inhibited pancreatic secretion.

Insulin-induced hypoglycaemia markedly stimulates pancreatic enzyme, bicarbonate and water secretion, and this effect is suppressed by antrectomy and antroneurolysis (315) and totally abolished by a complete vagotomy (104).

(f) Divalent ions. Magnesium sulphate and magnesium chloride placed in the upper intestine, are well known stimulants of pancreatic enzyme secretion (242) and there is evidence that this effect is mediated by CCK (131).

Experimental luminal hypercalcaemia stimulates pancreatic enzyme secretion in man (167). Experimental hypocalcaemia inhibits pancreatic secretion stimulated by CCK and secretin (412).

(g) Bile salts. See *Chapter IV, Section 4.1).*

1.3.3.5 The jejunal/ileal phase. Laugier and Sarles (218) found in the rat that iso-osmolar solution of oleic acid placed in the duodenum induced pancreatic secretion of water, bicarbonate and protein. When the infusion of oleic acid was terminated, pancreatic secretion fell slowly to basal levels, but 2-3 h later these workers observed that protein secretion became suppressed below basal levels. When oleic acid was placed straight into the ileum, protein secretion was immediately suppressed. Laugier and Sarles (218) suggested that oleic acid in the ileum released a hormonal factor with an inhibitory effect on the pancreas, and they were able to support this idea by cross-circulation studies.

An extract from the pig ileum has been purified (pancreatone, anticholecystokinin) and found to inhibit basal and CCK-stimulated pancreatic secretion of water and proteins in rats, when administered

parenterally (91). Harper *et al.* (159, 160) have confirmed in cats and Voirol (395) in man that intra-ileal infusion of oleic acid inhibits exogenously-stimulated pancreatic secretion.

1.3.3.6 The colonic phase. Hage *et al.* (151) observed that infusion of the proximal colon with oleic acid inhibited secretin stimulated pancreatic bicarbonate and protein secretion in conscious dogs, and Harper observed similar effects on secretin/pancreozymin stimulated pancreatic secretion in anaesthetised cats after extrinsic denervation of the colon and the pancreas. This indicated a humoral mechanism, which was both anti-secretin and anti-cholecystokinin in effect. Owyang *et al.* (289) reported recently that colonic perfusion of oleic acid inhibited pancreatic enzyme and bicarbonate secretion in man. While EAA and glucose placed in the colon had no effect on the pancreatic secretion, all three nutrients inhibited biliary secretion, suggesting differential sensitivity of pancreatic and biliary secretions.

1.3.3.7 The effect of exogenous peptides. (i) Secretin. (a) The effect on water and electrolyte secretion. Most recent studies on the effect of secretin on bicarbonate secretion have used a continuous i.v. infusion of the synthetic peptide (GIH). Doses as low as 0.03 CU $kg^{-1}h^{-1}$ of GIH secretin have recently been claimed to simulate peak plasma levels of secretin as observed after a meal (15–20 pmol/l) (417) (418), and to result in a detectable bicarbonate secretion (99, 417, 418). Until recently, studies have claimed that much higher doses were necessary to obtain physiological plasma levels (54, 150, 412) of secretin. This may underline the fact that the immunoassay for secretin is far from being established, and "physiological values" for plasma secretin have varied 100-fold between different centres during the last decade (158).

(b) The effect on enzyme secretion. It is difficult to estimate the effect of secretin on pancreatic enzyme secretion because of the "wash-out" phenomenon, which has repeatedly been reported to occur at the beginning of a secretin stimulation (9, 139) (see further *Section 4.4*). As Wormsley (412) has pointed out, there is no experimental proof that the initial increase in enzyme secretion is actually due to a "wash-out" of enzymes already secreted and lying around in the ductal system of the pancreas. Wormsley (412) has suggested that

the phenomenon is perhaps better understood by assuming that there is a transient stimulation by secretin of preformed zymogens from the pancreatic acinar cells, followed by an inhibition of this process by what is presumably a pharmacological action of secretin (412).

Probably the strongest experimental evidence that secretin has an effect on cellular release of enzymes in the pancreas is that acinar cells do have secretin receptors (312, 412).

This may explain why secretin at high doses inhibits pancreatic enzyme secretion, as it may occupy receptors which otherwise were bound by a more biologically active CCK.

From the physiologist's point of view, the effect of secretin on pancreatic enzyme secretion is at present considered minimal and probably only of theoretical interest (269).

(ii) Cholecystokinin. (a) The effect on enzyme secretion. 99% pure CCK (CCK-33) administered parenterally is a powerful stimulant to enzyme secretion in man (270). It is considered to stimulate both the synthesis and the discharge of enzymes (270).

According to a recent report from Walsh (398), the threshold for stimulation of pancreatic enzyme secretion is at plasma CCK-8 concentrations of 4-5 pmol/l, and approximately half-maximal response is at CCK-8 concentrations of 10 pmol/l.

These levels were, however, considerably higher than he measured in blood after a meal (398).

(b) The effect on bicarbonate secretion. There are reports which claim that pure CCK on its own in physiological doses stimulates water and bicarbonate secretion, at least in dogs (270, 357).

(iii) Gastrin. In man, gastrin is a stimulant of pancreatic enzyme secretion, and even at doses submaximal for gastric acid secretion (392). In dogs, gastrin is reported to be on molar basis one-third as effective as CCK in stimulating pancreatic enzyme secretion (412). While it is not known if a direct effect of gastrin on the pancreatic acinar cells is physiological or not, gastrin is physiologically considered to have an indirect effect on the pancreatic bicarbonate secretion through the stimulation of gastric acid and subsequently secretin release.

(iv) Motilin. In dogs, motilin is reported to stimulate protein secretion (412). Indirectly motilin may affect pancreas by enhancing gastric emptying (70), and by initiating the migrating myoelectric complex (180). Parenterally administered motilin suppresses secretin-induced bicarbonate secretion in dogs (201, 202).

(v) Glucagon. Sweeting (374) found in dogs that glucagon administered intravenously inhibited the pancreatic response to duodenal acidification, and Dyck found in man (106) that relatively small doses of glucagon (1.0 μg kg^{-1} to 5.0 μg kg^{-1}) produced a dose-related inhibition of pancreatic water secretion in response to secretin alone or secretin plus CCK. Necheles (275) (studies in dogs) and DiMagno (95) (studies in man) have also reported that i.v. glucagon inhibited pancreatic enzyme secretion, and this happened at similar plasma levels of glucagon as were obtained by a perfusion of the duodenum and the jejunum with EAA. DiMagno *et al.* (95) found this inhibition to be potent and not to be overcome by a pharmacological stimulus of CCK. Glucagon, however, had no effect on basal pancreatic secretion (95).

(vi) Pancreatic polypeptide. See *Chapter V.*

1.3.3.8 The splanchnic nerve system/adrenergic and anti-adrenergic drugs. Adrenergic nerve fibres have been found to pass to the acinar cells of the pancreas (357), and it is suggested that they may act there as inhibitors of pancreatic enzyme secretion. Secretin-stimulated pancreatic secretion is inhibited by β-adrenergic agonists (186) and recently catecholamines were found to inhibit pancreatic secretion even after their vasoconstricting effect had been abolished by α-receptor blockage (phenoxybenzamine) (158).

Most sympathetic nerve fibres, nevertheless, are closely linked to the blood vessels (periarterial plexuses), and their role there is to regulate blood flow. By regulating the blood flow to the pancreas, the splanchnic nerve system may play an important role in regulating the secretory function of the organ, as this might be controlled by blood-borne hormones and metabolites to a certain extent.

α-Adrenergic agonists suppress basal as well as stimulated pancreatic secretion, and this effect is probably due to vasoconstriction. Cutting the splanchnic nerves leads to an increase in pancreatic enzyme and bicarbonate secretion (381). The possible role of the sympatho-sympathetic reflexes in inhibiting pancreatic secretion

has gained little attention up to now, but there is evidence that the stimulation of the afferent sympathetic fibres from the lower end of the gut may inhibit pancreatic enzyme secretion (158).

1.3.3.9 Interaction between secretin and cholecystokinin. Henriksen and Worning (164) were probably the first to report that CCK augmented the effect of secretin on the bicarbonate secretion. Meyer *et al.* (252) supported this when they observed in dogs that the maximum bicarbonate response to secretin was increased by either i.v. CCK or the duodenal perfusion with *l*-phenylalanine. This suggested that both exogenous and endogenous CCK augmented the action of secretin. Similar findings were obtained by Konturek *et al.* (204) who found that amino acids and fat in the duodenum increased the secretin-induced bicarbonate response. The increase in secretin-induced bicarbonate secretion by CCK has been found to be at least 2 to 3-fold, suggesting that the interaction between secretin and CCK is in fact a potentiation, *i.e.* the combined effect of these two peptides is greater than the sum of the effect of each. These findings also suggest that a very small and transient increment of secretin may be able to stimulate considerably bicarbonate secretion when the secretion is also stimulated by a background stimulation of CCK and possibly other hormones (VIP, neurotensin ?). Very recently Fink and co-workers (119) reported in dogs that *l*-phenylalanine and digested bovine serum albumin enhanced secretin-induced pancreatic secretion.

Reports are more controversial as to whether secretin has an effect on CCK-stimulated enzyme secretion. Henriksen (164) and Meyer (252, 253) found some evidence for this. According to Wormsley (412), the effect in man of increasing the dose of secretin on a background dose of CCK is biphasic. When the dose rate of secretin is increased, while the dose of CCK is held constant, both bicarbonate and enzyme outputs increase with small doses of secretin, while large doses of secretin cause the output of bicarbonate still to increase but the output of enzymes is inhibited. However, Dembiński *et al.* (90) have reported in cats that a secretin dose up to 4 CU $kg^{-1}h^{-1}$ had no effect on the level of enzyme output stimulated by a peptone meal.

1.3.3.10 The mechanism of the pancreatic secretory response to intraluminal stimuli: a humoral effect or an enteropancreatic reflex?

Pavlov introduced the concept of long vago-vagal reflexes as a mechanism underlying the pancreatic secretory response to intestinal stimuli (295). Although his ideas were usually rejected, or considered of minor importance after the discovery of secretin, there were always leading authorities who claimed that the reflex hypothesis should be given more credit and that it might be the main regulatory mechanism (159, 381).

There is no disagreement that secretin (9, 150) and cholecystokinin (270) are powerful stimulants of pancreatic secretion when administered parenterally in pure forms. The disagreement lies in whether secretin and CCK are ever released into the blood in biologically active forms by intraluminal stimulants and, if so, in such an amount that they cause the powerful secretory responses of the pancreas seen after a meal (43).

Blood levels of secretin detected after a meal or infusion of acid are small and transient, and it has not yet been proven that they are biologically active. It has even been suggested that they were due to tissue damage caused by the experimental load of acid infused into the intestine (412).

The same applies to CCK. Although various molecular forms of CCK have been detected in blood (57, 247, 309), the assay technique is still in its infancy and plasma values vary greatly between different centres (32, 55, 57, 61, 158, 247, 256). Some workers have been unable to detect but marginal changes in plasma CCK after a meal (256, 398) and which are unlikely alone to explain stimulation of pancreatic enzyme secretion (see also *Section 1.3.2.6*).

Although still hypothetical, the concept of the enteropancreatic reflex is supported by abundant evidence of pancreatic enzyme and bicarbonate secretion being forcefully stimulated by excitation of efferent vagal nerves (381), and that vagotomy and anticholinergic drugs reduce the secretion (85, 236). The evidence for the afferent limb of the reflex arc comes from studies where, during duodenal perfusion of acid, glucose or amino acids, electrical impulses have

been recorded in the afferent fibres of the mesenteric nerves (14, 347). However, anatomically and physiologically the complete reflex arc has not yet been demonstrated.

Grossman and co-workers have recently published a series of studies which physiologically do support the existence of an enteropancreatic reflex. They demonstrated (354) in dogs with pancreatic fistulas and otherwise intact pancreas that when l-tryptophan or sodium oleate were rapidly injected into the duodenum, the latency period of the response of amylase secretion was 0.30 min for the amino acids and 0.33 min for the oleate, and this was significantly less than the latency period to CCK when injected intraportally of 0.53 min. Atropine and truncal vagotomy increased the latency period to l-tryptophan and the oleate 10-fold but had no effect on the latency period to intraportal CCK. These workers also made the observation that the latency period to luminal HCl was longer than to intraportal injection of CCK and that the response to HCl was only slightly and not significantly affected by atropine, indicating that the response to HCl was hormonally mediated (354, 355).

Recently, these results have been challenged by data from this same centre showing that an extrinsically denervated pancreas was no less responsive than the innervated pancreas to intestinally perfused stimuli (88).

At present the mechanism underlying the enteropancreatic responses seems to be neither solely humoral nor solely neurogenic, but an interaction between humoral and neurogenic agents, and considerable literature has accumulated in support of this.

Valenzuela *et al.* (393) reported that while atropine did not modify the amylase secretion stimulated by CCK-8, atropine markedly suppressed the basal enzyme secretion and the response to intraduodenal oleate, and this happened without changes in plasma CCK. This suggested that atropine neither interfered with the release of CCK nor the effect of CCK on the target organ, the pancreas, but that a part of the pancreatic response to luminal oleate was mediated by neurogenic pathways.

Konturek *et al.* (198) found that vagotomy markedly reduced the potentiation of secretin-induced bicarbonate secretion by CCK and caerulein, and Chey *et al.* (66) have reported in dogs that bicarbonate secretion, stimulated by duodenal acidification, was decreased by bilateral cervical vagotomy, but plasma concentrations of secretin, achieved by the duodenal acidification, were not affected.

They concluded that changes in cholinergic tone did not affect the release of endogenous secretin. This same group also reported recently in man (418) that i.v. atropine suppressed pancreatic secretion of bicarbonate and protein induced by low doses of secretin. I.v. atropine also suppressed bicarbonate secretion induced by intrajejunal perfusion of HCl, a perfusion which resulted in plasma secretin concentrations similar to those seen after a meal. However, atropine did not affect the increase in the plasma secretin concentration produced by the jejunal acidification, suggesting that atropine inhibited the pancreatic effect but not the intestinal release of secretin. These workers agreed therefore with Niebel (277), Preshaw (306), Solomon (362) and Wormsley (412), but disagreed with Konturek (200), Dembiński (90), Ertan (111) and Debas (85), who considered hormone release to be facilitated by the cholinergic tone.

Chey *et al.* (66) thought that the cholinergic tone made the pancreas more sensitive to hormones and secretion, but Dreiling *et al.* (104) found that total vagotomy did not influence the response of the pancreas to exogenous secretin. Malagelada *et al.* (241) observed, however, that after vagotomy and pyloroplasty the sensitivity of the pancreas to CCK was decreased but the sensitivity of the gall bladder to CCK was increased. Smith *et al.* (360) found insulin-induced hypoglycaemia to stimulate pancreas only in patients with intact nerve supply.

Local anaesthetics applied topically to the small intestinal mucosa in dogs strongly inhibits pancreatic secretion induced by duodenal acidification (269, 270).

1.3.3.11 The pancreatic secretion and duodenal motility. DiMagno *et al.* (96) have reported that volume and enzyme output of the pancreatic juice fluctuates in the fasting state in dogs and that it peaks during the phase III of the migrating myoelectric complex. Itoh *et al.* (181) have demonstrated interdigestive changes in the flow of pancreatic juice and fluctuations in the basal secretion in dogs, and that this was related to the gastric and duodenal motor activity. Keane *et al.* (192) demonstrated the cyclic pattern of pancreatic secretion into the duodenum in the fasting state with a maximum output during phases II and III of the migrating myoelectric complex. They also showed that the composition of the pancreatic fluid varied according to the different phases of the duodenal activity (193).

1.3.3.12 Difference in sensitivity of the pancreas and the biliary system to exogenous stimuli. Malagelada *et al.* (240), using the duodenal perfusion technique, reported that the pancreas could be stimulated by a threshold dose which was 8 times lower than a threshold dose for the gall bladder. More recent studies have not confirmed this. They have, indeed, shown that the dose-response curves of trypsin and bile salts to CCK were almost superimposable (235, 419, see also *Section 1.3.3.6*).

1.4 OVERVIEW

Based on the present knowledge, it is tempting to speculate that food, passing down the foregut, the stomach and the duodenum, stimulates the biliary and pancreatic secretion, while the content of the small intestine and the colon tend to inhibit it. The cephalic and foregut phases of this process may be mediated mainly by neurogenic pathways, while the effect of food in the duodenum is transmitted by a mechanism which is both neurogenic and humoral. The interplay between humoral and neural factors is still poorly understood, even after a century of active research. There is considerable evidence that the response in the ileum and the colon is mediated by humoral transmitters. However, until recently the regulatory effect of the lower small intestine and the colon on the pancreas and the biliary tract have received little interest. Little attention has also been paid to inhibitory effects on the biliary tract and the pancreas elicited from the more proximal parts of the intestine after a stimulation like a meal.

The aim of the studies presented here was however centred mainly around such inhibitory mechanisms, which might regulate biliary and pancreatic secretion in the postprandial and interprandial states, and the physiological and biochemical techniques necessary to assess the secretion under these circumstances.

CHAPTER II

THE DUODENAL PERFUSION TECHNIQUE, WITH GASTRIC ASPIRATION AND INERT RECOVERY MARKERS: AN EVALUATION OF FACTORS AFFECTING THE "STEADY STATE" CONDITIONS IN THE DUODENUM

2.1 INTRODUCTION

Duodenal perfusion techniques, with and without gastric aspiration, and using a variety of recovery markers, have been widely used to quantify biliary and pancreatic secretion in man (29, 45, 138). Although the same perfusion technique may apparently be used by several workers, in practice differences in procedural details invariably occur, which may affect the results. Each worker therefore has to evaluate his own perfusion technique under the experimental circumstances in which it is being used (42, 124).

The present chapter describes in detail the duodenal perfusion technique used in studies on biliary and pancreatic secretion described in the following chapters. Evaluation was carried out on the efficiency of the gastric and duodenal aspiration and the mixing of gastric and duodenal fluids. The effect of this on the steadiness of the "steady state" was estimated and the effects of perfusion flow rate on the "steady state" conditions.

2.2 MATERIALS AND METHODS

2.2.1 The experimental design and reduction of data

The retrospective evaluation of the perfusion technique was based on studies carried out consecutively in 66 normal subjects (ages 20-43 years). Fully informed, written consent was obtained from all volunteers in these studies and all experiments were approved by the Royal Postgraduate Medical School Research Ethics Committee. The studies were carried out to investigate the regulation of biliary and pancreatic secretion in man under various experimental conditions. The evaluation was limited only to the equilibration period in all studies, *i.e.* the initial 2 h perfusion period in every study during which time iso-osmolar saline alone was perfused through the duodenum, without or with a constant i.v. background stimulation of caerulein and secretin.* The equilibration period does not include the very first 30 min of duodenal perfusion/aspiration, where samples were usually not collected (for exception, see *Section 2.2.1.10*).

*Similar evaluation of the "steady state" during the "test periods" and the "recovery periods" were not carried out.

2.2.1.1 Efficiency of the gastric aspiration: recovery of phenol red from the stomach and the duodenum. The volume of gastric fluid, sampled every 10 min, was measured directly and the phenol red analysed as described in Section 2.2.7. The volume of fluid in the duodenal test segment was determined by calculations based on the equation described by Go *et al.* (138), and on ^{14}C PEG 4000 as a volume marker.

The recovery fraction for phenol red in every 10 min gastric and duodenal sample was estimated, and the average recovery for each study calculated and expressed as a mean ± SEM of all samples (n = 12) in every study.

2.2.1.2 Efficiency of the duodenal aspiration: recovery of PEG 4000 and ^{14}C PEG 4000 from the duodenum. PEG 4000 and ^{14}C PEG 4000 were measured in 10 min samples of the duodenal aspirate, as described in Section 2.2.7, and the recovery of PEG 4000 and ^{14}C PEG 4000, respectively, calculated for every 10 min sample as a percentage of the amount of the marker infused into the duodenum every 10 min.

Recovery was expressed as mean ± SEM of all samples (n = 12) for every study.

2.2.1.3 Assessment of duodeno-gastric reflux: the recovery of PEG 4000 and ^{14}C PEG 4000 from the stomach. The amount of PEG 4000 and ^{14}C PEG 4000 was measured in every 10 min gastric sample and the recovery calculated as a percentage of the amount of the marker infused into the duodenum every 10 min. The average recovery (± SEM) (n = 12) in each study was calculated for each of the two markers and the results plotted as a scattergram.

2.2.1.4 Assessment of jejuno-duodenal reflux. To assess the possible reflux of the duodenal aspirate infused into the jejunum, 2.2 x 10^4 dpm of ^{3}H PEG 4000 was added to the aspirate infused into the jejunum every 10 min. The [^{3}H] activity detected in the duodenal aspirate was calculated as a percentage of [^{3}H] infused every 10 min and finally expressed as mean ± SEM of all samples in each study.

2.2.1.5 The correlation between using ^{14}C PEG 4000 and PEG 4000 as reference substances to estimate "steady state" conditions in the duodenum. The coefficient of variation (CV) of the concentration of ^{14}C PEG 4000 and PEG 4000, respectively, in the duodenum over the 2 h equilibration periods

was calculated in all studies. The CV of ^{14}C PEG 4000 was plotted as a scattergram *versus* CV of PEG 4000.

2.2.1.6 The relationship between the efficiency of the gastric aspiration and the "steady state" of the volume marker ^{14}C PEG 4000. The efficiency of the gastric aspiration, estimated as the average percentage recovery of phenol red during the 2 h equilibration period, was plotted as a scattergram *versus* coefficient of variation of the volume marker ^{14}C PEG 4000 during the same period. As perfusion flow rate may have an effect on the "steady state" of the volume marker, only studies with the perfusion flow rate >5.0 ml min^{-1} and ≤10.0 were used in this study (n = 58).

2.2.1.7 The relationship between the efficiency of the duodenal aspiration and the "steady state" of the volume marker ^{14}C PEG 4000. The efficiency of the duodenal aspiration, estimated as the average percentage recovery of ^{14}C PEG 4000 during the 2 h aspiration period, was plotted as a scattergram *versus* coefficient of variation of the volume marker ^{14}C PEG 4000 during this period. As perfusion flow rate and gastric aspiration may have an effect on the "steady state" of the volume marker in the duodenum, only studies with flow rate >5.0 ml min^{-1} and ≤10.0 ml min^{-1}, and gastric aspiration efficiency of ≥70% were used in this evaluation.

2.2.1.8 The relationship between duodeno-gastric reflux and the "steady state" of the volume marker ^{14}C PEG 4000. The duodeno-gastric reflux, estimated as the average percentage recovery of ^{14}C PEG 4000 in the gastric aspirate, was plotted as a scattergram *versus* the steady state of ^{14}C PEG 4000 in the duodenal fluid, and the correlation between these two parameters estimated. The analysis was limited to studies with perfusion flow rate >5.0 ml min^{-1} and ≤10.0 ml min^{-1}, and gastric aspiration ≥70%.

2.2.1.9 The relationship between the perfusion flow rate and the "steady state" of the volume marker ^{14}C PEG 4000. The perfusion flow rate in ml min^{-1} was plotted as a scattergram *versus* the "steady state" of the volume marker ^{14}C PEG 4000 and the correlation between these two parameters estimated. The analysis was limited to studies with gastric aspiration ≥70%.

2.2.1.10 The time of appearance and disappearance of ^{14}C PEG 4000 in the duodenal fluid. The appearance and disappearance of ^{14}C PEG 4000 in the duodenal fluid was studied in two sets of studies, each at different perfusate flow rate: A) 4.1 ± 0.4 ml min^{-1}, SEM, n = 6; B) 9.0 ± 0.6 ml min^{-1}, SEM, n = 9. The studies lasted for 2½ h, and the samples were collected every 10 min, except for the first 30 min, where they were collected every 2.5 min. ^{14}C PEG 4000 was determined as dpm in every sample and plotted as geometric mean ± SEM *versus* function of time.

2.2.2 Instruments

Aspiration pumps for continuous gastric aspiration and continuous duodenal aspiration were obtained from Air Shields Inc, Hatboro, Philadelphia, Pennsylvania, USA. Accurate continuous i.v. infusion of caerulein and secretin, and gastric perfusion of phenol red were carried out using a Harvard compact infusion pump, Model 975 (Harvard Apparatus Company Inc, 150 Dover Road, Millis, Massachusetts 02054 - Sales distributor TEM Sales Ltd, Gatwick Road, Crawley, West Sussex RH10 2RG, England). Peristaltic pumps (Peripump multichannel, P-4202 and P-4205, Scientific Supplies Co Ltd, Scientific House, Vine Hill, London EC1, England) were used for continuous duodenal perfusion.

2.2.3 Chemicals

^{14}C Polyethylene glycol, mol. wt. 4000, 21.7 mCi/g in aqueous solution containing 3% ethanol, was purchased from Amersham International, Amersham, Bucks, England, and polyethylene glycol (1.2-^{3}H) mol. wt. 4000 (0.25 mCi/227.3 mg) from New England Nuclear, Boston, Massachusetts, USA. Polyethylene glycol, mol. wt. 4000, and phenol red were purchased from British Drug Houses Chemical Ltd, Poole, Dorset, England.

For information on secretin and caerulein, used as a continuous i.v. background stimulation in these studies, see *Section 4.2*.

All polyvinyl chloride and polyethylene chloride materials for perfusion/aspiration tubes were purchased from Portex®, Hythe, Kent, CT21 6JL, England.

2.2.4 Preparation of perfused solutions

PEG 4000 (5 g/l) together with ^{14}C PEG (5 µCi/l) prepared in NaCl (150 mmol/l) were used as non-absorbable duodenal reference markers. Phenol red (1.5 g/l in distilled water, pH 7.0) was used as a recovery

marker for gastric aspiration.

2.2.5 Preparation of infusion solutions

For preparation of caerulein and secretin infusion solutions for a background stimulation of biliary and pancreatic secretion, see *Section 4.2.5.*

2.2.6 Sample processing

Duodenal aspirates were pooled for 10 min on ice in darkness, aliquots saved in dark-brown bottles for analysis of PEG 4000, ^{14}C PEG 4000 and ^{3}H PEG 4000 and phenol red. The samples were kept frozen at -20°C until analysed, and never thawed more than twice before analysis. Gastric aspirates were pooled on ice for 10 min, and the samples kept frozen until analysed for phenol red, PEG 4000 and ^{14}C PEG 4000.

2.2.7 Analytical methods

PEG 4000 in the duodenal and gastric fluid was measured by the turbidimetric method of Hydén (172), as modified by Malawer and Powell (246). ^{14}C PEG 4000 in gastric and duodenal fluids was determined by liquid scintillation counting in a Packard Tri-Carb® 2660 counter. 0.5 ml of the sample was counted for 5 min in 10 ml of Patterson-Greene scintillation cocktail (293). Counts per minute (cpm) were corrected to disintegrations per minute (dpm) from standard curves using suitable quenched standards by picric acid (duodenal samples) or phenol red (gastric samples), respectively (*Appendix I* and *II*). ^{3}H PEG 4000 was simultaneously counted in samples from studies where this marker had been used as a tracer, and the spillover of [^{14}C] into the [^{3}H] channel corrected for by using automatically fixed discriminator settings (290). The maximum counting efficiency % (cpm/dpm x 100) for [^{14}C] and [^{3}H] was 92% and 43%, respectively.

The intra-assay reproducibility expressed as CV for determination of ^{14}C PEG 4000 in the duodenal fluid was 2.3% (n = 24), and the difference between duplicates was 1.0% ± 0.1% (SEM, n = 28). The corresponding figures for the modified Hydén method for determining PEG 4000 in the duodenal fluid were 3.0% (CV) (n = 24) and 4.2% ± 0.6 (SEM, n = 28), respectively.

Phenol red was analysed spectrophotometrically in an SP 500 Pye Unicam Spectrophotometer at wavelength 550 nm and 410 nm after alkalinisation (pH 12.3). The interference of other chromogens in the fluids was corrected for by using the formula:

$$ODcorr = OD550 - (0.13511 \times OD410) + 0.0039$$

as recommended by Baron (23). The recovery fraction was calculated as follows:

$$RF\% = \frac{ODcorr\ (sample) \times vol\ (sample)}{ODcorr\ (standard) \times vol\ (standard)} \times 100$$

Standard samples were obtained at the beginning and at the end of each study as the amount of phenol red infused per 10 min. Standard samples were diluted in approximately 50 ml of tap water and phenol red analysed as described before.

2.2.8 Statistical analysis

The data were evaluated by Student's *t*-test for paired or unpaired data, as appropriate, and least squares fit regression analysis (300).

2.2.9 The duodenal tube with jejunal re-infusion segment

The duodenal tube with jejunal re-infusion segment consisted of four tubes glued together (Fig. 1): (a) a transparent polyvinyl chloride tube of 3 mm inner diameter (Portex® reference code 800/000/225), and which at the distal end was provided with 20 aspiration holes (10 on each side) for continuous aspiration of the duodenal fluid; (b) a radio-opaque grade polyethylene tube (soft, low density, reference code 800/100/500, inner diameter 2 mm), which served as an air ventile close to the aspiration holes to ensure efficient continuous aspiration; (c) a radio-opaque grade polyethylene tube (hard, high density, reference code 800/100/300, inner diameter 1 mm) for infusion of the perfusate with the non-absorbable markers; (d) a polyethylene tube of the same type as described in (b) (2 mm inner diameter) for re-infusion of duodenal fluid into the jejunum. This tube reached 20 cm into the jejunum and was fixed to a solid extension of the previously described polyvinyl tube (a). A mercury bag of 13 g was fixed to the distal end of this tube to facilitate passage into the intestine. Radio-opaque markers were placed inside the tube wall at the site of the perfusion port, and just distal to the aspiration holes, to make correct location of the tube easier.

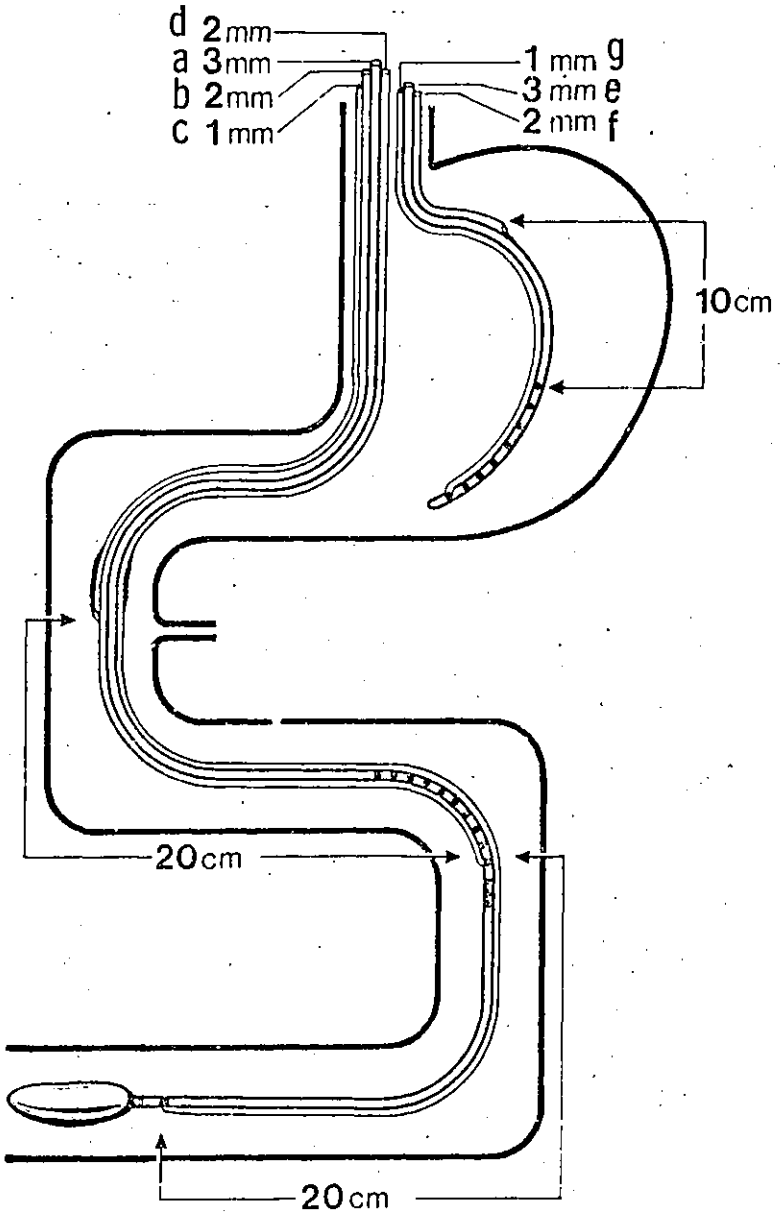


Fig. 1. A schematic diagram of the gastric tube and the duodenal perfusion/aspiration tube with a jejunal reinfusion segment.

2.2.10 The duodenal perfusion with jejunal re-infusion

After an overnight fast the duodenal tube was passed orally under fluoroscopic control into the duodenum and positioned so that the proximal perfusion port was opposite the ampulla of Vater and the aspiration holes for continuous suction of fluid were close to the ligament to Treitz (Fig. 1). The distance between the perfusion port and the aspiration holes (the mixing segment) was 15 cm (the most proximal hole) to 20 cm (the most distal hole). The jejunal segment reached 20 cm into the jejunum.

After the intubation, the volunteers rested comfortably for 30 min sitting in a chair, before the collection of fluid and the actual experiment started. All perfusion studies were carried out on the subjects in the sitting position to minimise duodeno-gastric reflux.

2.2.11 The gastric tube

The gastric tube consisted of three small tubes of material which has been described before (*Section 2.2.9*), *i.e.* the 3 mm polyvinyl tube (Fig. 1) (e) for gastric aspiration, and two polyethylene tubes (f, g), which served as an air ventile and an infusion tube for phenol red, respectively. The aspiration tube had 16 perforations (8 on each side) at the distal end for suction of fluid. The distance between the infusion port for phenol red and the moxt proximal aspiration holes was 10 cm.

2.2.12 The gastric aspiration

The gastric tube was introduced *per os* and positioned so that the distal end was in the antrum and the infusion port for phenol red in the middle of the body of the stomach. Phenol red was infused at 13.2 ml min^{-1}. The gastric aspiration was carried out by a continuous mechanical aspiration, interrupted frequently by effective manual suction.

2.3 RESULTS

2.3.1 Efficiency of the gastric aspiration: recovery of phenol red from the stomach and the duodenum

Based on the 2 h equilibration period only (12 samples), the recovery of phenol red from the stomach was on average 81.5% ± 8.5 (SEM, n = 12) with the range of 60.1% to 99.6%. The recovery of

of phenol red from the duodenal fluid was on average 15.7% ± 2.4% (SEM, n = 12) with the range of 1.4% to 42.9%. As can be seen from Appendix III, phenol red was overestimated at least in some of the studies (total recovery >100%).

2.3.2 Efficiency of the duodenal aspiration: recovery of ^{14}C PEG 4000 from the duodenum

The recovery of the duodenal marker ^{14}C PEG 4000 during the equilibration period was on average 50.4% ± 5.0 (SEM, n = 12) with the range of 17.9% to 85.1% (*Appendix III*).

2.3.3 Assessment of duodeno-gastric reflux: the recovery of ^{14}C PEG 4000 from the stomach

The recovery of ^{14}C PEG 4000 from the stomach (duodeno-gastric reflux) was on average 9.0% ± 1.9 (SEM, n = 12) with the range of 0.3% to 37.8% (*Appendix III*).

2.3.4 Assessment of jejuno-duodenal reflux: duodenal recovery of ^{3}H PEG 4000 infused into the jejunum

In the studies (n = 14) where the duodenal aspirate (>90%) was infused into the jejunum in every subsequent 10 min collection period throughout the studies, using ^{3}H PEG 4000 as a tracer, ^{3}H PEG 4000 was not detected in the duodenal aspirate in 8 of the studies. It was ≤5.4% of the infused total radioactivity in 5, and 32.1% ± 8.7 (SEM, n = 24) in one.

2.3.5 The correlation between using ^{14}C PEG 4000 and PEG 4000 in estimating "steady state" conditions in the duodenum

Fig. 2 shows that the correlation between the two volume markers, ^{14}C PEG 4000 and PEG 4000, in estimating the "steady state" in the duodenum was highly significant (r = + 0.7142, n = 66, P <0.0001). It shows that out of the 66 pairs of studies, 53 had CV <20%, 5 had CV <25% and >20%, and 8 had CV >25%. On average the CV in these studies was 13.1% ± 1.0 (SEM, n = 12) where ^{14}C PEG 4000 was used as a recovery marker, and 14.1% ± 2.5 (SEM, n = 12) where PEG 4000 was used as a recovery marker. The difference between these results was not significant (P <0.2).

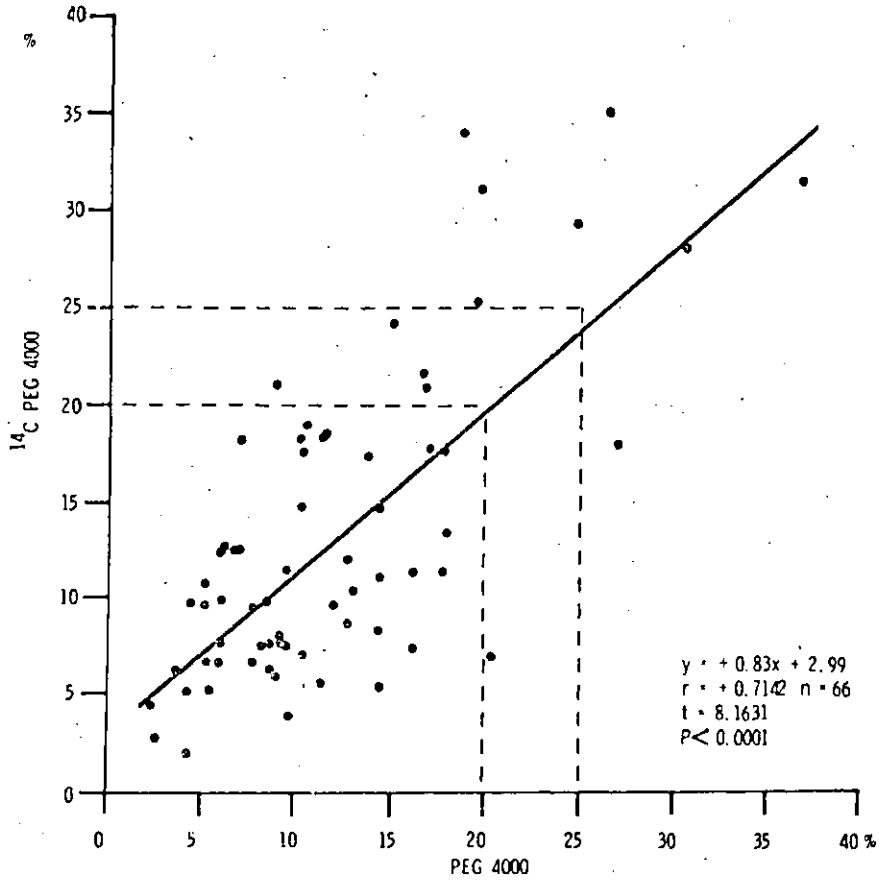


Fig. 2. A scattergram showing the least squares fit linear relationship between the "steady state" in the duodenum as estimated by the volume marker ^{14}C PEG 4000 (ordinate) or PEG 4000 (abscissa). The data are based on a 2 h equilibration period in 66 studies.

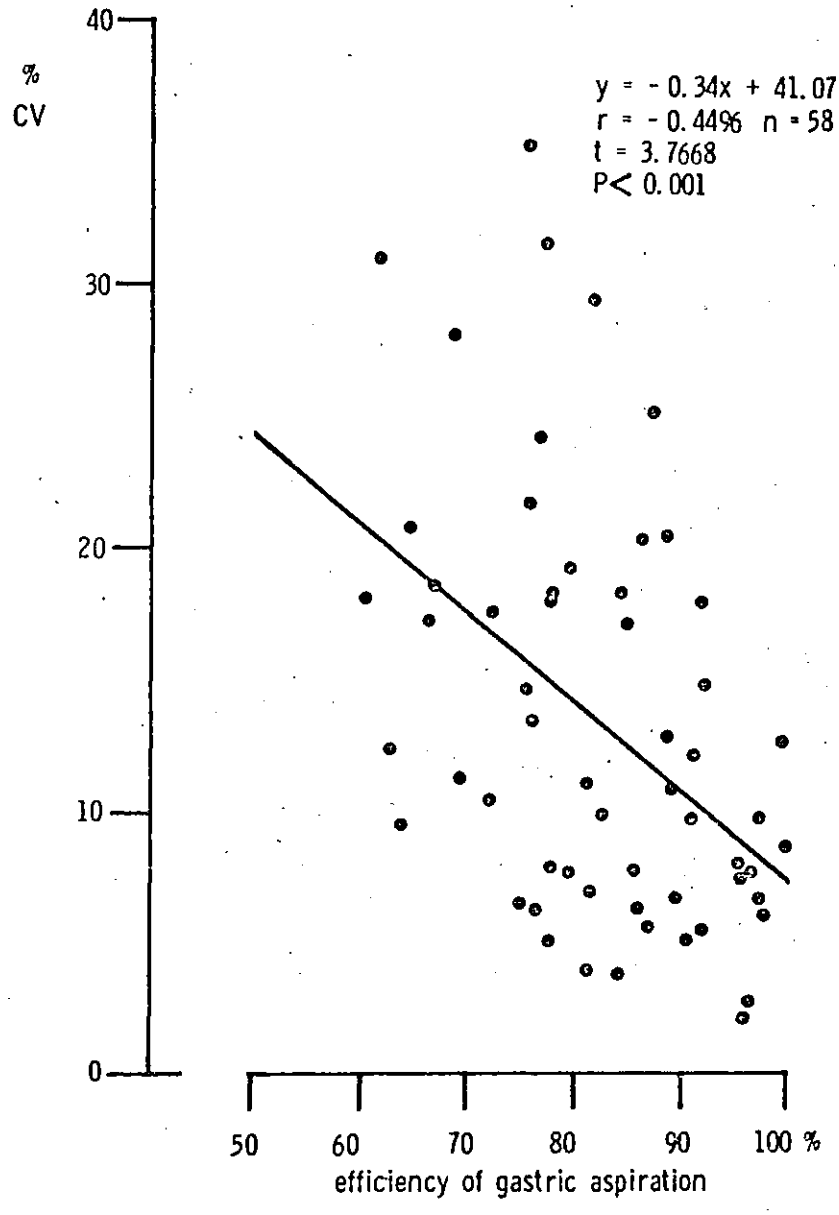


Fig. 3. A scattergram showing the least squares fit linear relationship between the efficiency of the gastric aspiration and the "steady state" of the volume marker ^{14}C PEG 4000 in the duodenum (expressed as a coefficient of variation of the conc. of the reference marker during the 2 h equilibration period). The data are based on a 2 h equilibration period in 58 studies, where the perfusion flow rate was >5.0 ml min^{-1} and $\leqslant 10.0$ ml min^{-1}.

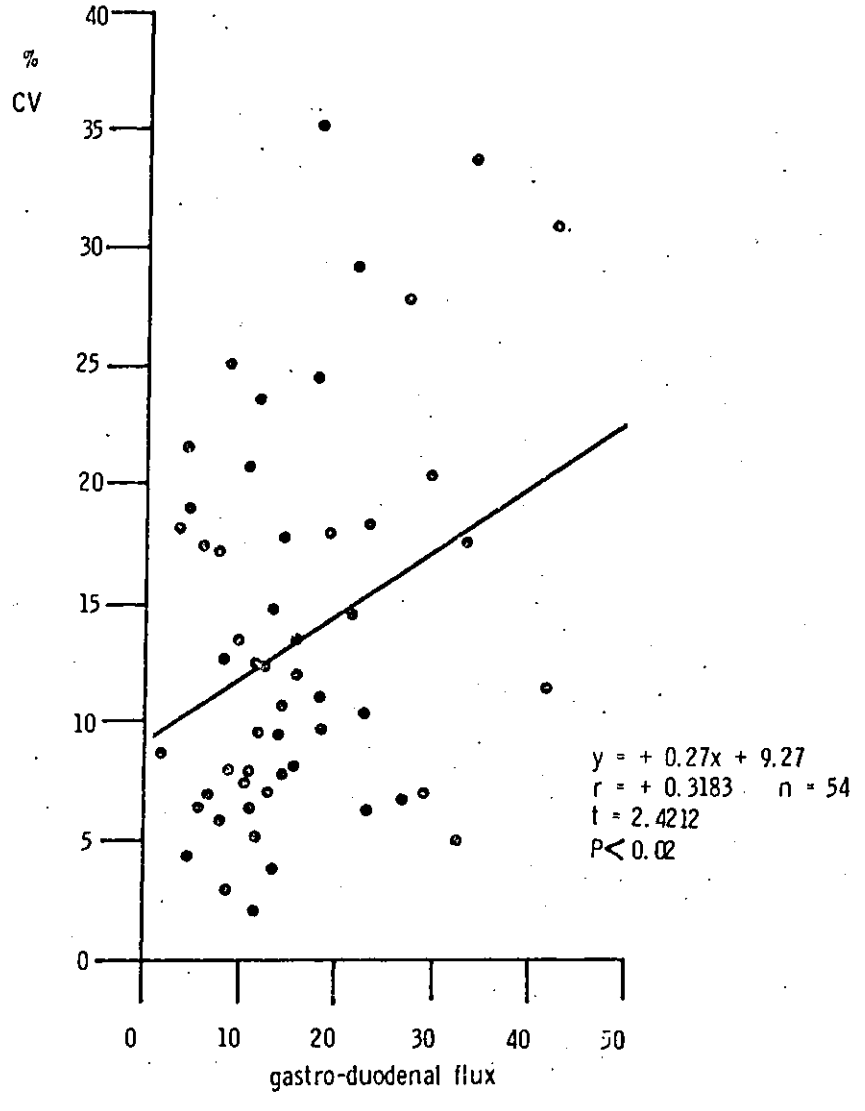


Fig. 4. A scattergram showing the least squares fit linear relationship between the amount of the gastric marker phenol red (PR) recovered from the duodenum (gastro-duodenal flux) and the "steady state" of the volume marker ^{14}C PEG 4000 in the duodenal test segment. The data are based on a 2 h equilibration period in 54 studies where the perfusion flow rate was >5.0 ml min^{-1} and ≤10.0 ml min^{-1}.

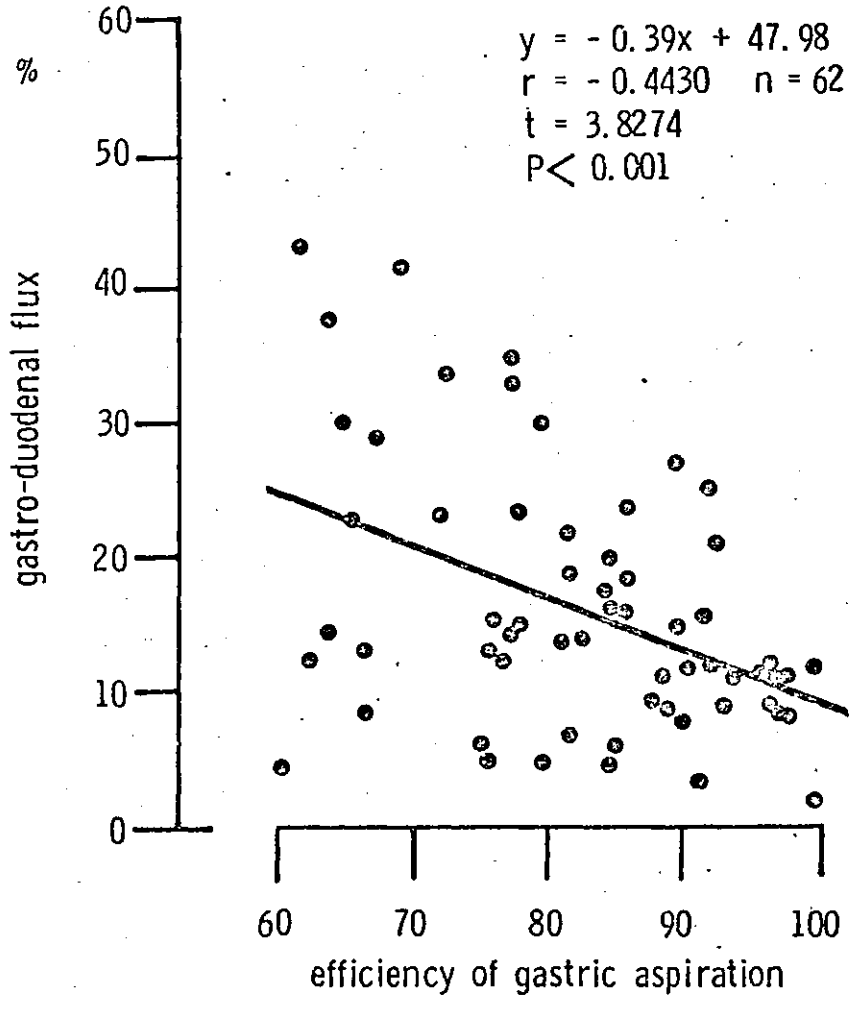


Fig. 5. A scattergram showing the least squares fit linear relationship between the efficiency of the gastric aspiration and the gastro-duodenal flux, estimated as the percentage recovery of the gastric marker phenol red from the duodenum. The data are based on a 2 h equilibration period in 62 studies.

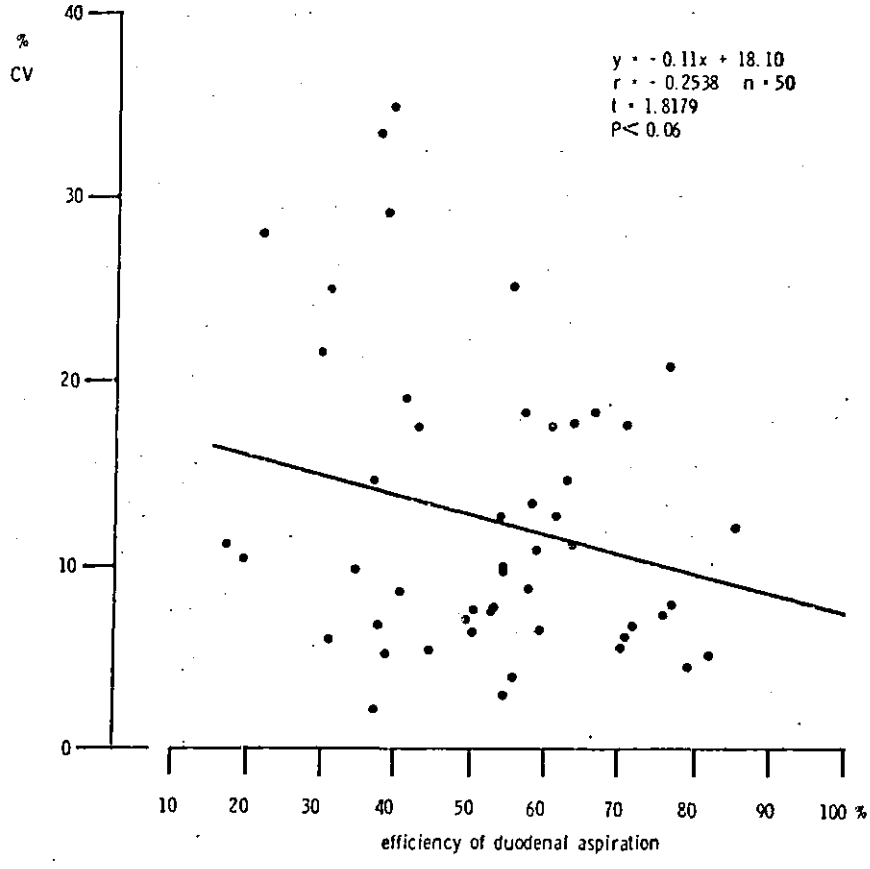


Fig. 6. A scattergram showing the least squares fit linear relationship between the efficiency of the duodenal aspiration and the "steady state" in the duodenum. The data are based on a 2 h equilibration period in 50 studies, where the perfusion flow rate was >5.0 ml min^{-1} and ≤10.0 ml min^{-1}, and the efficiency of the gastric aspiration ≥70%.

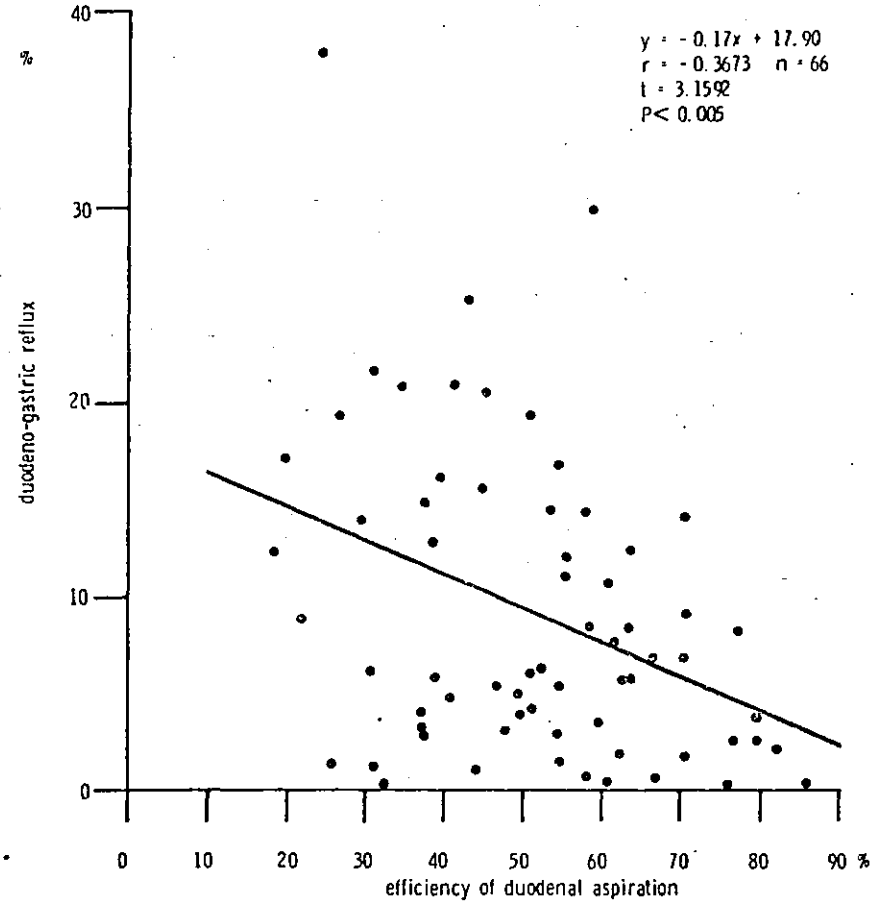


Fig. 7. A scattergram showing the least squares fit linear relationship between the efficiency of the duodenal aspiration and the duodeno-gastric reflux. The data are based on a 2 h equilibration period in 66 studies.

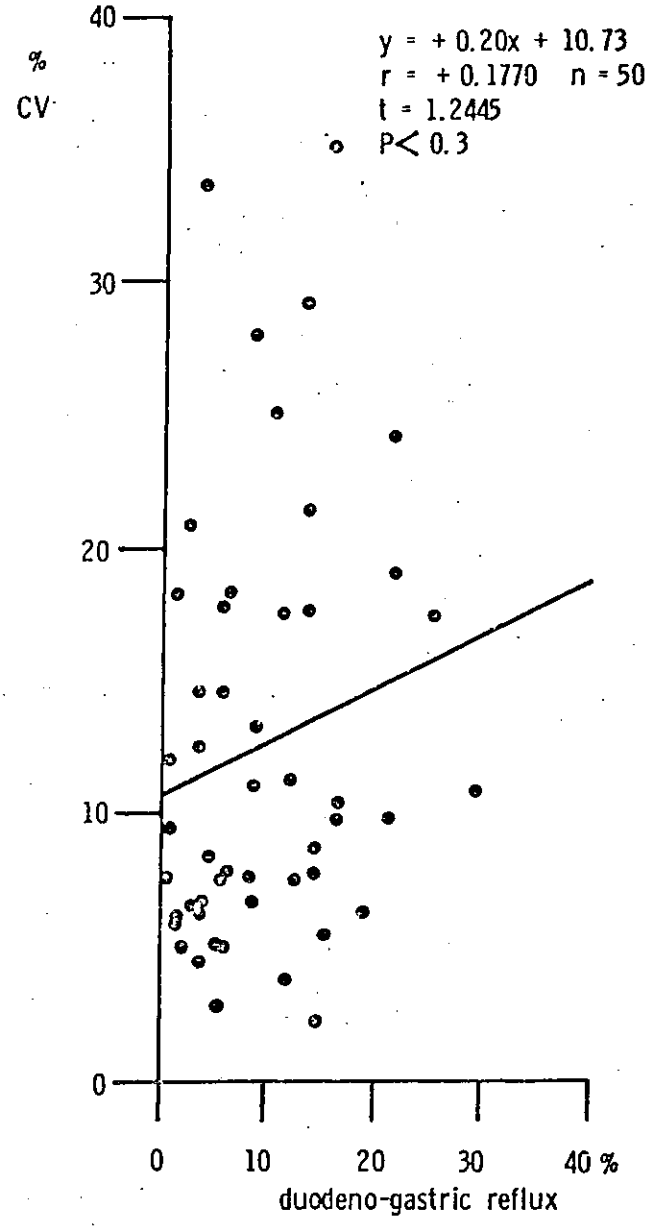


Fig. 8. A scattergram showing the least squares fit linear relationship between duodeno-gastric reflux estimated as the percentage gastric recovery of the duodenal marker ^{14}C PEG 4000 and the "steady state" in the duodenal test segment. The data are based on a 2 h equilibration period in 50 studies where the perfusion flow rate was >5.0 ml min^{-1} and ≤10.0 ml min^{-1}, and the efficiency of the gastric aspiration ≥70%.

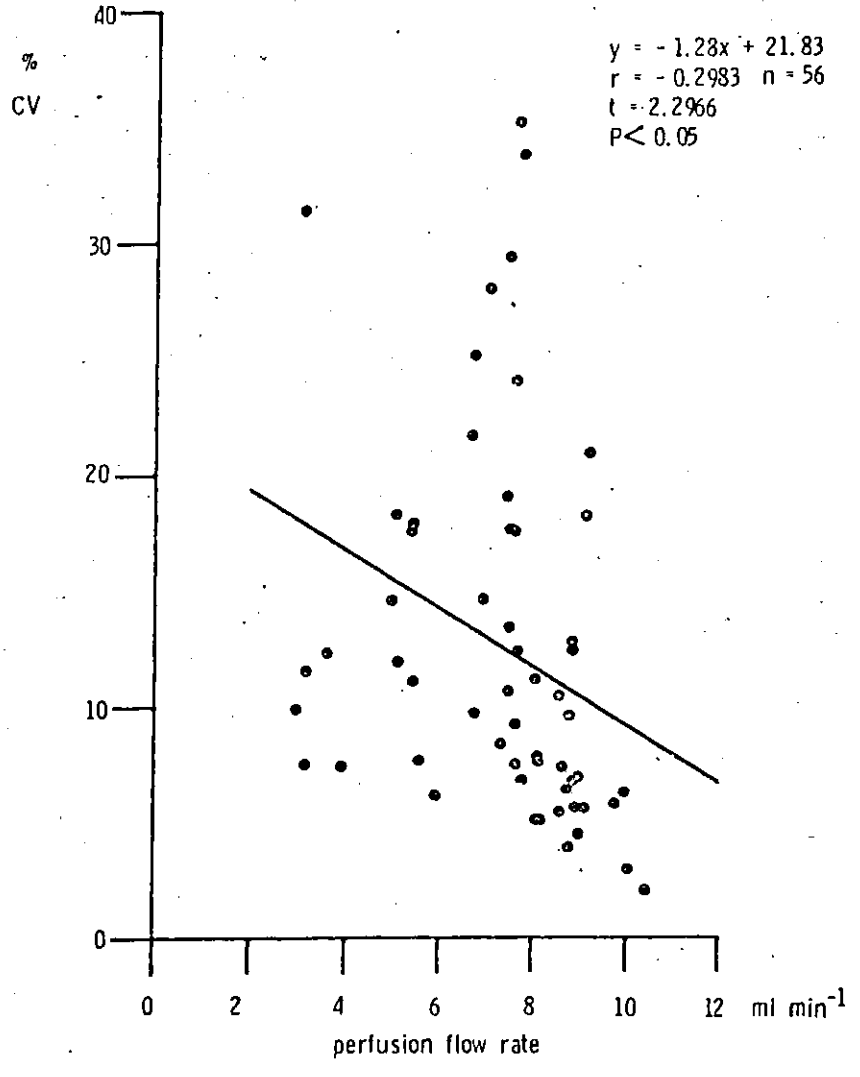


Fig. 9. A scattergram showing the least squares fit linear relationship between the perfusion flow rate and the "steady state" in the duodenal test segment. The data are based on a 2 h equilibration period in 56 studies, where the efficiency of the gastric aspiration was ⩾70%.

2.3.6 The relationship between the efficiency of the gastric aspiration and the "steady state" of the volume marker ^{14}C PEG 4000

Fig. 3 shows the significant correlation between the efficiency of the gastric aspiration and the "steady state" in the duodenum (r = - 0.4496, n = 58, P <0.001). Fig. 4 shows a weak but significant relationship between gastro-duodenal flux and the "steady state" in the duodenum (r = + 0.3183, n = 54, P <0.02). Fig. 5 shows that there was also a significant inverse correlation between the efficiency of the gastric aspiration and the amount of gastric fluid fluxed into the duodenum (P <0.001).

2.3.7 The relationship between the efficiency of the duodenal aspiration and the "steady state" of the volume marker ^{14}C PEG 4000

The correlation between the efficiency of the duodenal aspiration and the "steady state" was marginally significant (r = - 0.2538, n = 50, P <0.06) (Fig. 6). However, there was a clear correlation between the efficiency of the duodenal aspiration and the magnitude of the duodeno-gastric reflux (P <0.005), as shown in Fig. 7.

2.3.8 The relationship between the duodeno-gastric reflux and the "steady state" of the volume marker ^{14}C PEG 4000

A significant correlation was not found between duodeno-gastric reflux and the "steady state" in the duodenum (r = + 0.1777, n = 50, P <0.3) (Fig. 8). Fig. 8 also shows a great intercept on the ordinate, suggesting an independence of the "steady state" of the duodeno-gastric reflux.

2.3.9 The relationship between the perfusion flow rate and the "steady state" of the volume marker ^{14}C PEG 4000

Fig. 9 shows the significant relationship between the perfusion flow rate and the "steady state" (r = - 0.2983, n = 56, P <0.05). The efficiency of the gastric aspiration in these studies was ⩾70%.

2.3.10 The time of appearance and disappearance of ^{14}C PEG 4000 in the duodenal fluid

Figs. 10.A and B show that the reference marker ^{14}C PEG 4000 was detected in the duodenal fluid by the first 2.5 min sample collected in both groups of studies and that the concentration (dpm) of the

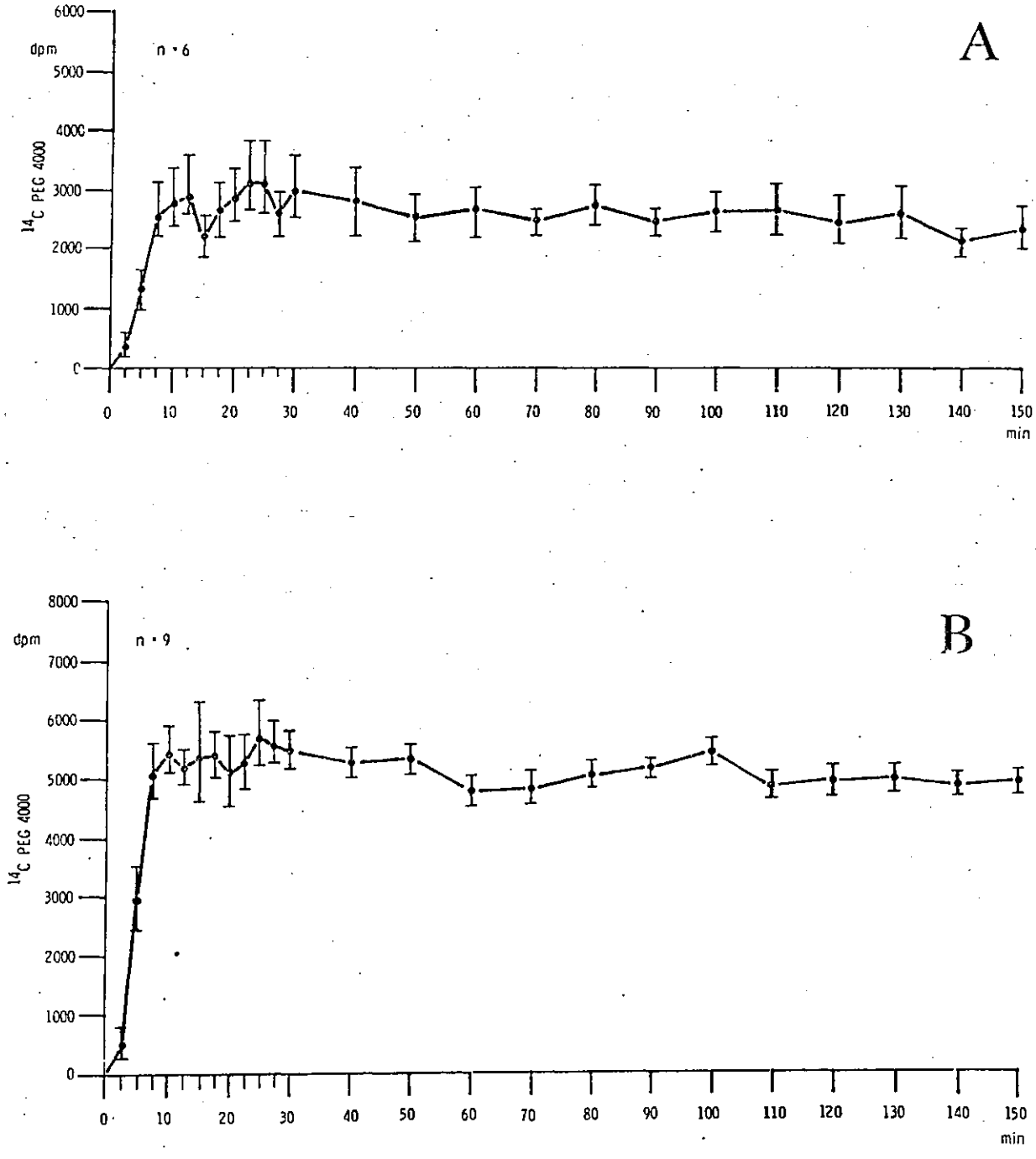


Fig. 10. The appearance and concentration levels (dpm) of ^{14}C PEG 4000 in the duodenal fluid in 2½ h perfusion studies. A: perfusion flow rate 4.1 ± 0.4 ml min^{-1}, SEM (n = 6); B: perfusion flow rate 9.0 ± 0.6 ml min^{-1}, SEM (n = 9). During the first 30 min of the studies samples were collected every 2.5 min, but thereafter every 10 min.

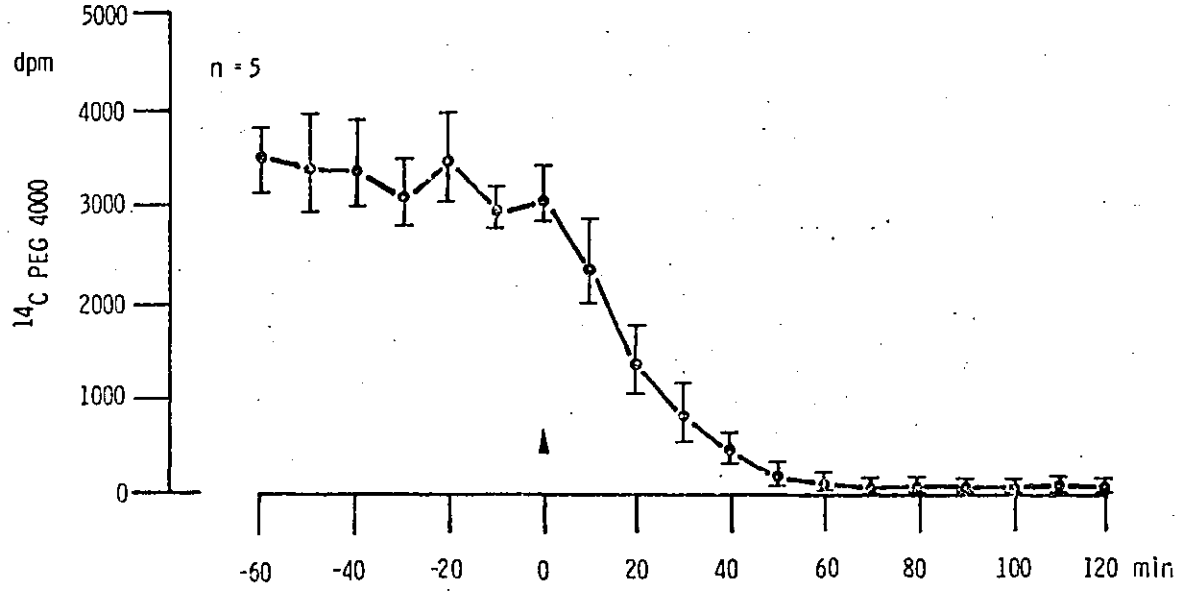


Fig. 11. The disappearance of ^{14}C PEG 4000 from the duodenal fluid in perfusion studies where the duodenal perfusion of the volume marker ^{14}C PEG 4000 ceased at hour 0 (arrow). After that the duodenum was perfused with n-saline for 2 h. Perfusion flow rate was 9.2 ± 1.1 ml min^{-1} (SEM, n = 5).

marker had reached a plateau by 10 min. It remained at that plateau throughout the studies with the "steady state" of 7.4% (CV) (flow rate 4.1 ± 0.4 ml min^{-1}) and 4.3% (CV) (flow rate 9.0 ± 0.6 ml min^{-1}, SEM), respectively. Fig. 11 shows the disappearance of the marker, and that it had virtually disappeared from the fluid by 1 h after cessation of the infusion of the marker into the duodenum.

2.4 DISCUSSION

Aspiration of fluid from the human duodenum *in vivo* can never be complete, and the fraction removed varies within and between studies. A reference marker, however, renders complete aspiration recovery unnecessary, and the duodenal perfusion technique used here is based on this principle. The duodenum was perfused at a constant rate with a marker solution to obtain steady concentration levels of the marker in the duodenum. Under ideal circumstances, where the marker is completely mixed and uniformly distributed within the duodenal fluid, the total volume of fluid in the duodenum can be calculated as the product of the volume of the fluid perfused into the duodenum per unit time and the ratio of the marker's concentration infused and aspirated (138).

In practice, a completely even distribution of the reference marker is not likely to be reached. It can only be approached. Peristaltic movement of the gut and variable luminal absorption and secretion inevitably upset the distribution of the marker within the fluid.

The only way to control how close to a uniform distribution of the reference marker one has reached in the duodenum is to demonstrate how constant the marker's concentration is in the aspirated samples. In the present studies this steadiness of the concentration of the reference marker PEG 4000 in the duodenal fluid was investigated and the possible effects of several factors on the steadiness evaluated. This was done by a retrospective analysis of the 2 h equilibration periods of the actual test studies which were carried out to investigate biliary and pancreatic secretion.

This analysis shows that on most occasions an efficient gastric and duodenal aspiration had been carried out during the equilibration period, and the duodeno-gastric reflux and gastro-duodenal flux were limited. The CV of the concentration of the marker varied between studies, but correlation analysis suggested that the "steady

state" was improved by efficient gastric and duodenal aspiration and high perfusion flow rate. That the gastric and duodenal aspiration did improve "steady state" was also supported by the correlation found between the magnitude of gastro-duodenal flux and the "steady state", the efficiency of the gastric aspiration and the magnitude of the gastro-duodenal flux, and the efficiency of the duodenal aspiration and the magnitude of the duodeno-gastric flux.

Although various publications mention their efficiency of gastric and duodenal aspiration, no detailed reports have yet been published on the recovery of gastric and duodenal markers in duodenal perfusion studies in man, except by Go *et al.* (138). The number of subjects in their report was considerably smaller than reported here (33 volunteers), and their report seems to be a selection from a larger material (138, 139). The recovery of the gastric marker from the stomach and the duodenum was similar in their study to that reported here. The magnitude of duodeno-gastric reflux was also similar in both series of studies. However, the recovery of the duodenal marker from the duodenum was considerably higher in the studies presented here (50.4% ± 5.0 *versus* 36.7% ± 3.3). This may be explained by the wide bore aspiration tube and the wide bore air ventile used in the present studies.

Go *et al.* (138) found that calculations of biliary and pancreatic outputs could be accurately performed without taking notice of gastro-duodenal flux, provided that the efficiency of the gastric aspiration was over 50%. In the present studies the efficiency of the gastric aspiration was 60% or more on all occasions. However, an inverse correlation was found between the magnitude of the gastric fluid which escaped into the duodenum and the "steady state" in the duodenum in these studies, indicating that even though gastric aspiration efficiency is over 60%, contamination by gastric fluid has an effect on the "steady state". Duodeno-gastric reflux was negligible in the present studies, which is in agreement with Go *et al.* (138) and it seemed not to have a detectable effect on the "steady state" conditions in the duodenum.

A good correlation was found between the two duodenal markers (radiolabelled and nonradiolabelled PEG 4000). No previous reports have dealt with this. Wingate *et al.* (405) compared PEG 4000 and ^{14}C PEG 4000 as water phase markers in the human jejunum and found that they gave a similar estimate of water absorption and secretion.

Their study, however, was based on a very small number of subjects and a different technique, using a balloon occlusive tube, which prevented fluid from the duodenum entering the jejunum.

Within the range of perfusate flow rate usually used in duodenal perfusion studies, it has been shown that the volume marker can be expected to reach a plateau by 10-15 min of perfusion in the duodenum and remain so for 2 h. This is in agreement with previous studies (138). To wash out the marker completely from the duodenum, at least 1 h is required. Based on these observations, the equilibration period of 2 h in the present studies should be more than adequate.

Phenol red was certainly overestimated in some of the studies (total recovery >100%). It is possible that this error was due to reflux of a bile-rich fluid into the stomach, which was not corrected for by the technique used here to correct for interference of chromogens. Alternatively, there may have been an overestimation of phenol red which actually escaped into the duodenum due to loss of duodenal marker from the duodenal fluid by reflux into the stomach. If this happened before adequate mixing with duodenal contents, this would have led to an overestimation of the duodenal fluid volume and an overestimation of phenol red in the duodenal segment. However, in the studies where the total recovery of phenol red was overestimated, the duodeno-gastric reflux of ^{14}C PEG 4000 was no greater than in the other studies, which makes the former explanation unlikely.

2.5 SUMMARY

(1) A retrospective evaluation was carried out on the steadiness of the concentration of the reference marker ^{14}C PEG 4000 in the duodenum in a large number of duodenal perfusion studies. The evaluation was limited to the 2 h equilibration periods in all studies. Studies were carried out with a wide bore duodenal aspiration tube and a wide bore air ventile, and on the subjects in a sitting position. A high duodenal perfusate flow rate and a long perfusion mixing segment were used to improve mixing with endogenous fluid. Collection periods were kept short to minimise fluctuations in marker concentration. Gastric aspiration was carried out in all studies.

(2) Carried out in this way, an efficient gastric and duodenal aspiration was obtained in most studies with limited gastro-duodenal flux and duodeno-gastric reflux.

(3) A "steady state" of 13.1% ± 1.0 (SEM, n = 66) was established in the duodenum. A positive correlation was found between the improvement of "steady state" on the one hand and the efficiency of the gastric aspiration, the efficiency of the duodenal aspiration, and the increase in flow rate of the perfusion fluid on the other hand. An inverse correlation was found between the "steady state" and the amount of gastric fluid fluxed into the duodenum.

(4) A good correlation was found between radiolabelled and unlabelled polyethylene glycol in estimating "steady state" in the duodenum.

CHAPTER III

PHYSICO-CHEMICAL STUDIES OF INDOCYANINE GREEN

3.1 INTRODUCTION

Indocyanine green (ICG) (Cardiogreen®) is a water soluble, non-toxic tricarbocyanine dye of a molecular weight 775 (52, 129) (Fig. 12). It was developed in the Kodak Research Laboratories by Heseltine and Brooker (52, 129) and introduced into clinical research by Fox *et al.* in 1956 (128). It has since been used extensively for measurement of blood flow through organs in man (56, 64, 222, 259, 310, 328, 402, 407) and animals (194, 368) by the indicator dilution technique (259). The λmax of ICG in blood is close to the isosbestic point of reduced haemoglobin and oxyhaemoglobin, which makes it possible to study the dilution of the indicator at varying pH and oxygen levels (129).

ICG is almost entirely confined to the blood compartment by protein binding (20, 128, 129, 185, 191, 307, 400), and it is rapidly and exclusively extracted from the blood by the liver and excreted into the bile chemically unchanged (22, 56, 64, 171). It has no enterohepatic circulation (56). Because of these biological properties, ICG has been used increasingly as a test substance for determination of hepatobiliary excretory function in man and animals (56, 64, 171, 194, 195, 208, 209, 294, 394, 402) and as a marker for estimation of biliary excretion (29, 105). It has been used in a radiolabelled form for these purposes (16, 17). ICG has been claimed to be satisfactory as an intestinal perfusion marker (123). Other cyanine compounds similar to ICG have been developed for clinical purposes (330).

Although ICG is a suitable substance for hepatobiliary studies, difficulties with its analysis in biological fluids occur, because like most cyanine-dye chromogens it is unstable on storage, especially in aqueous solutions (129, 134, 216, 258, 373) when a progressive loss of colour is observed, associated with the appearance of a fast-running degradation component on thin-layer chromatography (TLC) (22). The instability of ICG in aqueous solutions is affected by dilution, light, and by change in ionic strength of the solution (129, 134, 216, 390). Although ICG has been reported to be stable in methanol (304) and bile (20), this has not been studied in detail, and little is known of the effects of temperature on ICG (129, 134) and changes in pH (129, 187). The structure of the breakdown derivative is unknown (22).

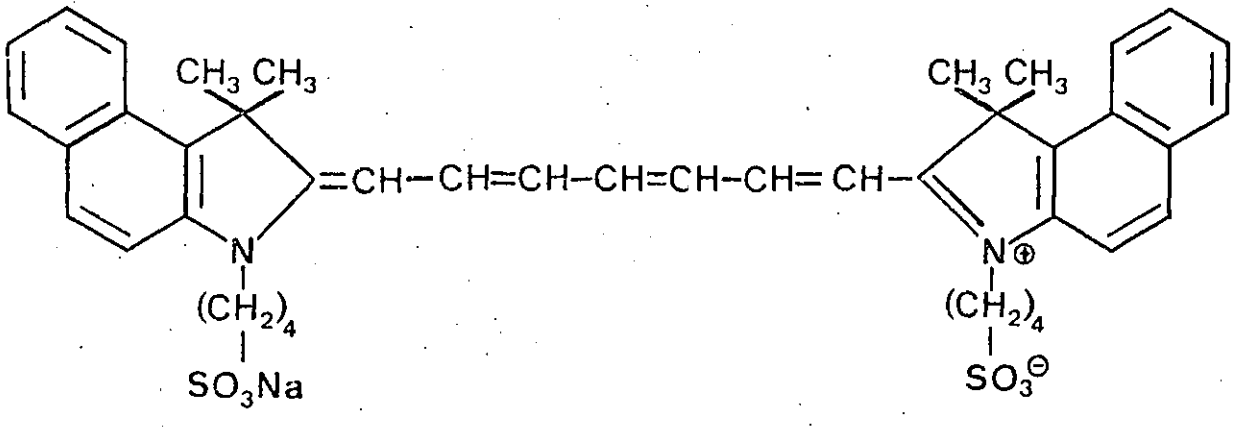


Fig. 12. The structural formula of indocyanine green: anhydro-3,3,3',3'-tetramethyl-1,1'-(sulfobutyl)-4,5,4',5'-dibenzoindotricarbocyanine hydroxide sodium salt. Molecular weight 775. Note, there are three vinyl groups (-CH=CH-) in the methine chain between the two dibenzoindole rings.

Because ICG was intended for use in human studies as a marker for biliary excretion into the duodenum, where pH and composition of fluid is variable, the effects of storage and changes in pH on the spectrophotometric absorbance of the compound were studied in various biological fluids. The effect of high temperature on the rate of decay of ICG in several organic solvents was also studied. The organic solvents and the temperature were chosen as they might be of value for tritium labelling ICG (Radiochemicals 1980, Amersham, England).

3.2 MATERIALS AND METHODS

3.2.1 Reagents and instruments

All reagents were of analytical grade (AR) from Merck or British Drug Houses unless otherwise stated. Human albumin was purchased from Sigma London (Sigma A 2386); ICG from Hynson, Westcott & Dunning Inc., Baltimore, Maryland 21201, U.S.A.; thin-layer chromatographic plates used were DC-Alufolien Kieselgel 60 F254, thickness 0.2 mm, Merck Art 5554 (E. Merck, D-6100 Darmstadt, F.R.G.). Spectrophotometric measurements were recorded on a Pye Unicam SP 800 Double Beam Recording Spectrophotometer and a Pye Unicam SP 500 Single Beam Spectrophotometer (Pye Unicam Ltd., Cambridge, England). Differential temperature studies were conducted in an autoclave from Tuttnauer Co. Ltd., Jerusalem, Israel.

3.2.2 Determination of the relationship between absorbance and concentration of ICG in various solvents

ICG (25 mg) was dissolved in manufacturer's solvent (triple distilled water, 25 ml) and diluted to the concentration of 10 µg/ml (1.29×10^{-2} mmol/l), 7.5 µg/ml (9.68×10^{-3} mmol/l), 5.0 µg/ml (6.45×10^{-3} mmol/l), 2.5 µg/ml (3.23×10^{-3} mmol/l), and 1.25 µg/ml (1.61×10^{-3} mmol/l) in the following solvents: methanol, 1.2-propanediol, *N*,*N*-dimethylformamide (DMF), single distilled water, human bile (bilirubin concentration 3.6 mg/ml, total bile salt concentration 12.0 mmol/l), boric acid-sodium borate buffer (pH 9.0), and human duodenal fluid (fasting, bilirubin free). Within 1 h of preparation the absorbance of ICG (λmax) in these solutions was measured in a double beam recording spectrophotometer against appropriate blanks, and the values were plotted on a graph as a function of concentration, as shown in Figs. 13A and B.

3.2.3 The rate of decay of ICG in various solvents: calculations of half-lives ($t_{\frac{1}{2}}$) and rate constants (k)

ICG (50 mg) was dissolved in the manufacturer's solvent (10 ml) and this solution was diluted to a concentration of 6.45×10^{-3} mmol/l (5 µg/ml) in the following solvents: methanol, 1.2-propanediol, DMF, single distilled water, human bile (bilirubin concentration 3.6 mg/ml, total bile salt concentration 12.0 mmol/l), 0.5% human albumin; and human duodenal fluid (fasting, bilirubin free). The absorption spectrum of each solution was scanned, from wavelength 600 nm to 850 nm, at <1 h, 12 h, 48 h, 7 days, 1 month, 3 months, 6 months and 1 yr after preparation, against appropriate blanks of solvent in a double beam recording spectrophotometer. Matched glass cuvettes of pathlength 1 cm were used. Solutions were kept in transparent sealed glass tubes in electric room light (300 lux) at room temperature during the period of storage.

Absorbance was plotted as a function of time, and half-lives ($t_{\frac{1}{2}}$) and rate constants (k) (24) were calculated, the latter according to the formula:

$$k = \frac{\log_{10} \frac{A_1}{A_2} \times 2.303}{T} ;$$

where A_1 = absorbance of ICG in a fresh solution at λmax;
A_2 = absorbance of ICG at λmax at the end of the observation period;
T = time of observation (in hours).

Absorbance curves were also plotted as a function of wavelength for each solvent at <1 h, 24 h, 1 wk, 1 month, 3 months and 6 months after preparation.

3.2.4 TLC of ICG stored for 6 months in various solvents

ICG at concentrations of 6.45×10^{-1} mmol/l (500 µg/ml) and 6.45×10^{-2} mmol/l (50 µg/ml) in the biological fluids and organic solvents described above was run on TLC plates at <1 h, 24 h, 48 h, 1 wk, 1 month, 3 months and 6 months after preparation, in the solvent system: pyridine:water:propan-1-ol:ammonia (5:4:20:0.4). 4 µl (500 µg/ml) and 40 µl (50 µg/ml) of solution was applied to the plates, respectively. Each run lasted 6 h and was carried out in total darkness at room temperature (20°C) after saturation of the development chamber for at least 20 min. Solutions were kept in transparent sealed glass tubes in electric room light (300 lux) at room temperature (20°C) during the whole period of storage.

R_f values were calculated for each solvent and expressed as mean values ± SD of the R_f values obtained during the observation time (6 months).

3.2.5 Effects of temperature on the rate of decay of ICG in various solvents: calculation of activation energy (Ea)

ICG (25 mg) was dissolved in manufacturer's solvent (25 ml) and immediately diluted to the final concentration of 6.45×10^{-3} mmol/l (5 µg/ml) in single distilled water, DMF, methanol and 1.2-propanediol. Aliquots were heated in sealed tubes to 120°C for 2 h in an autoclave. Samples were weighed before and after heating (weight change <0.01%). Absorbance spectra were obtained before and after heating in each organic solvent, and activation energy for decay in each solvent calculated (24), according to the formula:

$$Ea = \frac{R \times t_1 \times t_2 \times 2.303 \times \log_{10} \frac{k_2}{k_1}}{(t_2 - t_1)}$$

where R = gas constant (=1.9872 cal $deg^{-1}mol^{-1}$); t_1 = temperature before the heating in °K; t_2 = temperature during the heating in °K; k_1 = rate constant at 20°C; k_2 = rate constant at 120°C.

Absorbance was also plotted as a function of wavelength before and after heating for each organic solvent.

3.2.6 pH tolerance of ICG

ICG (25 mg) was dissolved in manufacturer's solvent (25 ml). 100 µl of this solution were diluted to 10 ml in appropriate buffers *(Appendix IV)* (81) giving a pH range from minus 0.5 to plus 13.0 in 17 consecutive samples of ICG at a final concentration of 1.25×10^{-2} mmol/l (10 µg/ml). The absorbance of each solution at λmax (775 nm) in these buffers was read at 1 h, 18 h and 48 h after preparation at room temperature (20°C), in a double beam spectrophotometer against appropriate buffer blanks and absorbance plotted as a function of pH.

3.2.7 Rate of decay of ICG at various pH's

ICG (25 mg) was dissolved in manufacturer's solvent (25 ml) and diluted in appropriate buffers (81) *(Appendix IV)*, at pH 1.6, 3.2, 5.0, 6.4, 9.0 and 12.6, respectively, and to a final concentration of ICG of 1.29×10^{-2} mmol/l (10 µg/ml). The absorbance of each solution was scanned over the wavelength range 600 nm to 1000 nm at <1 h, 8 h, 24 h and 4 days after preparation.

3.2.8 The dissociation constant of ICG (pKa)

The dissociation constant of ICG (pKa) was determined spectrophotometrically by the method of Flexser, Hammett and Dingwall (120, 184) using the equation:

$$pKa = pHe - \log \frac{de - dBH^+}{db - de}$$

The absorbance of ICG was measured at pH 7.4 at 775 nm, the position of maximum absorbance of the free base (db), and the absorbance of the conjugate acid (dBH^+) was measured at 775 nm at pH 1.0. The absorbance of the equilibrium mixture (de) was measured at 775 nm at pHe 3.2. This method of determining pKa is based on the assumption that at this concentration (1.29×10^{-2} mmol/l), ICG obeys Beer-Lambert's law (375) and this has been shown to be true by Gathje *et al.* (134).

3.2.9 Within-assay-precision and accuracy of measuring ICG in duodenal fluid and bile with and without methanol

ICG (25 mg) was dissolved in manufacturer's solvent (25 ml) and immediately diluted in human bile or bile containing 25% methanol. The final concentration of ICG was 10 µg/ml (1.29×10^{-2} mmol/l, bilirubin concentration of 1.2 mg/ml, total bile salt concentration 3.8 mmol/l and methanol 25%), 5 µg/ml (bilirubin concentration 0.6 mg/ml, total bile salt concentration 1.9 mmol/l and methanol 25%), and 2.5 µg/ml (bilirubin concentration 0.3 mg/ml, total bile salt concentration 0.8 mmol/l and methanol 25%), respectively. Samples of each concentration were divided into 20 aliquots for analysis. These concentrations of ICG represented the range used in the standard curve. Appropriate blanks were prepared for each sample by decolourisation of ICG by mixing 12 N HCl, distilled water and the sample together in the proportions: 1:1:2, which gave a final pH of <0.1. After storage in darkness for 30 min and millipore filtration of the samples and the blanks (Millipore®, pore size 0.45 µm), the absorbance of the samples was read against the blanks at λmax (805 nm for bile without methanol; 780 nm for bile with methanol). The same procedure was carried out for ICG at these concentrations in 145 mmol/l NaCl (0.9%) without methanol (λmax 775 nm) or with methanol (λmax 780 nm).

3.3 RESULTS

3.3.1 Relationship between absorbance and concentration of ICG in various solvents

In the organic solvents used, ICG obeyed Beer-Lambert's law within the range of concentration used (1.25 µg/ml to 10 µg/ml) (Fig. 13A). In biological fluids (Fig. 13B) results were, however, more variable. In bile and 0.5% human albumin a linear relationship was found up to a concentration of 7.5 µg/ml. In duodenal fluid a deviation from linearity occurred at concentration above 5.0 µg/ml. In boric acid-sodium borate buffer (pH 9.0) linearity was found over the whole concentration range.

3.3.2 Rate of decay of ICG in various solvents: calculations of half-lives ($t_{\frac{1}{2}}$) and rate constants (k)

Fig. 14 shows the rate of decay in various solvents up to 6 months after preparation. In human bile, duodenal fluid and distilled water, the decay curves were exponential, indicating that the decay was of a first order reaction. $t_{\frac{1}{2}}$ in distilled water, duodenal fluid and human bile were 1.4 days, 3.6 days and >1 year, respectively. In albumin an increase in absorbance was observed during the first 7 days of storage, after which ICG decayed in a linear fashion, similar to the decay observed in the organic solvents (zero order reaction). In the albumin solution $t_{\frac{1}{2}}$ was calculated as 81 days. In DMF, 1.2-propanediol and methanol, $t_{\frac{1}{2}}$ was 49 days, 90 days and >1 year, respectively.

The rate constants (k) (Table 1) in biological solvents correlated with $t_{\frac{1}{2}}$, being highest in distilled water and duodenal fluid and lowest in the organic solvents methanol and 1.2-propanediol.

The extinction coefficient (Table 1) was highest in methanol (2.34×10^5), lower in 1.2-propanediol (2.30×10^5), DMF (2.17×10^5), human bile (1.85×10^5), 0.5% human albumin (1.72×10^5) and distilled water (1.55×10^5), and lowest in the duodenal fluid (1.18×10^5).

The tinctorial strength of ICG in various solvents, estimated as the integrated area under the absorbance curve over the wavelengths 600 nm to 850 nm, was not constant, *i.e.* it was not offset by the shape of the curve in different solvents. The tinctorial strength had some correlation with ε, *i.e.* was highest where ε was highest. The fact that the tinctorial strength varied, suggests that ICG reacted with the solvent, and possibly by forming aggregates (147).

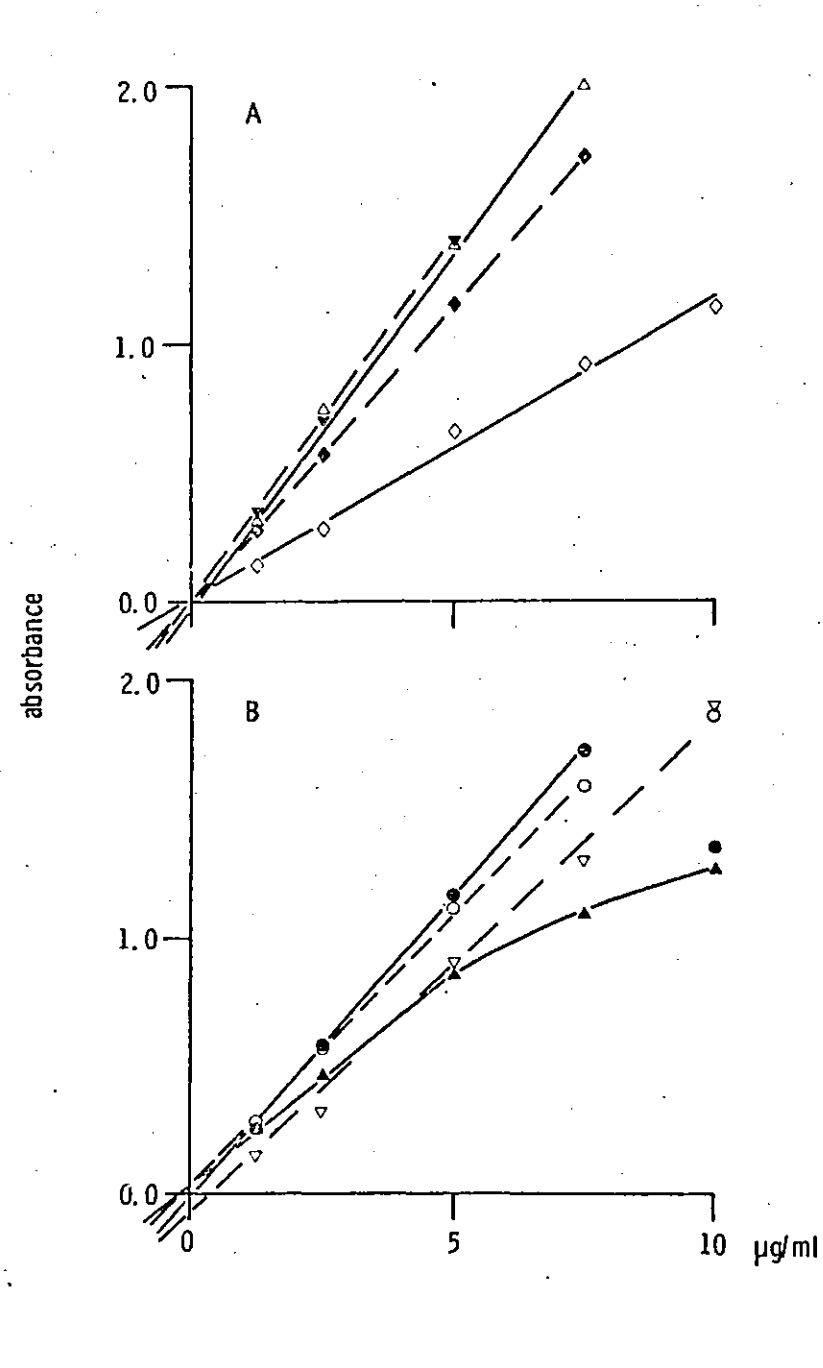


Fig. 13. The linear relationship between absorbance and concentration of ICG in various solvents (fresh preparations). A: ▼, methanol; △, 1.2-propanediol; ◆, dimethylformamide; ◇, distilled water. B: ●, human bile; ○, 0.5% human albumin; ▽, 0.025 M $Na_2B_4O_7$/0.1 M H_3BO_3 buffer (pH 9.0); ▲, human duodenal fluid free of bile (fasting).

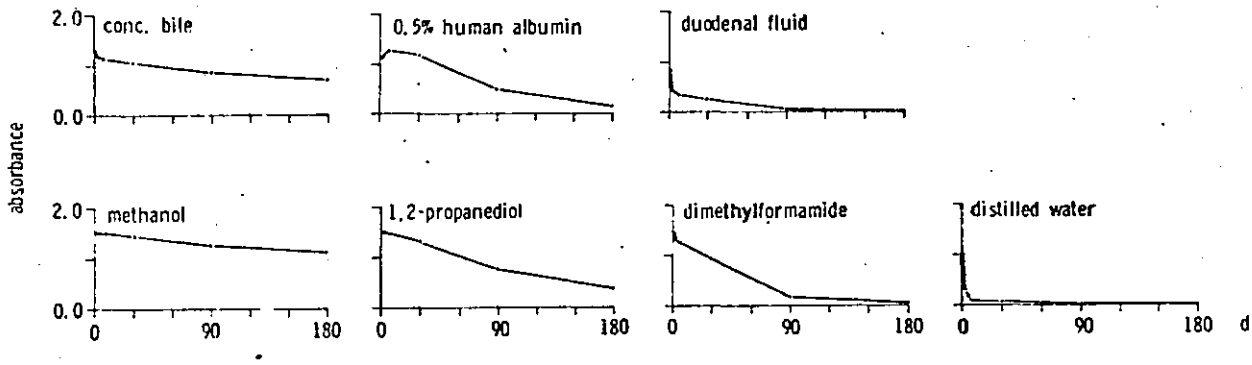


Fig. 14. Decay of ICG (6.45×10^{-3} mmol/l) plotted as a function of time during 6 months of storage in: human bile, 0.5% human albumin, human duodenal fluid free of bile (fasting), methanol, 1.2-propanediol, dimethylformamide, distilled water.

In fresh solutions (<1 h) λmax was 775 nm in both distilled water and duodenal fluid. There was a shift of λmax to 795 nm in 0.5% human albumin and to 805 nm in human bile. In the organic solvents investigated λmax was 780 nm to 785 nm.

Fig. 15 shows the absorbance spectra of ICG (6.45×10^{-3} mmol/l) in various biological fluids and organic solvents 1 h to 6 months after preparation. During this period there was a progressive fall in absorbance at λmax in all solutions as already has been expressed by $t_{\frac{1}{2}}$ and k. The absorbance curves became broad and flattened on storage, and the shoulder observed at this concentration of ICG at 700 nm to 750 nm disappeared.

During the 6 months of study λmax remained unchanged in methanol, DMF and 1.2-propanediol. There was a slight decrease in λmax during storage in bile (initial λmax 805 *versus* 800 nm during 1 to 6 months of storage), and in distilled water (initial λmax 775 nm *versus* 770 nm at the end of the observation period). In duodenal fluid and 0.5% human albumin there was, however, a shift to a higher λmax on storage (775 nm *versus* 785 nm for duodenal fluid, and 795 nm *versus* 800 nm for 0.5% human albumin).

3.3.3 Effects of temperature on the rate of decay of ICG in various solvents: calculation of activation energy (Ea)

As shown in Table 1, activation energy (Ea) was calculated for ICG in distilled water, DMF, methanol and 1.2-propanediol. This was based on the rate constants of ICG decay in these solutions at 20°C and during heating at 120°C for 2 h. Ea was low and close to zero on all occasions.

In Fig. 16 the absorbance spectra of ICG before and after heating (120°C) in these solvents are shown. The decomposition was minimal in methanol and 1.2-propanediol, in contrast to almost a complete decay in DMF and distilled water. No shift in λmax was observed during heating but the absorbance curves became broad and flattened as was observed in these solvents during long-term storage (Fig. 15).

3.3.4 TLC of ICG stored for 6 months in various solvents

Table 2 shows the R_f values of ICG at concentration 50 µg/ml in various solutions run on TLC during 6 months of storage. In bile, duodenal fluid and DMF only one component was observed during the whole observation period. R_f values were smaller in bile and duodenal

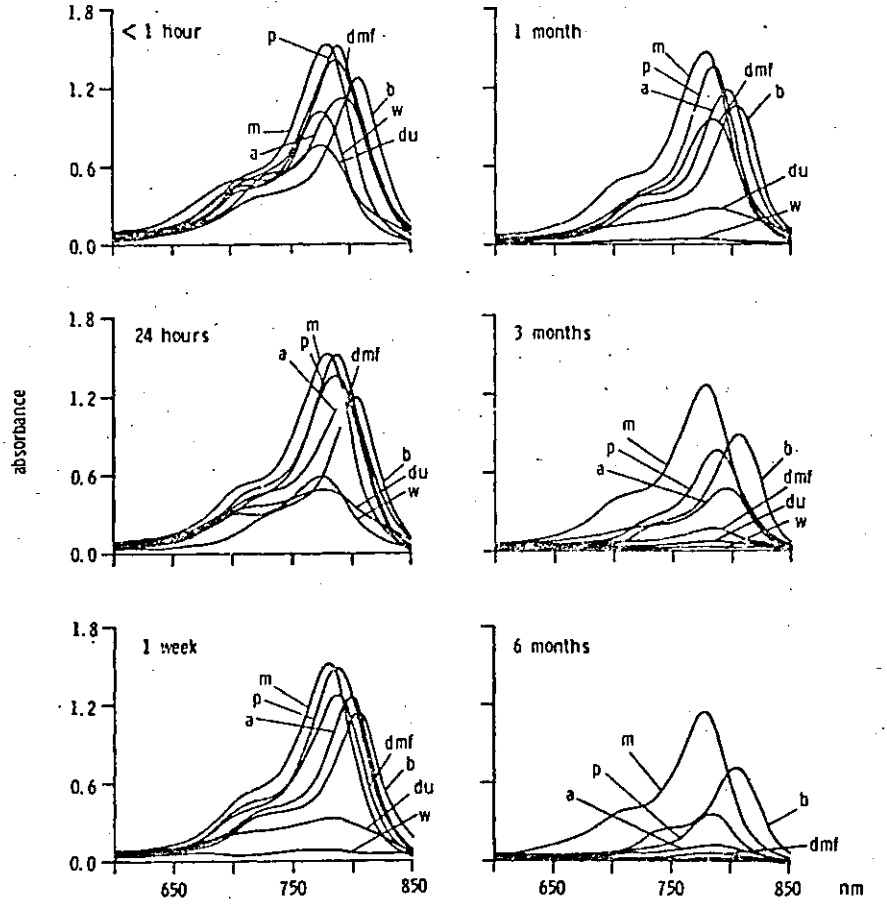


Fig. 15. Absorbance of ICG (6.45×10^{-3} mmol/l) during 6 months of storage in various solvents drawn as a function of wavelength (600-850 nm): a, 0.5% human albumin; m, methanol; p, 1.2-propanediol; dmf, dimethylformamide; b, human bile; w, distilled water; du, human duodenal fluid free of bile (fasting).

Table 1. Physicochemical characteristics of ICG in various solvents at constant pH.

	λmax nm	6.45×10^{-6} $\varepsilon_{20°C}$ 1 cm	Tinctorial strength§	k#	$t_{\frac{1}{2}}$ (days)	Ea Kjoules mol^{-1}
Human duodenal fluid	775	1.81×10^5	1.68	1.3×10^{-2}	3.6	–†
Single distilled water	775	1.55×10^5	1.00	2.3×10^{-2}	1.4	26.9
0.5% Human albumin	795	1.72×10^5	1.86	1.3×10^{-4}*	81.0	–†
DMF	785	2.17×10^5	2.12	1.8×10^{-3}	49.0	43.5
Methanol	780	2.34×10^5	2.43	5.6×10^{-4}	>1 year	15.6 θ
1.2-Propanediol	785	2.30×10^5	2.30	2.1×10^{-4}*	90.0	17.3 θ
Human bile	805	1.85×10^5	1.98	3.5×10^{-3}	>1 year	–†

*Calculations based on the period 7–30 days.

†Not carried out in biological fluids.

§Calculated as the area under the absorption curves relative to that in distilled water as a solvent.

#The unit of k for zero order reaction is mmol $dm^{-1}h^{-1}$ and the unit for k for first order reaction is h^{-1}. Conc. of ICG $6.45 \times 10^{-}$ $mmol^{-1}$.

θThe reaction practically did not occur.

Table 2. R_f of ICG (6.45×10^{-2} mmol/l) during 6 months of storage in various solvents.

	Human bile	Duodenal fluid	Distilled water		0.5% Human albumin		Methanol		DMF	1.2-Propanediol	
Fresh	0.24	0.30	0.39	0.48	0.40	0.49	0.43	0.51	0.60	0.49	0.53
24 hours	0.33	0.38	0.56	0.68	0.58	0.71	0.58	0.68	0.67	0.62	0.65
48 hours	0.29	0.31	0.40	0.57	0.51	-	0.52	-	0.67	0.58	0.61
1 week	0.29	0.33	-	0.58	0.51	0.63	0.55	-	0.66	0.58	0.60
1 month	0.34	0.39	-	0.64	0.46	0.69	0.54	0.68	0.66	0.60	0.64
3 months	0.34	0.35	-	-	0.40	-	0.41	-	0.66	0.56	0.58
6 months	0.34	-	-	-	-	-	0.53	-	-	0.63	-
$\bar{x}$	0.31	0.34	0.45	0.59	0.49	0.63	0.51	0.60	0.65	0.58	0.60
SD	0.04	0.04	0.10	0.08	0.08	0.10	0.05	0.09	0.03	0.05	0.04

fluid than in any other solvent studied. In distilled water, two components were observed from the beginning (<1 h from preparation). The fast-running component was initially small but after 48 h both components were of similar size and after 1 wk only the fast-running spot could be detected. In 0.5% human albumin, methanol and 1.2-propanediol, two components were observed but the spot of fast-running remained small all the time but was clearly separated from the main component. At the higher concentration of ICG studied (500 µg/ml), the R_f values proved to be similar. However, at this concentration two spots were observed in DMF.

3.3.5 pH tolerance of ICG

Fig. 17 shows the absorbance of ICG (1.29×10^{-2} mmol/l) plotted as a function of pH (range minus 0.5 to plus 13.0) <1 h, 18 h and 48 h after preparation. In the fresh preparation (<1 h), there was no difference in absorbance in pH range 5 to 11. At pH 11 to 12 and pH 5 to 6 there was a sudden decrease in absorbance and at pH <0.1 the absorbance was negligible (<5% of maximal). The colour of the solution changed immediately after preparation from an emerald green to a yellow-green at pH 1 to 4 and pH >11 and to a brown at pH <1.0. With storage for a few hours the solution at pH 1 to 4 became brown-coloured and solutions at pH >11 had become almost colourless. It is of interest that during 48 h of storage, ICG at pH range 8 to 10 remained relatively stable with a loss of only about 20% of its initial absorbance.

3.3.6 Rate of decay of ICG at various pH's: calculation of half-lives ($t_{\frac{1}{2}}$) and rate constants (k) at various pH's

Table 3 shows that $t_{\frac{1}{2}}$, k and ε are pH dependent. The values of $t_{\frac{1}{2}}$ varied from 1 to 2 h (pH 1.6 to 3.2) to more than 1 day (pH 9.0) and the rate constants varied from 1.23×10^{-1} (pH 1.6) to 1.31×10^{-2} (pH 9.0). The decay curves were in all cases exponential. The ε varied from 5.89×10^{4} to 1.32×10^{5}, being lowest at pH 1.6 and 12.6, and highest at pH 6.4, 9.0 and 5.0. The λmax was the same (775 nm) at all pH values studied. In Fig. 18 the decay curves of ICG at each pH are demonstrated at wavelength 660 nm and 850 nm. (The absorbance was studied up to wavelength 1000 nm in all cases but one was unable to detect any increase in absorbance at wavelength >850 nm during the time of study (4 days) at the concentration of ICG used (1.29×10^{-2} mmol/l.)

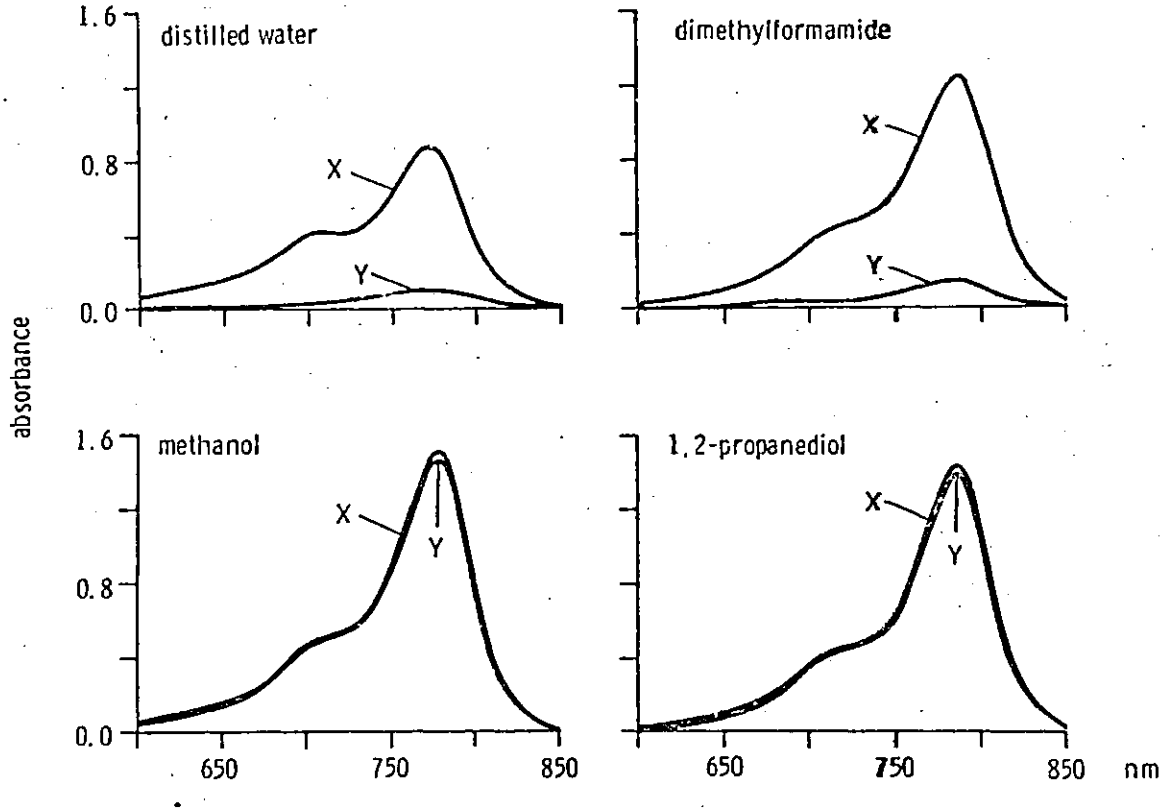


Fig. 16. Absorbance of ICG (6.45 x 10^{-3} mmol/l) before (X) and after (Y) heating (120°C for 2 h) in distilled water, dimethylformamide, methanol and 1.2-propanediol, drawn as a function of wavelength (600–850 nm).

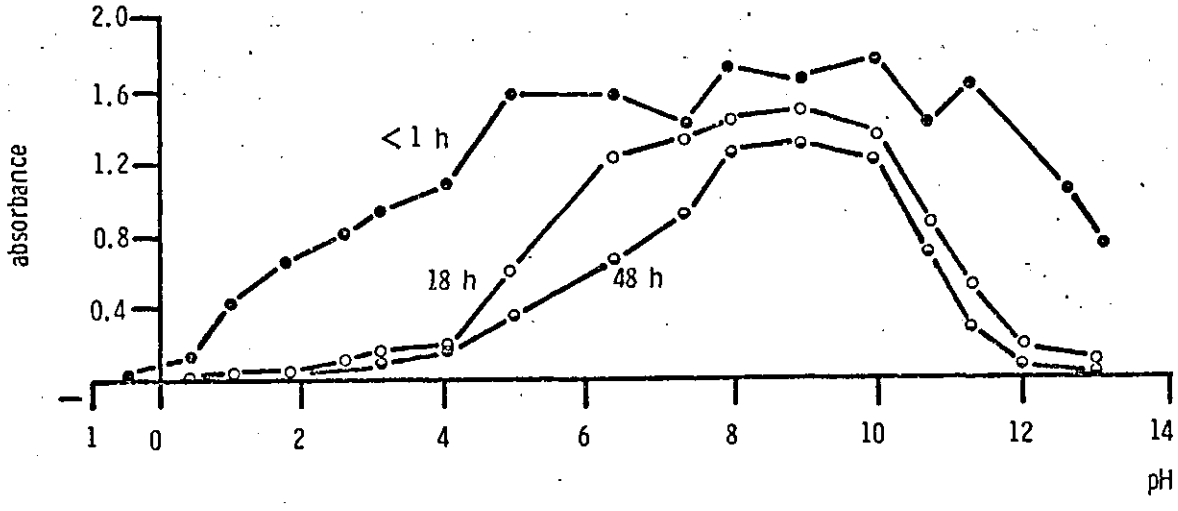


Fig. 17. Absorbance of ICG (1.29×10^{-2} mmol/l) at wavelength 775 nm plotted as a function of pH (17 different pH values, range minus 0.5 to plus 13.0), <1 h, 18 h and 48 h after preparation.

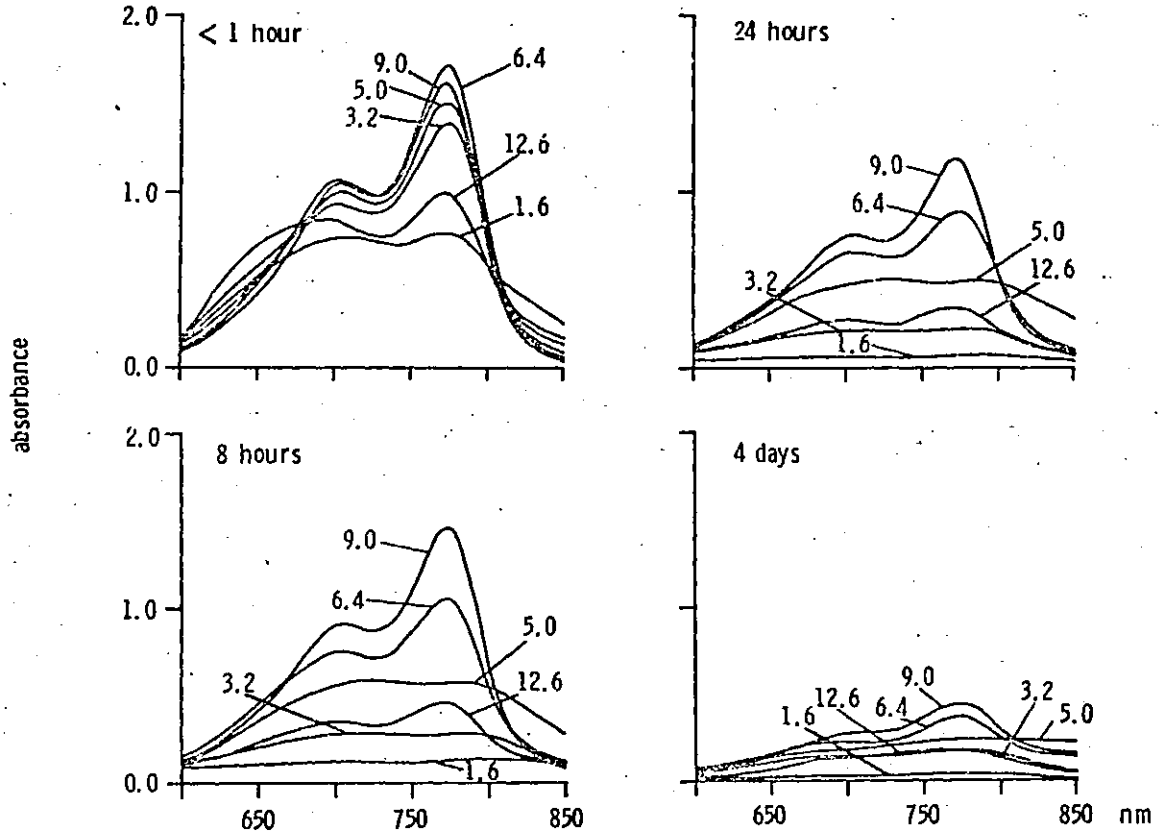


Fig. 18. Absorbance of ICG (1.29×10^{-2} mmol/l) at pH 12.6, 9.0, 6.4, 5.0, 3.2 and 1.6 drawn as a function of wavelength (600-850 nm) <1 h, 8 h, 24 h and 4 days after preparation. For solvents (buffers), see *Section 3.2.6.*

Table 3. Physicochemical characteristics of ICG in buffers at various pH.

pH	λmax* nm	ε 6.45×10^{-6} 20°C 1 cm	k†●	$t_{\frac{1}{2}}$ (hours)
12.6	775	7.60×10^{4}	8.24×10^{-2}	2.0
9.0	775	1.26×10^{5}	1.31×10^{-2}	>24.0
6.4	775	1.32×10^{5}	6.59×10^{-2}	12.8
5.0	775	1.16×10^{5}	4.66×10^{-2}	3.2
3.2	775	1.07×10^{5}	9.84×10^{-2}	1.2
1.6	775	5.89×10^{4}	1.23×10^{-1}	2.0

*In fresh solutions (<15 min after preparation).

†Based on the decay during the first 24 h after preparation.

●The unit for k is h^{-1}.

Table 4. Within-assay-precision (n = 20) and accuracy of measuring ICG in normal saline and human bile with or without 25% methanol.

	0.9% NaCl			0.9% NaCl + 25% methanol			Human bile			Human bile + 25% methanol		
ICG g/ml	2.5	5.0	10.0	2.5	5.0	10.0	2.5	5.0	10.0	2.5	5.0	10.0
$\bar{x}$	2.8	5.7	10.7	3.1	6.0	9.9	2.0	5.1	8.3	2.9	5.8	11.8
SEM	0.13	0.16	0.13	0.05	0.17	0.18	0.06	0.07	0.03	0.02	0.03	0.13
CV	20.0	12.3	5.5	7.0	12.7	8.2	14.5	6.0	1.8	2.5	2.7	5.0
Range	1.7-3.5	3.9-6.4	9.4-11.6	2.8-3.7	5.4-7.5	8.5-11.8	1.6-2.8	4.4-5.8	8.2-8.6	2.8-3.2	5.3-6.0	10.8-12.

The pKa of ICG was calculated as:

$$pKa = 3.2 - \log \frac{0.92 - 0.44}{1.49 \times 0.92} = 3.27$$

de, dBH and db were obtained from data presented in Fig. 17.

3.3.7 Within-assay-precision and accuracy of measuring ICG in 0.9% saline and bile with and without methanol

Table 4 shows the within-assay-precision and accuracy of measuring ICG at concentration 2.5, 5.0 and 10.0 µg/ml, respectively (n = 20) in 0.9% NaCl, 0.5% NaCl with 25% methanol, human bile, and human bile with 25% methanol. In 0.9% NaCl with or without methanol there was a recovery of 114% to 124% at the lower concentrations (2.5 µg/ml and 5.0 µg/ml) and CV (coefficient of variation) was 7% to 20%. In human bile only there was a tendency to some underestimation (average recovery 80%, 102%, 83%), but CV was low except at 2.5 µg/ml (14.5%). In human bile with 25% methanol, there was an average recovery of 116% to 118%, but CV was low (2.5% to 5.0%)

3.4 DISCUSSION

Although the biological properties of ICG have ensured its widespread use in diagnostic medicine and clinical research, its physicochemical properties have not been well defined and its accurate analysis in biological fluids is not without difficulties. The relationship between absorbance and concentration of ICG is linear in some but not all solvents. ICG is unstable in aqueous solutions and shows variable stability in other solvents. Instability increases with temperature in some but not all solvents and pH markedly affects the rate of degradation. Accuracy and precision of analysis similarly vary from one solvent to another, even when determinations are made at λmax, appropriate temperature and pH, and on freshly prepared solutions. Nevertheless, analysis of ICG in different solutions is possible on both freshly prepared and stored samples providing the physicochemical properties are understood and appropriate precautions are taken. The technique of analysing ICG in plasma has been validated and the solvents used in this study were selected because of interest in measuring ICG in intestinal fluid containing varying proportions of bile pigments, bile salts, electrolytes and protein, and the need to store samples and standard solutions in the laboratory prior to analysis.

As has been discussed recently by Landsman *et al.* (216) a linear relationship between absorbance and concentration of ICG in human serum and plasma is generally accepted (13, 216, 328), although it has been disputed by some workers (350). In the present study a linear relationship has been found over the test concentration range of ICG (1.25 to 10 µg/ml) in organic solvents. In biological fluids, however, linearity was found only up to 7.5 µg/ml for 0.5% human albumin and bile and up to 5 µg/ml for duodenal fluid.

The instability of ICG in dilute aqueous solutions was confirmed in this study. The $t_{\frac{1}{2}}$ for decay of ICG (6.5×10^{-3} mmol/l) in distilled water was 1.4 days.

In duodenal fluid the $t_{\frac{1}{2}}$ was estimated as 3.6 days, which was greater than expected since electrolytes have been found to promote ICG degradation (134, 216). Protein content of the duodenal fluid may have opposed the ionic effect (134). ICG was relatively stable in DMF, 0.5% human albumin and 1.2-propanediol ($t_{\frac{1}{2}}$ 49, 81 and 90 days respectively), but was most stable in methanol and human bile ($t_{\frac{1}{2}}$ >1 yr). The decay of ICG was exponential in biological fluids apart from 0.5% human albumin where an increase in absorbance was observed over the first 7 days. This increase in absorbance of ICG in albumin on storage has been observed before (134). It has also been observed in distilled water at high concentrations of ICG (134, 216, 350). High concentrations of ICG, and protein binding, are both suggested to promote an aggregate formation of ICG in aqueous solution and this has been thought to increase the absorbance of ICG and cause a shift in λmax to a higher wavelength (22, 29, 64, 134).

Although there was a decrease in absorbance of ICG in bile to some extent at the beginning of the storage, this quickly slowed down and ICG remained relatively stable in bile to the end of the 1 yr observation period ($t_{\frac{1}{2}}$ >1 yr). This cannot be explained by stabilisation by protein in bile as there was no change in stability of ICG in protein precipitated bile (protein was precipitated by pure methanol, the methanol evaporated and the residue reconstituted in distilled water (unpublished data)). The decay pattern of ICG in albumin was also different from that of bile (Fig. 14). It is suggested that the stability of ICG in bile is due to other constituents of bile such as bile pigments, lipids or bile salts.

The extinction coefficient (ε) of ICG was lower in duodenal fluid than in water, which is in agreement with previous observations that electrolytes decrease the extinction coefficient of ICG in a concen-

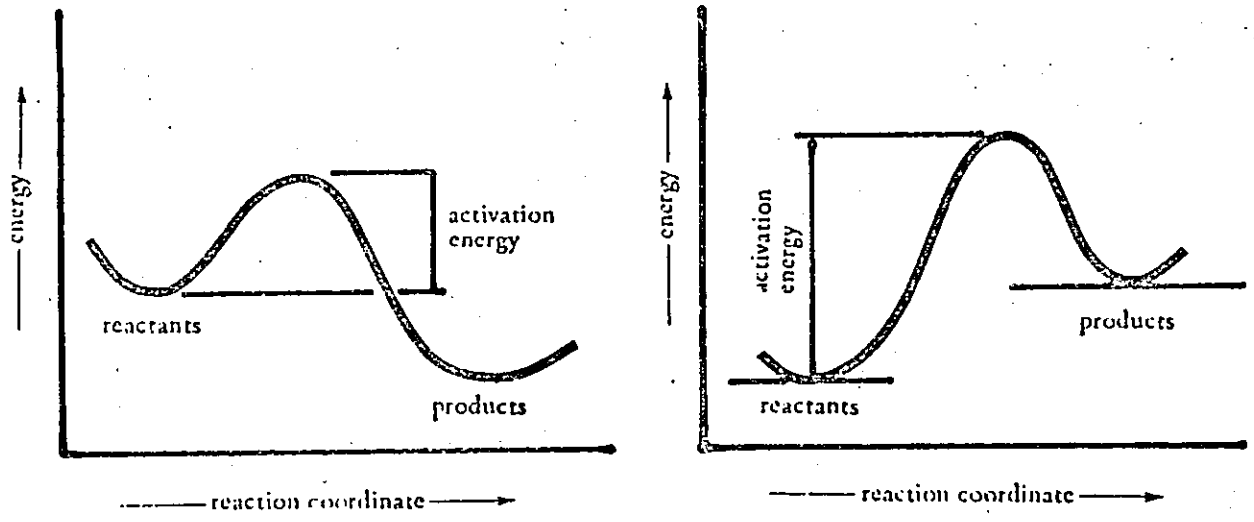


Fig. 19. A schematic explanation of activation energy.

tration-dependent fashion (129). Extinction coefficients in bile and 0.5% albumin were higher than in aqueous solutions but the highest values were found in organic solvents.

The abrupt deviation of ICG from the Beer-Lambert's law in strong bile may be due to the fact that the bile salt concentration was certainly above the critical micelles point and this may have lead to aggregate formation between bile salts and ICG. This is supported by the shift to a λmax of a higher wavelength in this solution than in the solution containing ICG and bile salts of lower concentration. However, according to Baker (20), an increase in bile salt concentration causes an increase in extinction coefficient of ICG, which is the opposite to what was observed here.

On TLC, ICG behaved differently in different solutions (Table 2). In distilled water two components were observed from the very beginning at both concentrations studied (500 µg/ml and 50 µg/ml). These two components were well separated. After 3 months the slower component could not be seen at either concentration (Table 2). This is comparable with the results of Barbier and de Weerdt (22), who used a similar technique and found R_f for the slower component to be 0.47 in freshly prepared water. They found that the slower component had totally disappeared 4 months after preparation. They used, however, a more concentrated solution of ICG (5 mg/ml) than used here. In bile and duodenal fluid there was only found one component and in these media R_f was smaller than in distilled water. In albumin one observed two components from the beginning, but the faster fraction (R_f 0.63) always remained small.

The activation energy (Ea) (Fig. 19) was studied in the organic solvents by heating ICG in the solutions under investigation at 120°C for 2 h. Ea was low for all solutions used in the differential temperature studies, indicating that there is a little energy barrier to overcome for the decomposition reaction to occur, and that in fact this is lower than the thermal energy available at room temperature (40 to 60 Kjoule).*

The effects of pH on the absorbance of ICG have previously been studied in part by Fox and Wood (129) and Jones Owen (187), who were unable to see any changes in absorbance of ICG in water or plasma at pH 6.0 to 8.0. In the present study it has been found that above pH 11 and below pH 5 there is a sudden decrease in absorbance and the colour of ICG changes to light brown. The colour change in acidic and basic solutions can be restored at least partly to green

*This applies only to ICG in water and DMF, since practically no reaction occurred in methanol and 1.2-propanediol (Table 1), i.e. the compound was not "activated" in these solvents at that temperature.

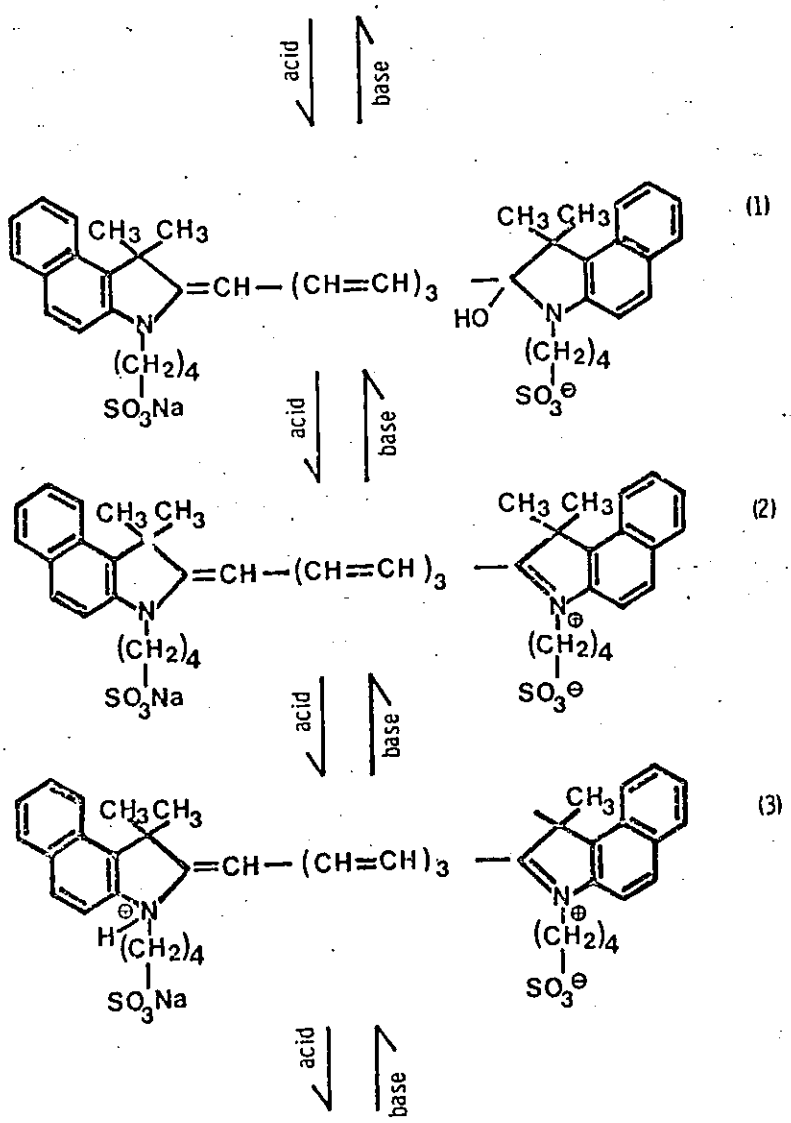


Fig. 20. Suggested reversible reactions of ICG in acid and alkaline media.

colour by changing the pH to neutral again. At pH <0.1 the absorbance of ICG has become negligible, and this has been used to correct for interference of bile pigments when ICG is measured in bile, by using decolourised samples in strong acids as blanks (29).

This effect of pH may be explained by the fact that many ionic dyes react with water at high pH values to form initially a 'carbinol' type base, which is colourless (Fig. 20 (1)). This compound may either degrade further to colourless compounds, or it can regenerate the original cationic dye (Fig. 20 (2)) on acidification. Similarly, the more hypsochromic colour in acid may result from protonation which gives the dicationic species (Fig. 20 (3)).

The λmax was found to be at lowest wavelength in water and duodenal fluid free of bile (775 nm), higher in organic solvents (780 to 785 nm) and highest in 0.5% human albumin (795 nm), and concentrated bile (805 nm). Previous workers have also found variability in λmax of ICG in different solvents (13, 20, 64, 129, 187, 216, 350).

There is a disagreement about λmax for ICG in the same solvent. For ICG in distilled water λmax has been reported to be 770 nm (134), 775 nm (22, 129, 258), 780 nm (13, 20, 187, 216, 327, 350), 785 nm (22, 273), and 790 nm (304) and for bile 800 nm (105) and 805 nm (20, 174). Various reports have also been published on λmax of ICG in saline and plasma (13, 22, 129, 134, 174, 184, 216, 304, 328, 368).

The present study has shown that λmax for ICG is not only dependent on the nature of the solvent in question, but varies also with the concentration of ICG in the solvent, pH of the solvent, and the time ICG has been stored in the solvent. The shape of the absorbance curve also changes with change in concentration of ICG and new absorbance peaks may develop on storage (134, 216, 373).

3.5 SUMMARY

Various physicochemical studies were carried out on the tricarbocyanine dye indocyanine green in biological fluids and organic solvents. Indocyanine green obeys the Beer-Lambert's law within the concentration range of 1.25 µg/ml to 10.0 µg/ml in distilled water, methanol, dimethylformamide, 1.2-propanediol and buffers (pH 9.0), but only to 7.5 µg/ml in human bile and 0.5% human albumin, and only to 5.0 µg/ml in human duodenal fluid. The extinction coefficient of indocyanine green is highest in methanol (2.34×10^5) and lowest in

duodenal fluid (1.18×10^5). The λmax varies from 775 nm (distilled water and duodenal fluid) to 805 nm (human bile). Indocyanine green is stable in human bile and methanol ($t_{\frac{1}{2}}$ >1 yr), less stable in 1.2-propanediol, 0.5% human albumin and dimethylformamide ($t_{\frac{1}{2}}$ 90, 81 and 49 days respectively), and least stable in duodenal fluid and distilled water at room temperature ($t_{\frac{1}{2}}$ 3.6 days and 1.4 days respectively). Indocyanine green is thermolabile in some solvents (decomposes at 120°C in distilled water or dimethylformamide) but not in others (remains stable in methanol and 1.2-propanediol). It is also labile at pH <5 and >11, but more stable at pH 8 to 11, and has a pKa of 3.27. The decomposition product of indocyanine green (as yet unidentified) is detected on thin-layer chromatography as a fast-running component (R_f 0.59 to 0.65 *versus* 0.45 to 0.58 for the non-decomposed product). Indocyanine green can be measured with precision (CV <5%) and accuracy of 106-127% in bile containing methanol. The activation energy of ICG in organic solvents was low (<50 Kjoules mol^{-1}).

CHAPTER IV

LUMINAL EFFECTS OF SODIUM TAUROCHOLATE AND ESSENTIAL AMINO ACIDS ON BILIARY AND PANCREATIC SECRETION IN MAN

4.1 INTRODUCTION

Factors which inhibit biliary and pancreatic secretion, and which may contribute to the overall regulation of the delivery of bile and pancreatic juice into the duodenum, are not well understood in man. One such factor may be the presence of bile salts in the duodenum, since previous workers have shown that perfusion of the human duodenum with the pure bile salt sodium taurocholate may inhibit biliary bilirubin secretion (239). Duodenal bile salts may also affect pancreatic secretion but various effects have been reported in human and animal studies by different workers (125, 161, 239, 243, 251, 284, 380, 410).

In the present investigations several aspects of the effects of the bile salt sodium taurocholate (STC) on biliary and pancreatic secretion were studied in man. Firstly, the effects of STC in the duodenum on the secretion were determined under basal conditions. Secondly, the effects of the bile salt on exogenously (caerulein/secretin) and on endogenously (luminal EAA perfusion) stimulated biliary and pancreatic secretion were studied. This approach was adopted in an attempt to distinguish between the effects of the bile salt in the duodenum and the effect at the level of the target organs (the biliary system and the pancreas).

In order to study whether there was a relationship between the concentration of the bile salt in the duodenum and the effect on the biliary secretion, dose-response studies were carried out with different concentrations of the bile salt against different levels of exogenous biliary stimulation with caerulein. To investigate further the mechanism of the effect of STC on the biliary excretion, an attempt was made to identify separately the role of the gall bladder in bile delivery by comparing the responses in healthy volunteers with intact gall bladders with responses in cholecystectomised subjects, and the possible role of the sphincter of Oddi by monitoring pancreatic secretion as well in all studies. Finally, plasma levels of gastrin, motilin, pancreatic polypeptide and gastric inhibitory polypeptide were monitored in some of these studies to identify whether any of these candidate peptide hormones from the upper gastrointestinal tract

and the pancreas were affected during the bile salt perfusion, and which might therefore have been involved in the observed responses.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

The experiments were planned to study the effects of duodenal perfusion with STC on basal and stimulated biliary and pancreatic secretion in man. Duodenal perfusion with EAA was used to provide an endogenous stimulus for biliary and pancreatic secretion, and caerulein and secretin were administered by continuous intravenous infusion as exogenous stimuli for the secretion. The doses were adjusted so that they might simulate postprandial or interprandial secretory stimulation. To separate the effects on the gall bladder from the effects on the sphincter of Oddi, studies were carried out both on healthy subjects with gall bladders and on cholecystectomised subjects. The studies were carried out either with bilirubin or ICG as biliary markers. The use of ICG allowed plasma levels of the biliary marker to be assessed during the studies and the possible effect of the bile salt on hepatic uptake and excretion to be estimated.

4.2.1.1 Effects of STC and EAA on basal secretion: studies using bilirubin as a biliary marker. The effects of STC and EAA on basal biliary and pancreatic secretion were studied in five healthy volunteers (ages 21-24 yrs). After 2 h of duodenal perfusion with NaCl (145 mmol/l) (equilibration period), STC (10 mmol/l) was perfused for 4 h, followed by perfusion with EAA (78 mmol/l) and STC (10 mmol/l) in combination for 1 h. In a separate group of control studies, the prolonged effects of EAA (78 mmol/l) on the basal secretion were studied in five volunteers (ages 20-24 yrs). The EAA perfusion lasted for 3 h and was preceded by an equilibration period of 2 h, when the duodenum was perfused with NaCl (145 mmol/l) alone. In a third group of studies (12 subjects, ages 20-25 yrs), the basal bilirubin and trypsin secretion were monitored for 6 h during duodenal perfusion with NaCl (145 mmol/l) alone.

4.2.1.2 Effects of STC and EAA on exogenously stimulated secretion: studies using bilirubin as a biliary marker. The effects of STC and EAA on exogenously stimulated biliary and pancreatic secretion were studied in eight volunteers (ages 23-30 yrs), who received an

intravenous infusion of caerulein (2.5 ng $kg^{-1}h^{-1}$) and secretin (0.025 CU $kg^{-1}h^{-1}$) for 6 h. During this time the duodenum was perfused for the first 2 h with NaCl solution (145 mmol/l) alone (equilibration period). This was followed by a perfusion with STC (10 mmol/l) for 1 h, followed by a perfusion with EAA (78 mmol/l) and STC (10 mmol/l) in combination for 1 h, followed during the final hour with a perfusion of EAA (78 mmol/l) alone.

As a control study, duodenal perfusion with NaCl (145 mmol/l) alone was carried out for 6 h in 12 volunteers (ages 20-25 yrs), who received similar caerulein/secretin stimulation as described above.

4.2.1.3 Effects of STC on exogenously stimulated secretion: dose-response studies in healthy subjects using ICG as a biliary marker. The effects of STC on exogenously stimulated biliary and pancreatic secretion were studied in healthy subjects by using secretin and varying doses of caerulein as exogenous stimuli. In five subjects (ages 20-26 yrs) caerulein (5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$) were infused i.v. for 6 h. During the first $2\frac{1}{2}$ h (equilibration period) of these studies, the duodenum was perfused with NaCl (145 mmol/l) alone. This was followed by a duodenal perfusion with STC (15 mmol/l) for 2 h (test period) followed by NaCl (145 mmol/l) alone for $1\frac{1}{2}$ h (recovery period). In a separate series of studies (3 subjects, ages 21-24 yrs) a duodenal perfusion with STC (30 mmol/l) was carried out against the same background stimulation of caerulein and secretin as described before.

In another series of studies the background stimulation of caerulein was increased to 10 ng $kg^{-1}h^{-1}$ (2 subjects) or 20 ng $kg^{-1}h^{-1}$ (2 subjects) combined with the same dose of secretin (0.1 CU $kg^{-1}h^{-1}$), using luminal STC (15 mmol/l) as described before.

Control studies were carried out in five healthy subjects (ages 25-35 yrs), where the duodenum was perfused with NaCl (145 mmol/l) for 6 h using a background stimulus of i.v. caerulein (5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$). For comparison see basal studies on ICG (Fig. 38, p. 167).

4.2.1.4 Effects of STC on exogenously stimulated secretion in cholecystectomised subjects: studies using ICG as a biliary marker. The effects of STC on biliary and pancreatic secretion were studied in five cholecystectomised subjects (ages 34-56 yrs). These subjects had undergone cholecystectomy more than 6 months before the perfusion studies were carried out. On no occasion had sphincteroplasty or exploration of the common bile duct been carried out. In these

subjects the duodenum was perfused for 2 h with STC (15 mmol/l) after an equilibration period of 2 h. This was followed by a recovery period with NaCl (145 mmol/l) for 1 h. As a background stimulus, i.v. infusion of caerulein (5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$) was used.

4.2.2 Recovery of the gastric marker (phenol red) from the stomach and duodenum

Basal studies using bilirubin as a biliary marker (4.2.7.1). NaCl control group: recovery from the stomach 75.7% ± 2.4 (SEM, n = 12) and the duodenum 13.8% ± 2.4 (n = 12); STC test group: recovery from the stomach 80.4% ± 6.1 (n = 5) and the duodenum 21.6% ± 4.5 (n = 5); EAA test group: recovery from the stomach 72.9 ± 4.9 (n = 5) and the duodenum 22.3% ± 5.8 (n = 5).

Caerulein/secretin stimulated studies using bilirubin as a biliary marker (4.2.7.2). NaCl control group: recovery from the stomach 87.6% ± 2.9 (n = 12) and the duodenum 11.6% ± 2.6 (n = 12); STC test group: recovery from the stomach 75.1% ± 5.3 (n = 8) and the duodenum 24.5% ± 3.2 (n = 8) (p <0.01 compared with control group 11.6% ± 2.6).

Caerulein/secretin stimulated studies using ICG as a biliary marker: healthy subjects (4.2.7.3). NaCl control group: recovery from the stomach 80.8% ± 8.1 (n = 5) and the duodenum 10.0% ± 2.0 (n = 5); STC test group: recovery from the stomach 79.2 ± 3.6 (n = 12) and the duodenum 22.8% ± 3.9 (n = 12) (p <0.05 compared with control group 10.0% ± 2.0).

Caerulein/secretin stimulated studies using ICG as a biliary marker: cholecystectomised subjects (4.2.7.4). Recovery from the stomach 82.4% ± 2.5 (n = 5) and the duodenum 10.0% ± 2.0.

4.2.3 Recovery of the duodenal marker (PEG 4000 or ^{14}C PEG 4000) from the duodenum and the stomach

Basal studies using bilirubin as a biliary marker (4.2.7.1)(PEG 4000). NaCl control group: recovery from the duodenum 54.2% ± 7.0 (SEM, n = 12) and the stomach 10.3% ± 2.9 (n = 12); STC test group: recovery from the duodenum 49.2 ± 4.0 (n = 5) and the stomach 17.1% ± 4.2 (n = 5);

EAA test group: recovery from the duodenum 53.9% ± 12.8 (n = 5) and the stomach 22.5% ± 11.3 (n = 5).

Caerulein/secretin stimulated studies using bilirubin as a biliary marker (4.2.7.2) (PEG 4000). NaCl control group: recovery from the duodenum 59.2% ± 6.5 (n = 12) and the stomach (4.4% ± 1.1 (n = 12); STC test group: recovery from the duodenum 52.1% ± 8.7 (n = 8) and the stomach 11.8% ± 4.4 (n = 8).

Caerulein/secretin stimulated studies using ICG as a biliary marker: healthy subjects (4.2.7.3) (^{14}C PEG 4000). NaCl control group: recovery from the duodenum 60.7% ± 10.1 (n = 5) and the stomach 7.7% ± 3.3 (n = 5); STC test group: recovery from the duodenum 54.4% ± 3.8 (n = 12) and the stomach 7.5% ± 1.6 (n = 12).

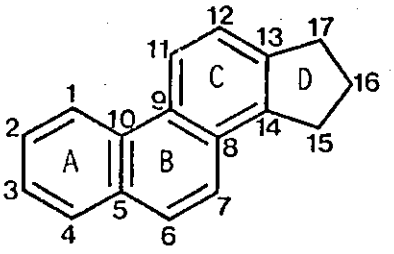
Caerulein/secretin stimulated studies using ICG as a biliary marker: cholecystectomised subjects (4.2.7.4) (^{14}C PEG 4000). Recovery from the duodenum 67.0% ± 8.6 (n = 5) and the stomach 9.2% ± 3.6 (n = 5).

4.2.4 Chemicals/instruments

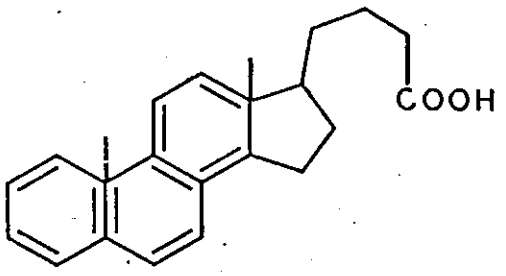
Pure natural porcine secretin, 3500 clinical units (CU) per mg, stabilised in cysteine hydrochloride, was obtained from GIH Research Unit, Chemistry Department, Karolinska Institute, Stockholm, Sweden (Professor Victor Mutt) distributed by Kabi Diagnostica, Fack, Nyköbing, Sweden. Caerulein (Caeruletide®) 5 µg/ml, batches TF/20400, TF/20523 and TF/21257) was provided by Farmitalia Ltd, Kingmaker House, Station Road, Barnet, Herts EN5 1NU, England.

Essential amino acids (EAA) were purchased from Sigma London Chemical Co Ltd, Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, England. Sodium taurocholate was synthesised from taurine (British Drug Houses Chemicals Ltd, Poole, Dorset, England) and cholic acid (Sigma London Chemical Co Ltd) using EEDQ (N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline, ethyl-1,2-dihydro-2-ethoxy-1-quinoline-carboxylate) (Aldrich Chemical Co Inc, The Old Brickyard, New Road, Gillingham, Dorset SP8 4JL, England) as a coupling agent.

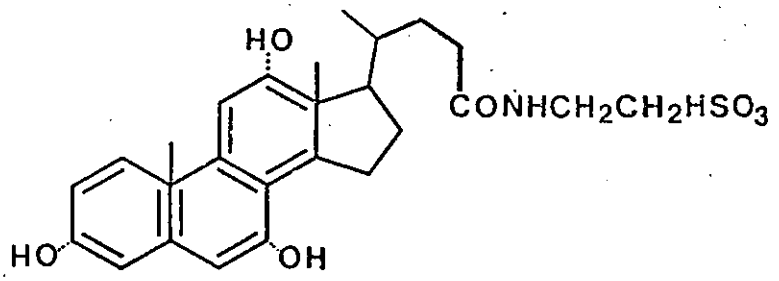
Synthesis of sodium taurocholate. Sodium taurocholate (STC) (Fig. 21) was synthesised in the laboratory as described by Lack *et al.* (214) with the following modifications: after boiling the mixture of taurine, cholic acid and EEDQ for 24 h, ethanol was evaporated from



cyclopentanophenantrene



5 β - cholanic acid



taurocholic acid

Fig. 21. Sterically the fusion of the A/B ring is *cis* (the methyl group at C_{10} and the hydrogen atom at C_5 do both have a β-orientation). The B/C and C/D rings have *trans* (allo) fusions (the hydrogen atom at C_8 is β-orientated but the hydrogen atom at C_9 is α-orientated; the hydrogen atom at C_{14} has α-orientation but the methyl group at C_{13} has β-orientation).
cis = on this side; *trans* = on the other side;
α-orientation = away from the observer;
β-orientation = towards the observer.

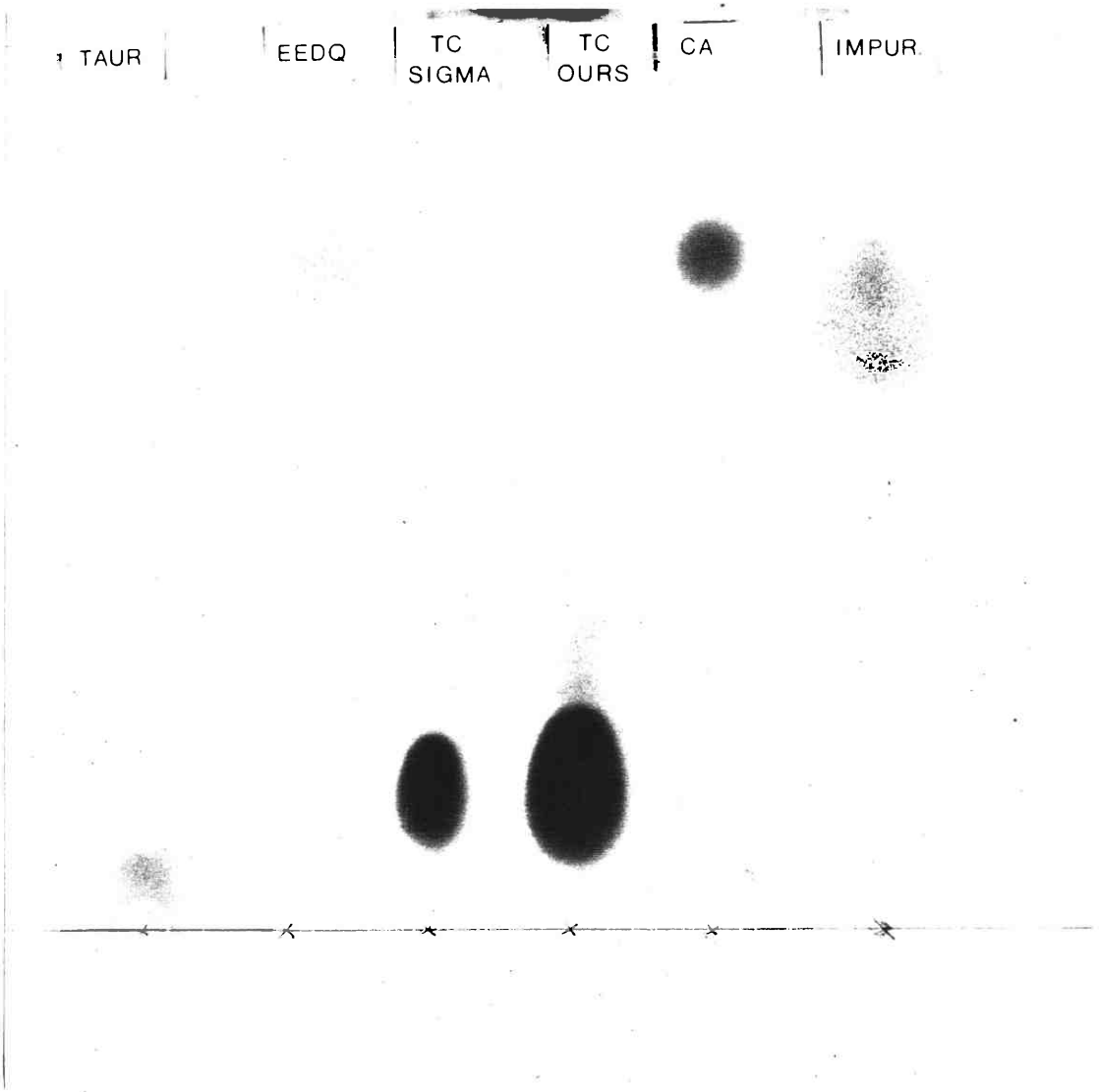


Fig. 22. A TLC of taurine, EEDQ, STC standard (Sigma), STC produced in the laboratory for the present studies, and cholic acid.

the mixture in a rotary vacuum evaporator. The residue was transferred to a separating funnel, dissolved in water and washed three times with dichloromethane. The aqueous extract was then freeze-dried. This freeze-dried material was dissolved in ethanol and precipitated by addition of ethyl acetate and finally allowed to crystallise over several days at -20°C. On thin layer chromatography the product moved as a single spot identical to a Sigma standard (Sigma T4009) (see Fig. 22).

4.2.5 Preparation of infusion solutions

10 ml of sterile NaCl solution (145 mmol/l) containing 200 µmol/l of human albumin were injected into a glass vial containing 75 CU of secretin in a solid form. After a thorough shaking, an appropriate amount of secretin and caerulein (in liquid form, 5 µg/ml), were measured and transferred to a 50 ml plastic syringe. This was diluted further by sterile NaCl (145 mmol/l), containing human albumin (200 µmol/l) so that the following doses were achieved when the parenteral infusion was carried out at the flow rate of 0.11 ml min^{-1}:

1) caerulein 2.5 ng $kg^{-1}h^{-1}$ & secretin 0.025 CU $kg^{-1}h^{-1}$ in the studies described in Section 4.2.1.2;
2) caerulein 5.0 ng $kg^{-1}h^{-1}$ and secretin 0.1 CU $kg^{-1}h^{-1}$ in the studies described in Sections 4.2.1.3 and 4.2.1.4;
3) caerulein 10.0 ng $kg^{-1}h^{-1}$ and secretin 0.1 CU $kg^{-1}h^{-1}$ in the studies described in Section 4.2.1.3;
4) caerulein 20.0 ng $kg^{-1}h^{-1}$ and secretin 0.1 CU $kg^{-1}h^{-1}$ in the studies described in Section 4.2.1.3.

ICG was dissolved in the manufacturer's solvent and diluted further by sterile NaCl (145 mmol/l) containing human albumin (200 µmol/l) infused at a dosis of 0.25 mg $kg^{-1}h^{-1}$ at a flow rate of 0.11 ml min^{-1}. The syringe and the infusion line containing ICG were covered with an aluminium paper to protect ICG from direct light.

4.2.6 Preparation of perfusion solutions

Perfusates of STC (10 mmol/l, 15 mmol/l or 30 mmol/l), EAA (78 mmol/l) and a mixture of STC and EAA, were prepared immediately before use, made iso-osmolar (290 mOsm kg) by NaCl and adjusted to pH 6.3 by NaOH and HCl, and warmed up to 37°C before use. All perfusates contained PEG 4000, 5 g/l, as a duodenal reference marker. In studies described in Sections 4.2.1.3 and 4.2.1.4 the perfusion solution also contained ^{14}C PEG 4000 as a reference marker.

Phenol red was dissolved in NaCl (145 mmol/l) and diluted to the final concentration of 1.5 g/l for gastric perfusions.

4.2.7 Perfusion technique

This technique is described in detail in Sections 2.2.9 to 2.2.12. Re-infusion of duodenal aspirate was not carried out in these studies.

4.2.8 Analytical methods

Bilirubin in the duodenal aspirates was measured by the method of Powell (305). Trypsin was measured in a semi-automatic recording titrator (Radiometer Corp., Copenhagen, Denmark), using *p*-tosyl-l-arginine methyl ester (Sigma No. T4626) as a substrate dissolved in a solution of 0.04 mmol/l NaCl and 0.02 mmol/l $CaCl_2$ (36). As a titrant, 0.01 mol/l NaOH was used. Results were expressed in international units (IU), 1 IU of enzyme activity being the enzyme activity which catalysed the hydrolysis of 1 µmol of substrate per min at pH 8.2 at 30°C. Total bile salts were measured enzymatically by hydroxy steroid dehydrogenase (Sigma No. H7127, Type 1), using β-nicotinamide adenine dinucleotide (β-NAD) (Sigma No. N7129) as a co-enzyme and 4 mol/l hydrazine hydrate (BDH) as a ketone trapping agent in 50 mmol/l tetra-sodium pyrophosphate buffer (BDH) (pH 9.5). Samples were incubated for 40 min at 30°C and read in a Gilford spectrophotometer at 340 nm against blanks. Chenodeoxycholic acid (Sigma No. C9377) was used as a standard (36).

Bicarbonate was measured by addition of hydrochloric acid, boiling and back-titration. pH was measured in a Pye Unicam pH-meter. For measurement of PEG 4000, see Section 2.2.7. For analysis of ICG in serum and duodenal fluid, see Section 3.2 and (36).

Motilin, gastrin and GIP were measured by Dr. N.D. Christofides.* Motilin was measured with *N*-terminal reacting porcine antiserum (75), which detected both large and small molecular forms of motilin in plasma, and showed no cross-reactivity with other hormones. Changes of 3 pmol of motilin/l of plasma could be detected with 95% confidence. GIP was measured with a *C*-terminal-reacting porcine antiserum which detected large and small molecular forms of GIP in plasma without cross-reacting with other hormones (327). Changes of 2.5 pmol/l of plasma were detected with 95% confidence. Gastrin was measured with antiserum raised against human gastrin, which detected changes in gastrin plasma levels of 2 pmol/l with 95% confidence (317). PP was measured by Dr. T.E. Adrian.* PP was measured with an antiserum raised against bovine pancreatic polypeptide. The assay detected changes in PP concentrations of 8 pmol/l with 95% confidence (36).

*Endocrine Unit, R.P.M.S., Hammersmith Hospital.

4.2.9 Statistics

Statistical analysis was performed by using Student's *t*-test for paried or unpaired data, as appropriate. Since the data on biliary and pancreatic secretion and gastrointestinal hormones were not normally distributed, but approximately log normal, all statistical evaluation of these data was done on log values. The data are expressed in the text, tables and figures as means (geometric mean) ± SEM (in parentheses), the geometric mean ± SEM being calculated on the log values and then transformed to a linear scale by taking anti-logs (300). Except where otherwise stated the data on biliary and pancreatic secretion represent the mean (geometric mean) per 10 min ± SEM of the hour or hours in question.

4.3 RESULTS

4.3.1 Effects of STC and EAA on basal secretion: studies using bilirubin as a biliary marker

The results of these studies are shown in Fig. 23. Basal bilirubin secretion fell promptly from 389 µg/10 min (262-585, SEM, n = 5) during the hour immediately preceding STC perfusion to 23 µg/10 min (14-38, n = 5) (p <0.001) during the second half of the first STC perfusion hour (Fig. 23A). During the following 3 h of STC perfusion, a progressive increase in bilirubin secretion was observed, the bilirubin secretion during the fourth hour of STC perfusion being 333 µg/10 min (195-578, n = 5) and not significantly different from the basal levels. During subsequent perfusion for 1 h with EAA/STC in combination, bilirubin secretion rose markedly above these levels to 1195 µg/10 min (740-2095, n = 5) (p <0.01) (Fig. 23A), suggesting that a gall bladder emptying had occurred.

A slight increase in trypsin secretion was observed during the 4 h of STC perfusion (Fig. 23B), eventually reaching 1510 IU/10 min (1210-1734, n = 5) (hour 4) *versus* 1250 IU/10 min (992-1591, n = 5) (basal p = 0.05). When STC and EAA were perfused together, a marked increase above these levels was observed to 2455 IU/10 min (1780-2965, n = 5) compared with 1250 IU/10 min (992-1591, n = 5) (basal p <0.01).

Bicarbonate secretion did not change during the 4 h of STC perfusion (Table 5) but during the final hour when EAA and STC were perfused together, a trivial but statistically significant increase in bicarbonate secretion was observed (p <0.05).

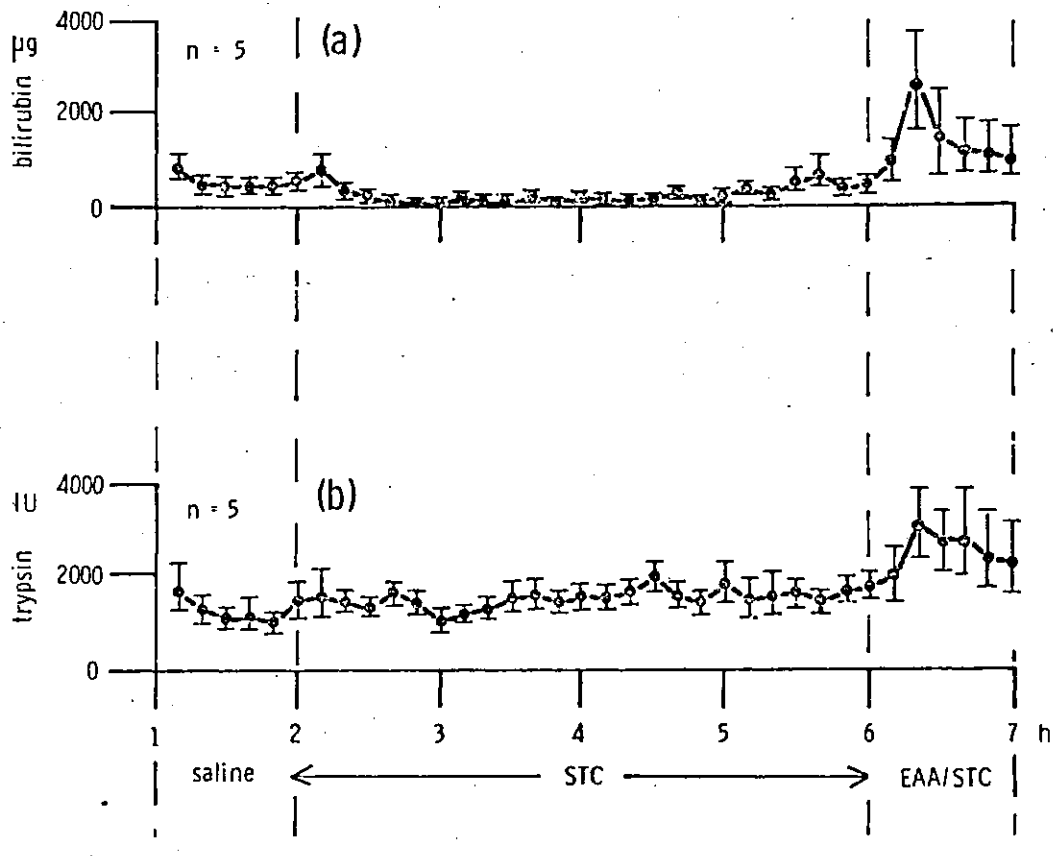


Fig. 23. Duodenal recovery of (A) basal bilirubin secretion and (B) trypsin secretion in five fasting normal subjects during 1 h duodenal perfusion of NaCl solution (145 mmol/l: saline) followed by 4 h perfusion of STC (10 mmol/l) followed by 1 h perfusion of EAA/STC (78/10 mmol/l). Results are shown as geometric means ± SEM (bars) (n = 5) for every 10 min period.

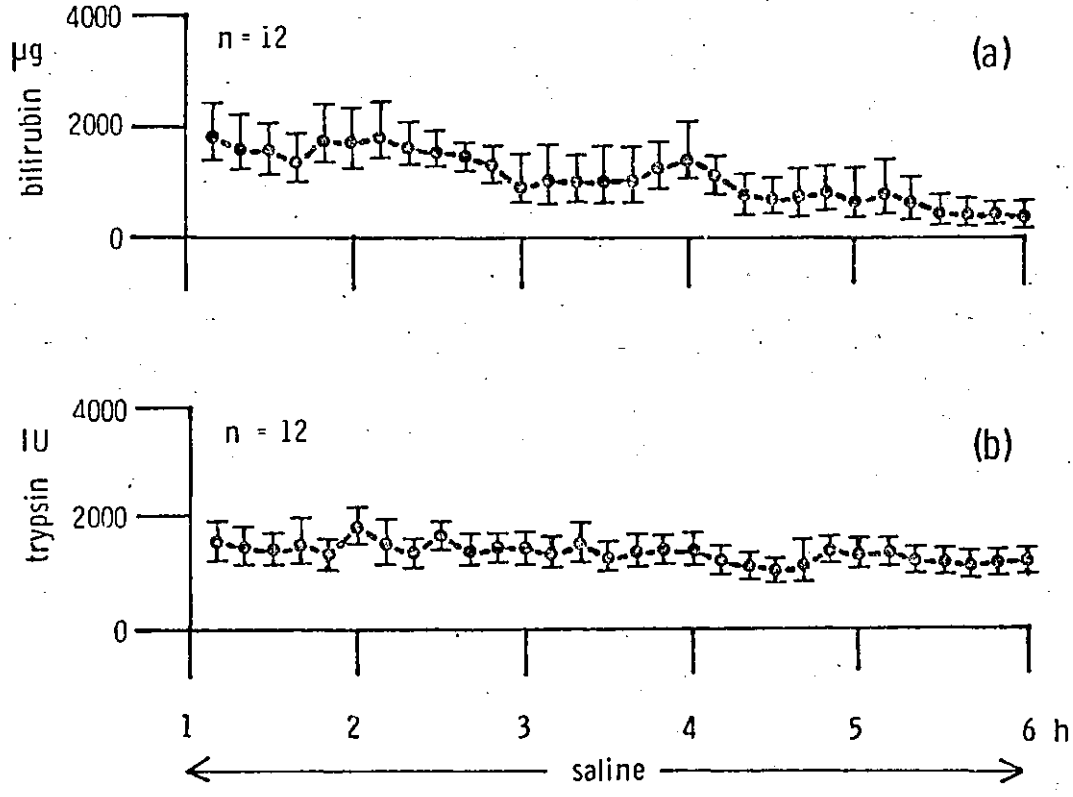


Fig. 24. Duodenal recovery of (A) basal bilirubin secretion and (B) basal trypsin secretion in twelve fasting subjects during 6 h duodenal perfusion of NaCl solution (145 mmol/l: saline). Geometric means ± SEM (bars) are shown (n = 12).

The concentration of total bile salts in the duodenum during the 4 consecutive hours of STC perfusion were 6.4 mmol/l (5.8-7.0), 6.4 mmol/l (6.3-6.5), 6.2 mmol/l (5.3-7.2) and 6.0 mmol/l (5.0-7.3), respectively, compared with 0.7 mmol/l (0.3-1.0) during the basal period when NaCl (145 mmol/l) was perfused alone.

In the control group no abrupt changes in bilirubin secretion were observed although there was a downward trend in bilirubin secretion during the 6 h of NaCl (145 mmol/l) perfusion (Fig. 24). However, a wide variation in basal bilirubin secretion was observed between the control group and the STC test group. Inherent individual variation in basal bilirubin secretion is a likely explanation to this, rather than differences in marker recovery (see Sections 4.2.2 and 4.2.3).

In the control studies receiving a prolonged (3 h) perfusion of EAA (Fig. 25A), bilirubin secretion rose from 486 µg/10 min (257-927, n = 5) during the basal period to a peak value of 2727 µg/10 min (2428-3055, n = 5) 20 min after the beginning of EAA perfusion (p <0.02). Subsequently, bilirubin secretion fell to a plateau of 1392 µg/10 min (1055-1716, n = 6) during the second and third hours of EAA perfusion (Fig. 25A) (p <0.05, compared with basal). During the first 30 min of EAA perfusion peak output of trypsin was 4919 IU/10 min (4028-6008, n = 5) compared with 1679 IU/10 min (1062-2674, n = 5) in the basal period (p <0.05) (Fig. 25B). Trypsin secretion fell subsequently to a plateau level of 2733 IU/10 min (1968-3764, n = 5) during the second and third hours of EAA perfusion. Bicarbonate secretion did not change in these studies (Table 5).

4.3.2 Effects of STC and EAA on exogenously stimulated secretion: studies using bilirubin as a biliary marker

Exogenously stimulated bilirubin secretion was 2628 µg/10 min (2179-3189, n = 8) during the hour immediately preceding STC perfusion (total bile salt concentration 1.1 mmol/l, 0.7-1.8, n = 8), but decreased to 393 µg/10 min (214-734, n = 8) (p <0.01) during the STC perfusion hour (total bile salt concentration 6.8 mmol/l, 5.7-8.1, n = 8) (Fig. 26A). When EAA was added to the STC perfusate, bilirubin secretion reached a peak output of 6668 µg/10 min (4699-9462, n = 8) at 20 min (p <0.01), compared with the pre-STC hour), after which bilirubin secretion fell towards initial levels. A subsequent perfusion with EAA alone did not increase bilirubin secretion further, which remained at 2747 µg/10 min (2828-3254, n = 8) (p <0.05, compared with pre-STC hour).

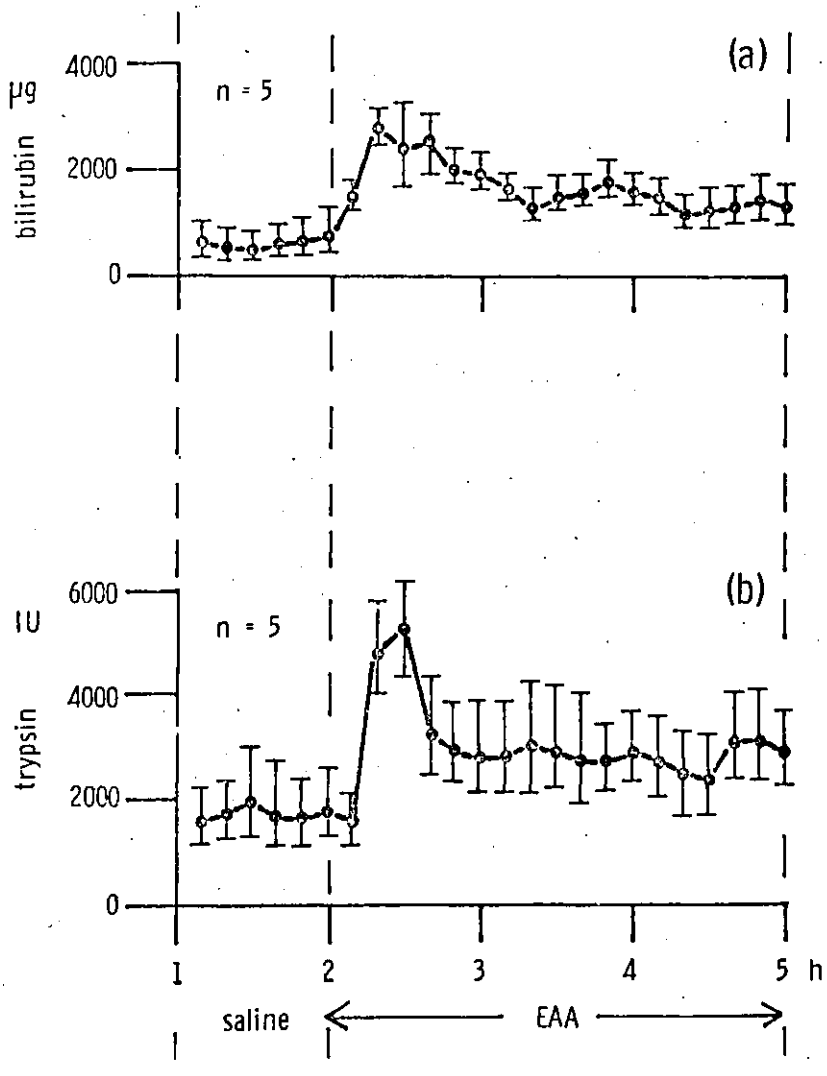


Fig. 25. Duodenal recovery of (A) bilirubin secretion and (B) trypsin secretion in five normal fasting subjects during 1 h duodenal perfusion of NaCl (145 mmol/l: saline) followed by 3 h perfusion of EAA (78 mmol/l). Results are shown as geometric means ± SEM (bars) (n = 5) for every 10 min period.

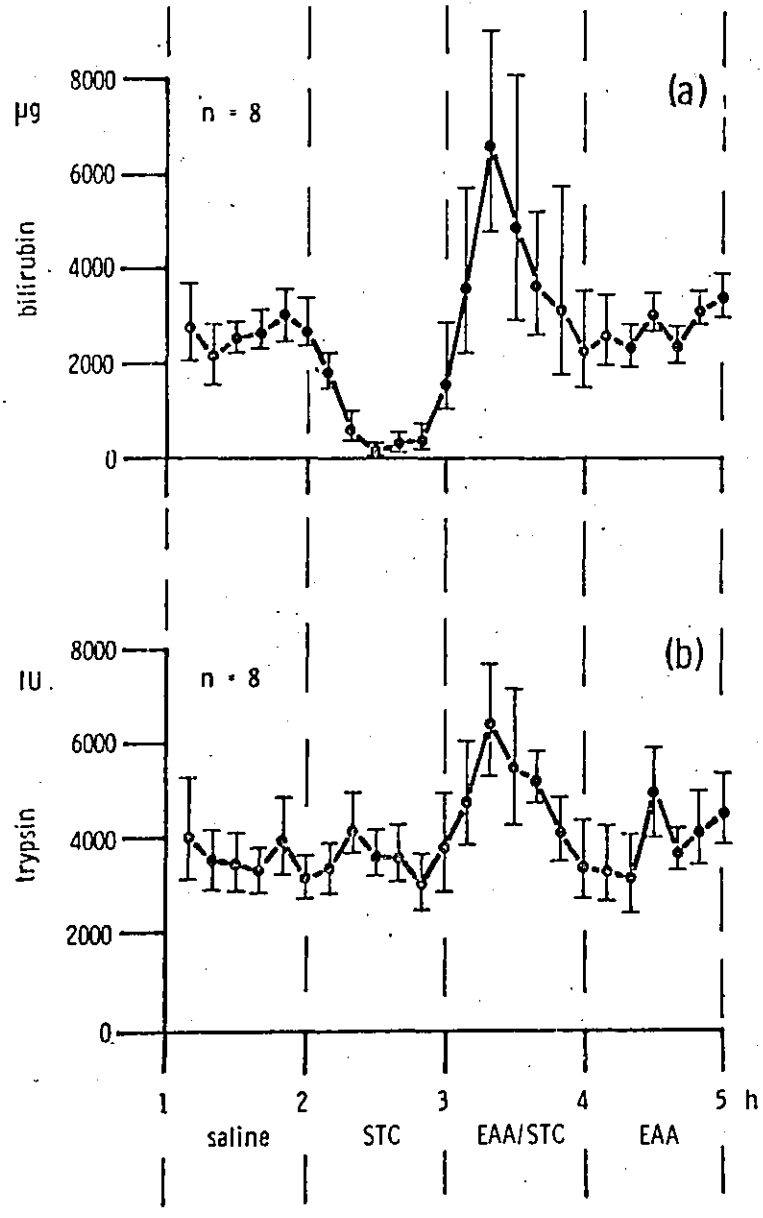


Fig. 26. Duodenal recovery of intravenous caerulein (2.5 ng $h^{-1}kg^{-1}$) and secretin (0.025 CU $h^{-1}kg^{-1}$) stimulated bilirubin secretion (A), and trypsin secretion (B) in eight fasting normal subjects during 1 h duodenal perfusion with NaCl solution (145 mmol/l: saline), followed subsequently by 1 h perfusion of STC, EAA/STC and EAA respectively. Results are shown as geometric means ± SEM (bars) (n = 8) for every 10 min period.

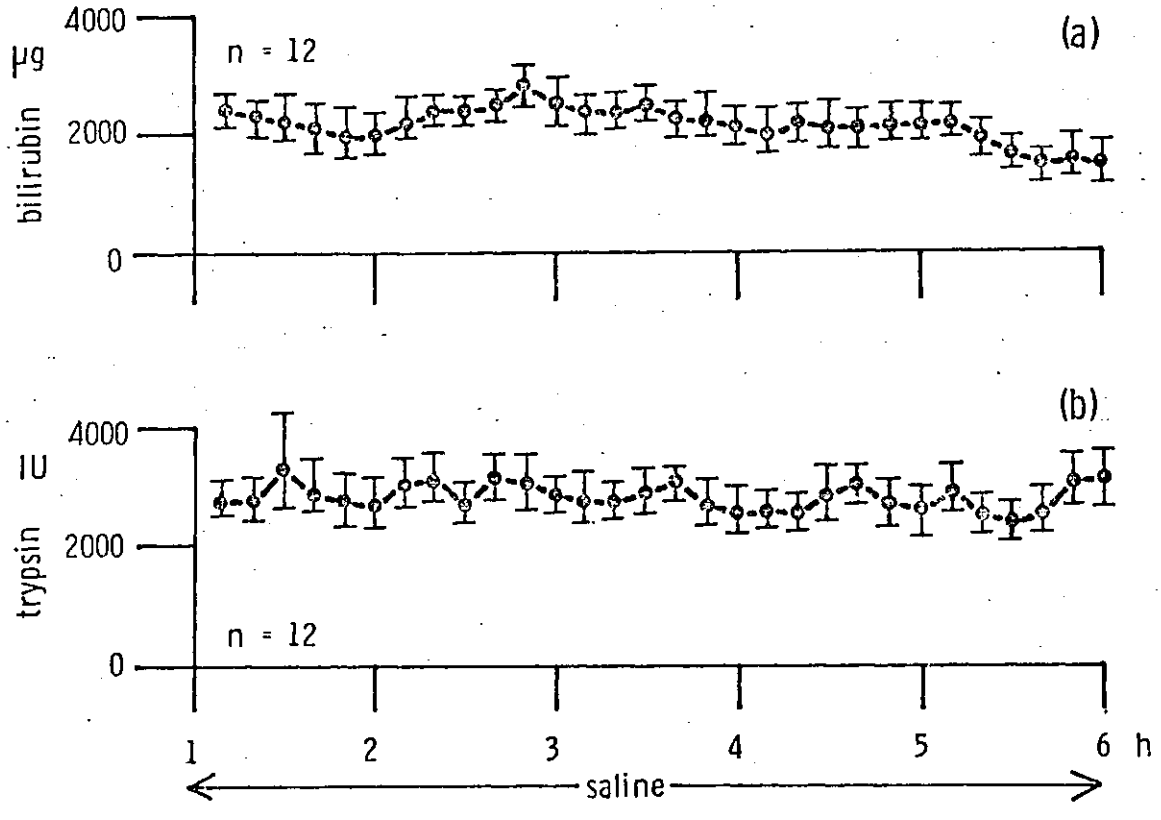


Fig. 27. Duodenal recovery of intravenous caerulein (2.5 ng $h^{-1}kg^{-1}$) and secretin (0.025 CU $h^{-1}kg^{-1}$) stimulated bilirubin secretion (A), and trypsin secretion (B) during 6 h duodenal perfusion of NaCl solution (145 mmol/l: saline). Geometric means ± SEM (bars) are shown (n = 12).

Table 5. Total amount of bicarbonate (μmol/10 min) in the duodenal fluid in the basal and stimulated studies using bilirubin as a biliary marker.

Perfusion fluid: / Duration:	Equilibration period, NaCl 145 mmol/l, 1 h	Test period, STC 10 mmol/l, 1 h or 4 h*	Test period, EAA/STC 78/10 mmol/l,* 1 h	Test period, EAA 78 mmol/l, 1 h or 3 h*
Basal studies (n=5)	77 (45-139)	128 (86-196)	252† (220-290)	-
Basal studies (n=5)	132 (78-236)	-	-	151 (85-256)
Caerulein/secretin stimulated (n=8)	1437 (785-2329)	2848†(1462-5548)	2213†(1801-4531)	1351 (680-2683)

*STC was perfused for 4 h in the basal studies. EAA was perfused for 3 h in the basal studies.

†$P \leq 0.05$ difference from NaCl control perfusion.

Figures express geometric means ± SEM (in parentheses).

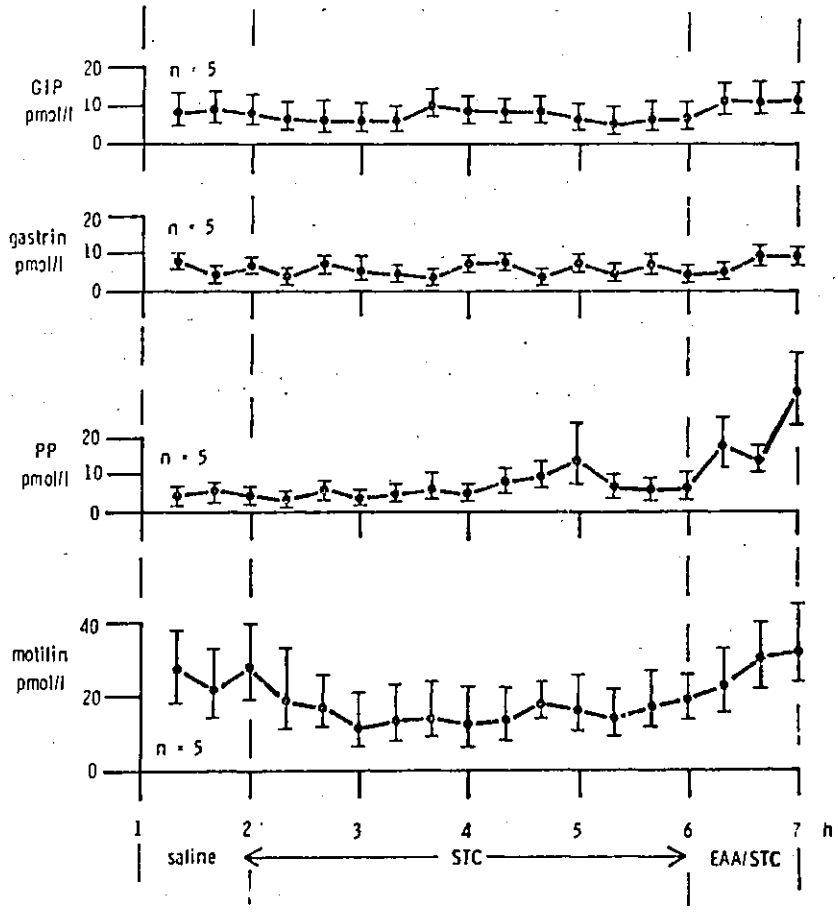


Fig. 28. Plasma levels of GIP, gastrin, PP and motilin (pmol/1) during duodenal perfusion with NaCl solution (145 mmol/1: saline), STC and EAA/STC respectively. Basal studies. Results are shown as geometric means ± SEM (bars) (n = 5) for every 20 min period.

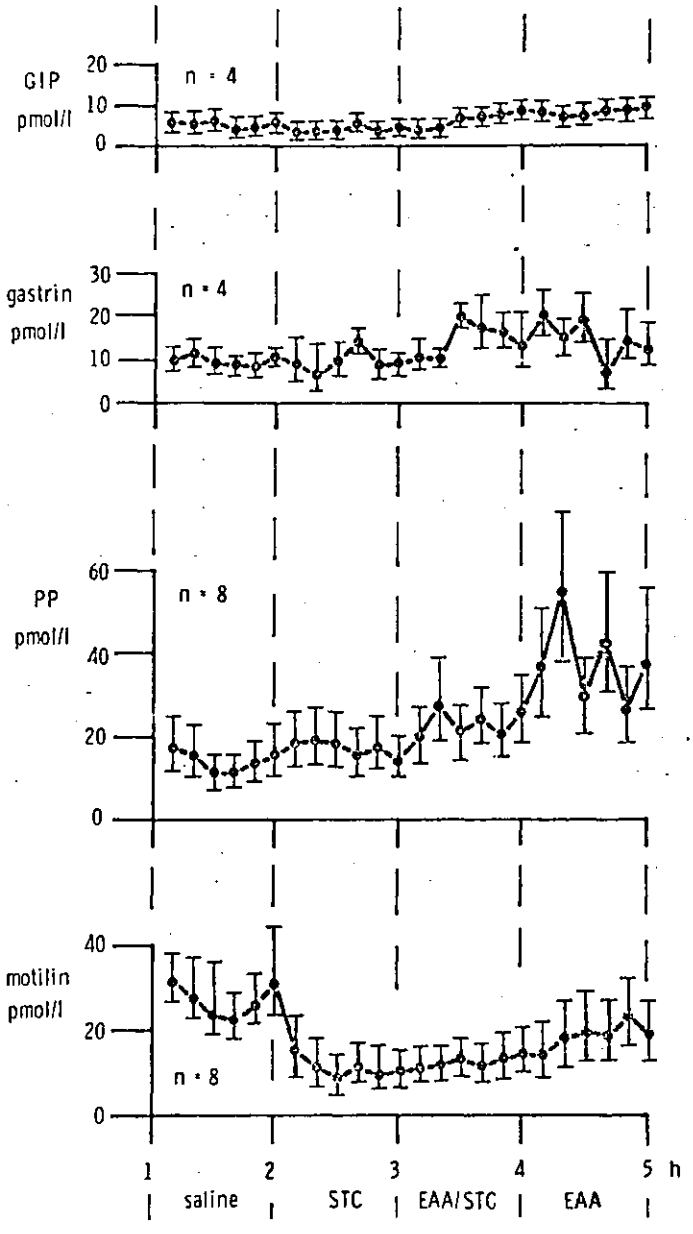


Fig. 29. Plasma levels of GIP, gastrin, PP and motilin (pmol/l) during duodenal perfusion with NaCl solution (145 mmol/l: saline), STC, EAA/STC and EAA alone respectively. Caerulein/secretin-stimulated studies. Geometric means ± SEM (bars) (n = 4 or n = 8) for every 10 min period.

Exogenously stimulated trypsin secretion did not change during perfusion with STC alone (3600 IU/10 min, 3019-4303, n = 8, *versus* 3515 IU/10 min, 2905-4261, n = 8, pre-STC hour p >0.05) (Fig. 26B). During the combined EAA/STC perfusion, trypsin secretion rose transiently at 20 min to 6295 IU/10 min (5164-7674, p <0.02, compared with pre-STC hour), falling before the EAA/STC perfusion was completed and failing to rise again during perfusion with EAA alone (Fig. 26B). A slight increase in bicarbonate secretion was observed during the perfusion of STC alone and EAA/STC in combination compared with the initial NaCl perfusion ($p \leq 0.05$) (Table 5).

4.3.3 Plasma levels of gastrointestinal hormones

Plasma levels of gastrin, GIP and PP did not change during STC perfusion in either the basal or exogenously stimulated studies. However, EAA in combination with STC, or perfused alone, revealed an increase in plasma PP levels (Figs. 28 & 29) (p <0.05). Plasma levels of motilin were significantly suppressed (p <0.001) in both basal and exogenously stimulated studies during STC perfusion (Figs. 28 & 29); however, levels rose again when EAA were added to the perfusate.

4.3.4 Effects of STC on exogenously stimulated secretion: dose-response studies in healthy subjects using ICG as a biliary marker

Results from the control studies of stimulated biliary excretion of ICG are shown in Fig. 30. ICG was recovered in the duodenum within 30 min of i.v. infusion. In most individuals the excretion of ICG into the duodenum had reached a relatively stable level after 2 to 3 h of i.v. infusion and remained so throughout the studies.

The effect of STC on caerulein/secretin stimulated ICG excretion in subjects with gall bladders is shown in Figs. 31 to 33 (individual data). Using caerulein stimulation of 5 ng $kg^{-1}h^{-1}$, secretin of 0.1 CU $kg^{-1}h^{-1}$ and STC of 15 mmol/l, the biliary recovery of ICG was reduced in this group from 78% (71-85), just prior to the bile salt perfusion, to 40% (33-49) (p <0.025) during the bile salt perfusion period with a maximum mean suppression to 26% (p <0.001) (Fig. 31). In the recovery period, when NaCl was perfused alone, the excretion of ICG rose to 120% (115-125). Serum levels of ICG had reached a plateau after 1 h of i.v. infusion (Fig. 34), and remained at plateau level (926 ± 91 µg/l, SEM, n = 5) throughout the studies. The total bile salt concentration in the duodenum rose from 3.3 mmol/l (3.0-3.7)

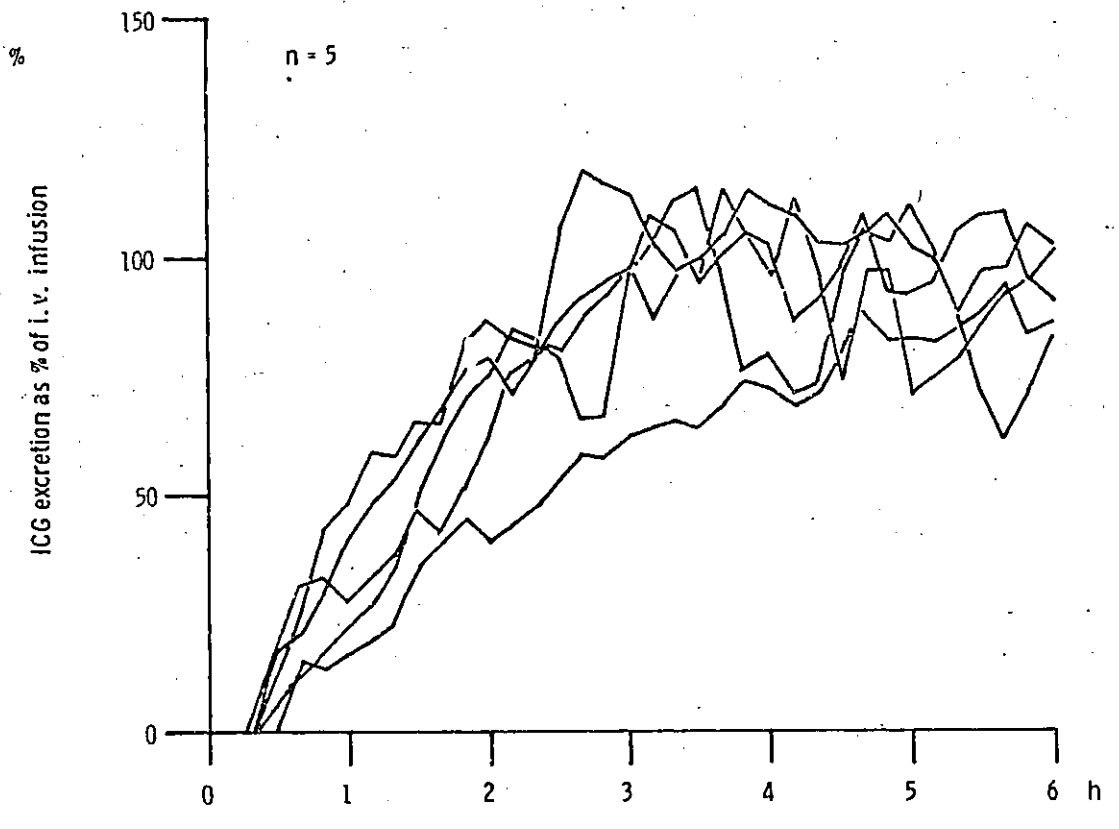


Fig. 30. Biliary excretion of ICG in healthy subjects with gall bladders (individual data) during 6 h duodenal perfusion of NaCl (145 mmol/l: saline). The biliary excretion was stimulated by i.v. caerulein (5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$).
For comparison see basal studies on ICG (Fig. 38, p. 167).

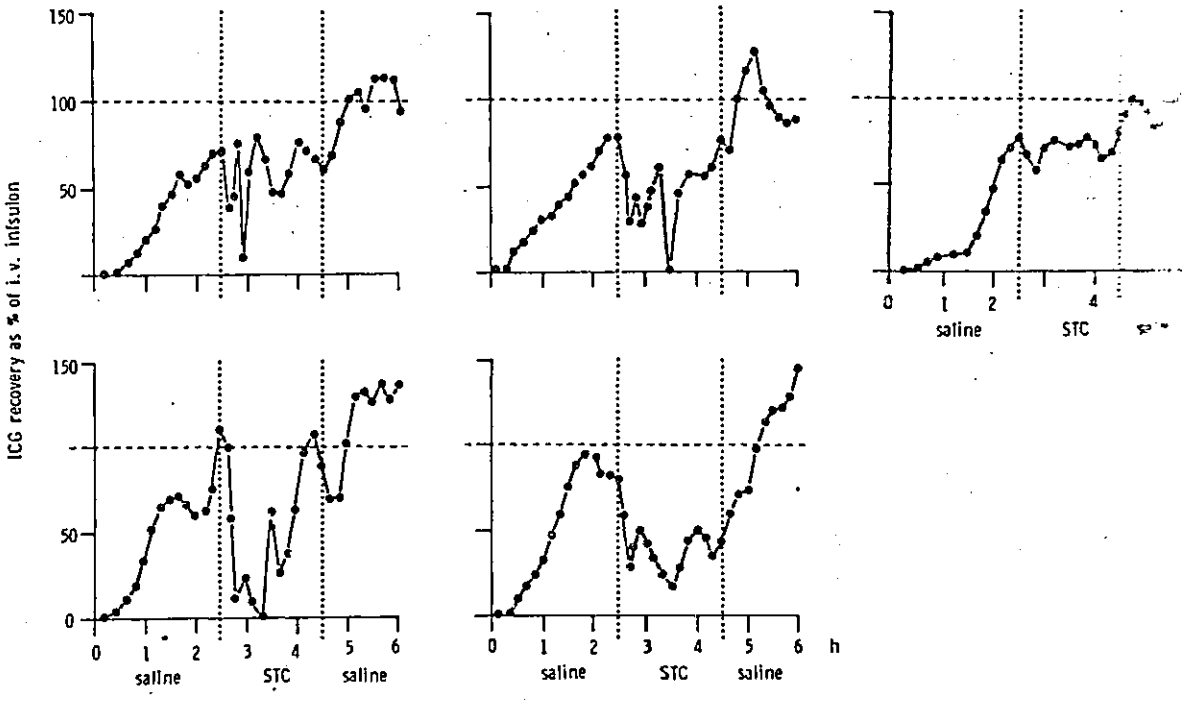


Fig. 31. Biliary excretion of ICG in five healthy subjects with gall bladders (individual data) during duodenal perfusion of NaCl (145 mmol/l: saline) and STC (15 mmol/l). The biliary excretion was stimulated by i.v. caerulein (5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$).

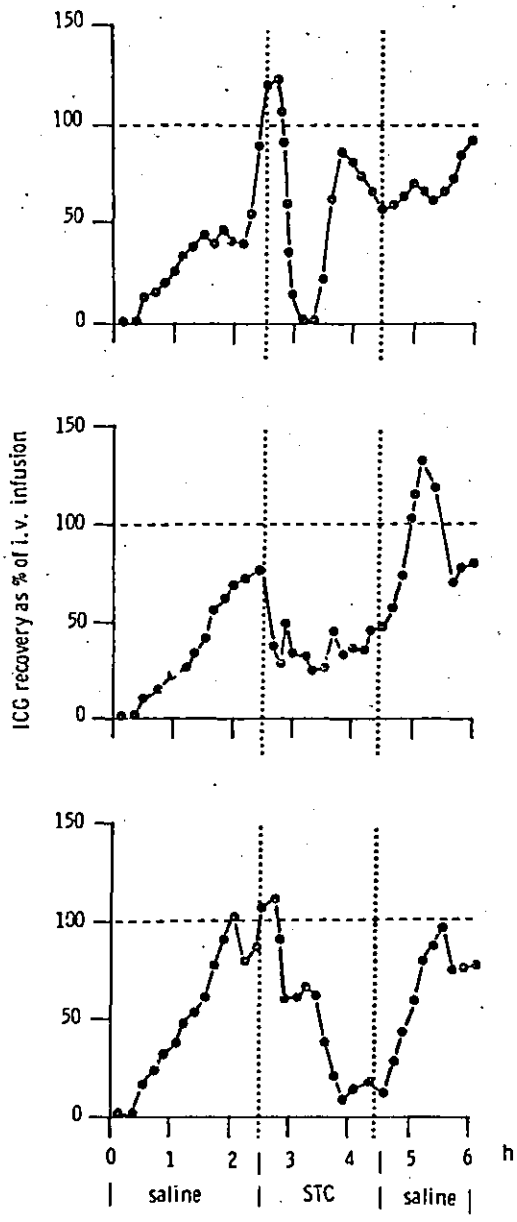


Fig. 32. Biliary excretion in three healthy subjects with gall bladders (individual data) during duodenal perfusion with NaCl (145 mmol/l: saline) and STC (30 mmol/l). A background stimulation of caerulein (5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$) was used.

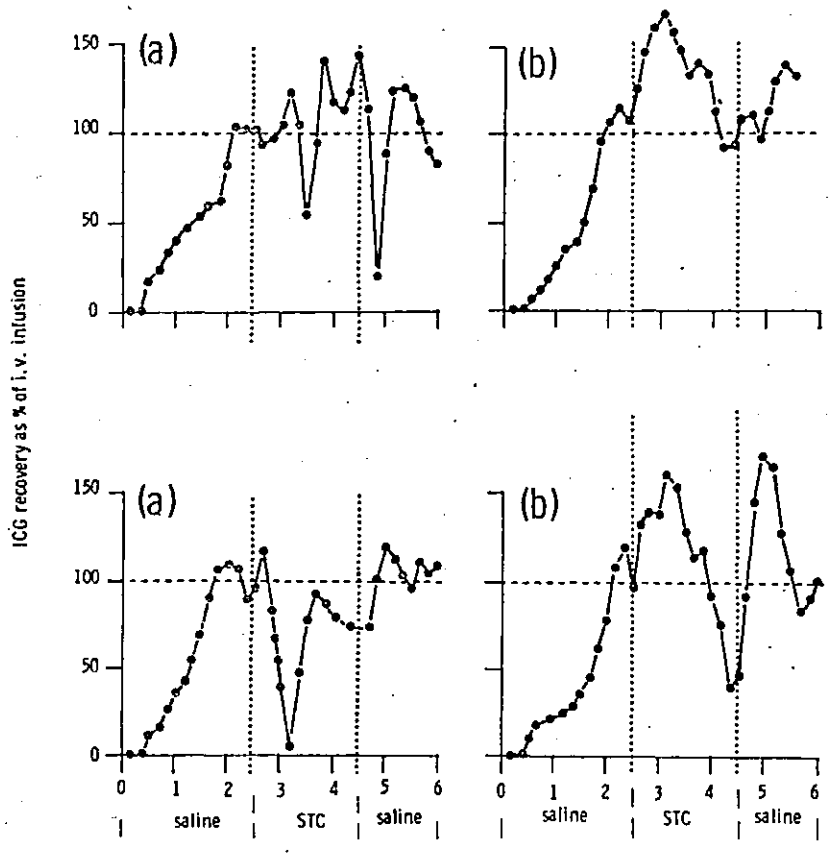


Fig. 33. Biliary excretion of ICG in healthy subjects with gall bladders (individual data) during duodenal perfusion of NaCl (145 mmol/l: saline) and STC (15 mmol/l). The biliary excretion was stimulated by: A, i.v. caerulein 10 ng $kg^{-1}h^{-1}$ and secretin 0.1 CU $kg^{-1}h^{-1}$; B, i.v. caerulein 20 ng $kg^{-1}h^{-1}$ and secretin 0.1 CU $kg^{-1}h^{-1}$.

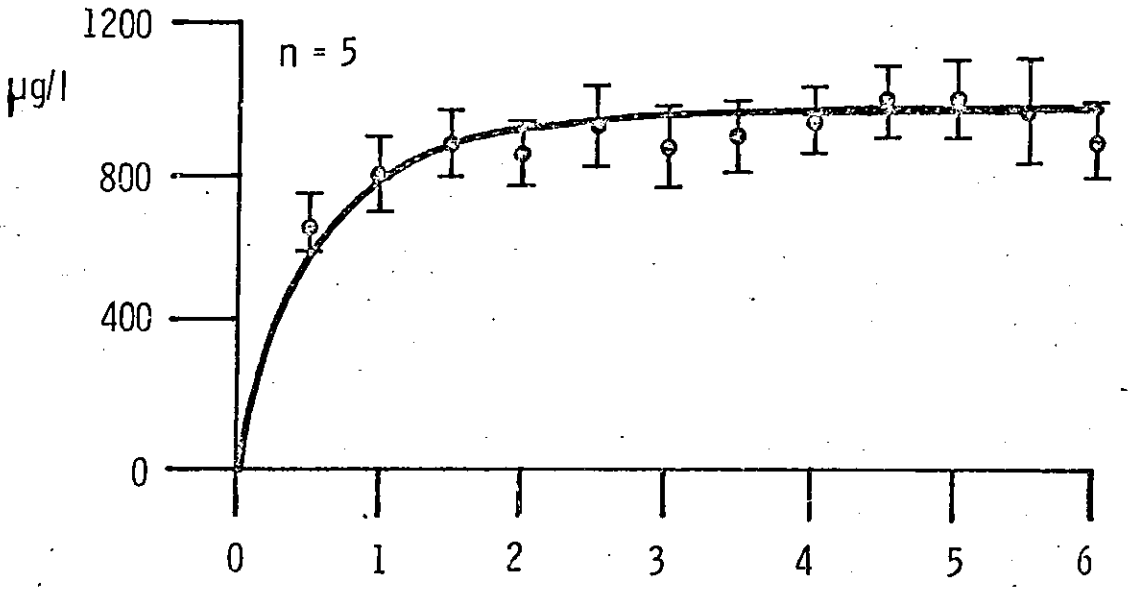


Fig. 34. Serum levels of ICG in five healthy subjects with gall bladders during duodenal perfusion of NaCl (145 mmol/l: saline) or STC (15 mmol/l). See further legend to Fig. 31.

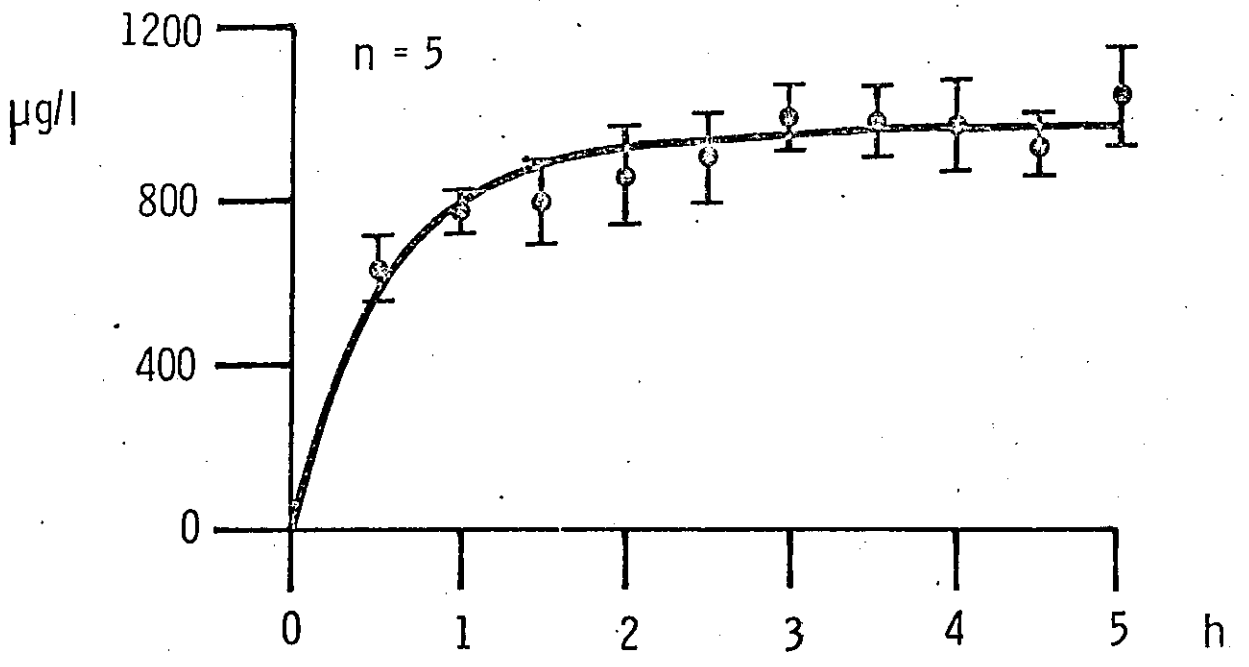


Fig. 35. Serum levels of ICG in five cholecystectomised subjects during duodenal perfusion of NaCl (145 mmol/l: saline) or STC (5 mmol/l). See further legend to Fig. 36.

to 12.7 mmol/l (11.3-14.0) during the perfusion of STC, with a fall to 2.2 mmol/l (1.8-2.4) in the recovery period.

Using the same exogenous stimulus of caerulein and secretin as before, but 30 mmol/l of STC, ICG excretion was reduced to 32% (30-33), with a maximum mean suppression to 10% during the period of STC perfusion (Fig. 32). The total bile salt concentration in the duodenum rose from 2.6 mmol/l (2.2-3.8) to 23.7 mmol/l (16.0-28.2) with a fall to 5.5 mmol/l (3.6-8.3) in the recovery period (Table 6).

In the studies where caerulein stimulation was increased to 10 ng $kg^{-1}h^{-1}$ (Fig. 33A), a suppression of biliary excretion could still be detected. At 20 ng $kg^{-1}h^{-1}$ dose (Fig. 33B), however, a gall bladder emptying occurred during the period of bile salt perfusion in both subjects studied. These results suggest that duodenal perfusion with STC (15 mmol/l) can suppress biliary excretion when caerulein infusion rate does not exceed 10 ng $kg^{-1}h^{-1}$.

Total volume of fluid in the duodenum did not significantly change during the period of bile salt perfusion (Table 7).

No change in trypsin secretion was observed in the studies where the perfusate of 15 mmol/l STC was used (Table 8). However, in the subjects where 30 mmol/l STC was used, results suggested that secretion of trypsin increased slightly during the period of STC perfusion (Table 8).

Bicarbonate did not change during the bile salt perfusion, neither at concentration 15 mmol/l or 30 mmol/l (Table 9). Nor were there any significant changes in pH of the duodenal fluid (aspirated at the distal end of the duodenum (Table 10).

4.3.5 Effects of STC on exogenously stimulated secretion in cholecystectomised subjects: studies using ICG as a biliary marker

The effects of duodenal perfusion of STC (15 mmol/l) on the biliary excretion in cholecystectomised subjects is shown in Fig. 36. Occasional transient falls in ICG output were observed during the bile salt period (Fig. 36) but this was quantitatively less than in the subjects with intact gall bladders (Fig. 31). However, in the recovery period ICG excretion increased to 135% (108-169), indicating that bile was held up in the biliary tract during the STC perfusion. Serum ICG had reached a plateau after 1 h of i.v. infusion and remained at that level during the studies (Fig. 35).

During the 2 h of STC perfusion, the concentration of bile salts in the duodenum was on average 15.5 mmol/l (15.0-16.0) in comparison with 4.6 mmol/l (4.1-5.4) during the 1 h equilibration period immediately before the bile salt perfusion,

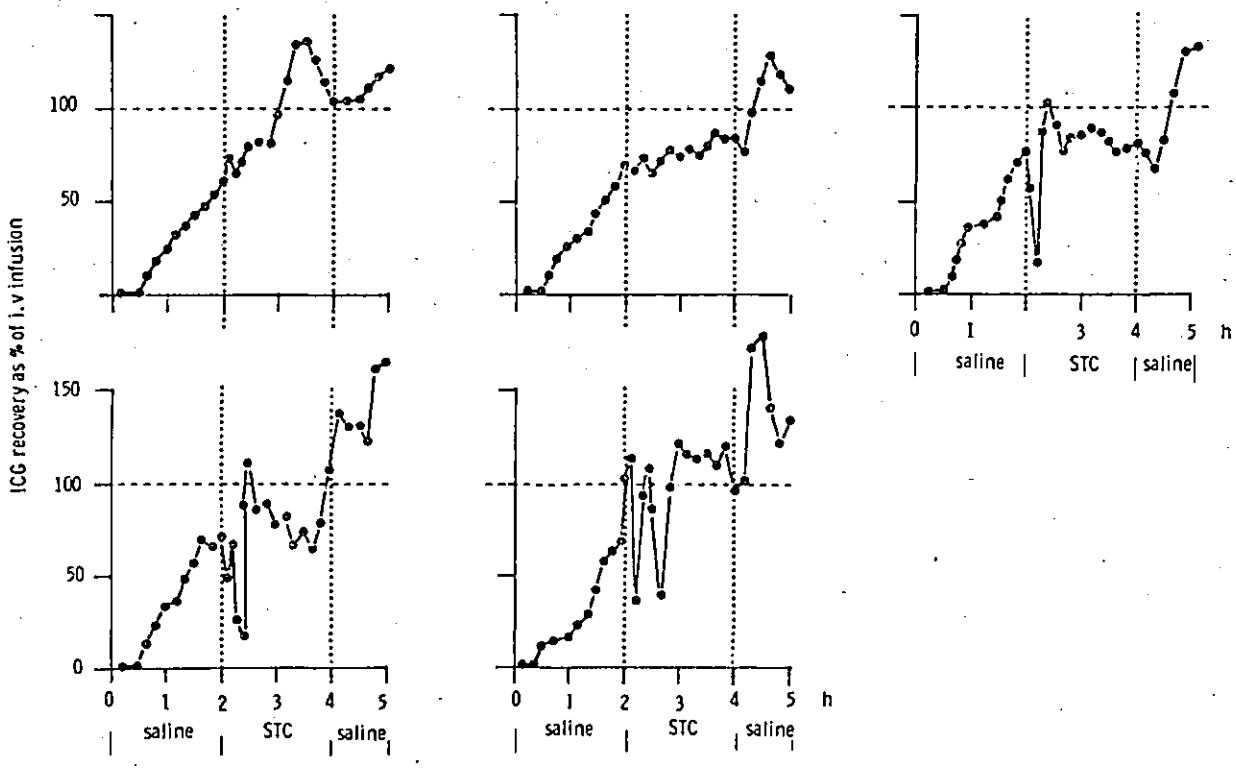


Fig. 36. Biliary excretion of ICG in five healthy cholecystectomised subjects (individual data) during duodenal perfusion with NaCl (145 mmol/l) and STC (15 mmol/l). The biliary excretion was stimulated by i.v. caerulein 5 ng $kg^{-1}h^{-1}$ and secretin 0.1 $kg^{-1}h^{-1}$.

Table 6. Conc. of total bile salts in the duodenum (mmol/l) during duodenal perfusion of NaCl (145 mmol/l) without or with STC. Caerulein/secretin stimulated studies using ICG as a biliary marker.

Perfusion fluid: Duration:	Equilibration period, NaCl 145 mmol/l, 1 h	Test period, STC 15 or 30 mmol/l*, 2 h	Recovery period, NaCl 145 mmol/l, 1 h
Control subjects (n=5)	4.3 (3.8-4.9)	2.1† (1.8-2.5)	2.2 (1.8-2.4)
Healthy subjects (n=5)	3.3 (3.0-3.7)	12.7 (11.3-14.0)	6.0 (5.4-6.7)
Healthy subjects (n=3)	2.6 (2.2-3.8)	23.7 (16.0-28.2)	5.5 (3.6-8.3)
Cholecystectomized subjects (n=5)	4.6 (4.1-5.4)	15.5 (15.0-16.0)	5.9 (5.3-6.5)

*STC 15 mmol/l perfusion was used in the studies on cholecystectomized subjects and healthy subjects (n=5). In the studies on healthy subjects (n=3) STC 30 mmol/l was perfused.

†In the studies on control subjects NaCl (145 mmol/l) alone was perfused throughout.

Figures express geometric means ± SEM (in parentheses).

Table 7. Total volume of fluid (ml) in the duodenum during duodenal perfusion of NaCl (145 mmol/l) without or with STC. Caerulein/secretin stimulated studies using ICG as a biliary marker.

Perfusion fluid: Duration:	Equilibration period, NaCl 145 mmol/l, 1 h	Test period, STC 15 or 30 mmol/l,* 2 h	Recovery period, NaCl 145 mmol/l, 1 h
Control subjects (n=5)	141 (135-148)	154 (147-164)	143 (133-152)
Healthy subjects (n=5)	165 (141-197)	122 (109-139)	145 (122-172)
Healthy subjects (n=3)	124 (105-144)	126 (102-155)	124 (106-143)
Cholecystectomized subjects (n=5)	118 (109-127)	96 (84-109)	132 (112-156)

* Legend, see Table 6.

Figures express geometric means ± SEM (in parentheses).

Table 8. Total amount of trypsin (IU/10 min) in duodenal fluid during duodenal perfusion of NaCl (145 mmol/l) without or with STC. Caerulein/secretin stimulated studies using ICG as a biliary marker.

Perfusion fluid: Duration:	Equilibration period, NaCl 145 mmol/l, 1 h	Test period, STC 15 or 30 mmol/l*, 2 h	Recovery period, NaCl 145 mmol/l, 1 h
Control subjects (n=5)	3704 (2972-4624)	4071 (2972-5017)	3807 (3048-4583)
Healthy subjects (n=5)	3978 (3516-4506)	3102 (2786-3427)	3581 (3199-4010)
Healthy subjects (n=3)	2421 (1791-3277)	4457 (3105-6396)	2525 (1563-4235)
Cholecystectomized subjects (n=5)	2299 (1950-2716)	2306 (1919-2773)	2460 (2218-3442)

*Legend, see Table 6.

Figures express geometric means ± SEM (in parentheses).

Table 9. Total amount of bicarbonate (μmol/10 min) in the duodenal fluid during duodenal perfusion of NaCl (145 mmol/l) without or with STC. Caerulein/secretin stimulated studies using ICG as a biliary marker.

Perfusion fluid: Duration:	Equilibration period, NaCl 145 mmol/l, 1 h	Test period, STC 15 or 30 mmol/l*, 2 h	Recovery period, NaCl 145 mmol/l, 1 h
Control subjects (n=5)	5012 (4613-5441)	6086 (5861-6314)	5757 (5458-6067)
Healthy subjects (n=5)	6886 (5613-8450)	5093 (4198-6176)	6081 (5129-7205)
Healthy subjects (n=3)	3319 (2415-4562)	2832 (2118-3785)	2328 (1795-3022)
Cholecystectomized subjects (n=5)	3542 (3126-3999)	3191 (2838-3899)	3540 (2218-5654)

*Legend, see Table 6.

Figures express geometric means ± SEM (in parentheses).

Table 10. pH of duodenal fluid during duodenal perfusion of NaCl (145 mmol/l) without or with STC. Caerulein/secretin stimulated studies using ICG as a biliary marker.

Perfusion fluid: Duration:	Equilibration period, NaCl 145 mmol/l, 1 h	Test period, STC 15 or 30 mmol/l*, 2 h	Recovery period, NaCl 145 mmol/l, 1 h
Control subjects (n=5)	8.02 (7.95-8.09)	7.94 (7.91-7.98)	8.10 (8.04-8.17)
Healthy subjects (n=5)	7.90 (7.70-8.11)	7.99 (7.84-8.15)	7.98 (7.84-8.13)
Healthy subjects (n=3)	8.09 (8.06-8.12)	8.11 (8.02-8.20)	8.00 (7.91-8.09)
Cholecystectomized subjects (n=5)	7.98 (7.96-8.00)	8.08 (8.01-8.11)	8.15 (8.11-8.18)

*Legend, see Table 6.

Figures express geometric means ± SEM (in parentheses).

and 5.9 mmol/l (5.3-6.5) during the recovery period afterwards (Table 6).

Total volume of fluid in the duodenum did not significantly change during these studies (Table 9).

No significant changes in trypsin or bicarbonate secretion were observed in these studies nor were there any changes in duodenal pH (Tables 8 to 10).

4.4 DISCUSSION

It has been demonstrated in these studies that during duodenal perfusion with the bile salt STC in man, the biliary excretion was suppressed.

The biliary suppression was observed both in the basal state and when the biliary excretion was stimulated by a continuous parenteral infusion of caerulein and secretin. However, when the background stimulation by caerulein was increased towards the levels of the cholecystokinetic stimulation expected after a fatty meal (60), or the duodenum was perfused with a mixture of EAA known to have a submaximal cholecystokinetic effect (138), the inhibitory effect on the biliary excretion was abolished, at least when the total bile salt concentration was kept within the limits which can be expected postprandially.

When these studies were repeated under the same experimental circumstances in cholecystectomised subjects, the inhibiting effect during STC perfusion was quantitatively less than in the patients with intact gall bladders. This suggests that the suppressive effect of STC on the biliary excretion in normal subjects was due in part to an increase in gall bladder storage. This is supported by the fact that in the studies carried out with ICG as a biliary marker, blood levels of ICG remained stable during the bile salt perfusion, which suggests that the hepatic uptake of ICG was not affected.

The inhibition of the biliary markers was observed as early as 10 to 20 min after the beginning of the STC perfusion, which makes it unlikely that it was caused by a direct action on the hepatic secretion by absorbed STC passing through the liver. Bile salts, including STC, are reported to act as choleretics in man (136, 188), and indeed the gradual increase in bilirubin secretion after the initial inhibition in the prolonged (4 h) STC perfusion may reflect this.

Current views on the regulation of biliary excretion stress the possible role of the sphincter of Oddi in promoting gall bladder storage (19, 135). Indeed, cyclical changes in the sphincter of Oddi tone have been recorded (135), and related to other events during fasting, such as the interdigestive migrating myoelectrical activity of the gut (96, 296). However, the inhibitory effect of STC seems to be exerted predominantly on the gall bladder, and does not seem to influence pancreatic secretion, as would be expected if its effect was mediated via increased tone of the sphincter of Oddi.

Bile salts (125, 161, 410) and bile (284, 286, 287) have been reported by several workers to stimulate pancreatic secretion in man and animals, and this might be due to secretin release, induced by the bile salts (153, 154). Others have, however, reported inhibitory effects by bile salts on pancreatic secretion (239, 243). Species difference (153, 161) and different effects of different bile salts (125, 154, 410) may explain the discrepancy found between these studies. It has however repeatedly been shown by the studies presented here that STC, placed in the duodenum, does not suppress pancreatic enzyme secretion.

The concentration of total bile salts in the duodenum in these studies was well within the range of total bile salt concentration which has been reported after a meal (115, 116). However, perfusion of the duodenum with STC alone is not physiological. This particular bile salt was selected since it had previously been used to study basal and stimulated biliary and pancreatic secretion in man (239). In these studies, by Malagelada and co-workers, basal pancreatic secretion remained unchanged during STC perfusion, as stressed by the authors, but bilirubin secretion was reduced, although this seems not to have been appreciated (239). Therefore, there appears to be an agreement on the effects of STC on basal biliary and pancreatic secretion.

Malagelada *et al.* (239) did not use i.v. cholecystokinin or cholecystokinin analogue as a background stimulation in their studies, except at a high dose and in combination with intraluminal stimulation with amino acids and oleic acids. STC did not reduce bile secretion stimulated by this combined background stimulus, which certainly was supraphysiological (239, 240, 243). This is in keeping with the lack of effect of STC when large doses of caerulein were infused in the present studies.

The inhibition of biliary excretion of ICG by STC (15 mmol/l) in the duodenum was overcome by increasing the caerulein stimulus, and at the highest caerulein dose level studied (20 ng $kg^{-1}h^{-1}$), a dose known to contract the gall bladder to a similar extent as a fatty meal (60), prompt gall bladder emptying was observed in spite of STC perfusion.

The mechanism by which bile salts in the duodenum may promote biliary inhibition remains obscure. Motility and secretory functions of the biliary tract and the pancreas are probably regulated by both hormonal and neural factors, evoked mainly by stimuli from inside the gastrointestinal tract (136, 357). Although cholecystokinin and secretin may mediate enterobiliary and enteropancreatic responses, and certainly infusion of pure peptides are powerful stimuli, increases in cholecystokinin and secretin-like immunoreactivity in the blood after luminal stimulation have not yet been proven to be biologically adequate to account for the secretory responses to meals (342, 398, 413). Furthermore, the apparently selective inhibitory effect of STC on the biliary system demonstrated here would be unlikely to result from suppression of secretion of either of these hormones. Exclusively neural pathways for enterobiliary and enteropancreatic reflexes have not been defined though a rich autonomic innervation is evident and secretory functions of these organs can be stimulated or inhibited by this system (136, 357).

Of the gastrointestinal hormones measured during STC perfusion, only motilin showed changes coincident with suppression of the biliary output. Motilin enhances gastric emptying (73), activates duodenal motility (180), and increases gall bladder pressure (8, 180). Suppression of plasma motilin concentration may therefore be expected to facilitate gall bladder relaxation and promote gall bladder storage of bile. Motilin levels rise modestly and transiently in man during duodenal acidification (262) and standard hospital meals (72, 74, 77, 262, 321), but may also be reduced below basal levels postprandially (72, 221, 322). Duodenal alkalinisation is said to suppress motilin in plasma (262). However, luminal bile salt concentrations have not been correlated with plasma motilin in these studies so that the postprandial significance of STC suppression of motilin concentration to the postprandial events in the duodenum is not clear.

Domschke *et al.* (102) have reported that bile instilled in the duodenum released motilin in man and that taurocholate (15 mmol/l) released motilin from the human duodenal mucosa *in vitro*. Somatostatin

suppressed the motilin release induced by taurocholate (102), suggesting that the release of motilin was not due to unspecific cellular damage caused by the bile salt. These contradictory results are difficult to interpret. Contradictory results on motilin release from different laboratories have been suggested to be due to species differences and different assay techniques (72, 77, 130, 221, 321).

The existence of a hitherto unknown inhibitory hormone or hormones, which were able to antagonise the actions of CCK on the gall bladder, the biliary tract and the pancreas, have been speculated for a long time, and although the information is still scanty there is an increasing support for this hypothesis (218, 325). The inhibition of the action of circulating caerulein on the gall bladder, reported here, might have been mediated through such an inhibitory factor released into the blood by the bile salts in the duodenum.

The short-lived or phasic trypsin secretory response to EAA during STC perfusion was also observed during control studies with EAA perfusion alone. This pattern of enzyme response to EAA has been observed before and attributed to a "wash-out" of stored enzymes (139, 239), or to a stimulation of glucagon release by absorbed EAA and a secondary inhibition of enzyme secretion (95). Although frequently referred to, the "wash-out" effect has actually never been proven to exist (412), and in the studies presented here it is unlikely to explain the phasic enzyme response to EAA in the studies receiving the prolonged exogenous caerulein/secretin stimulation, which would have depleted enzyme stores. It should also be pointed out in these studies that the trypsin secretion during EAA/STC and EAA perfusion, superimposed on caerulein/secretin stimulation, did never fall below the plateau obtained by caerulein and secretin, which suggests that the mechanism of EAA and caerulein/secretin-induced secretion is different.

Although STC had no definite effect on pancreatic trypsin secretion, nor any effect on basal bicarbonate secretion, a modest increase in caerulein/secretin stimulated bicarbonate output was observed. Other workers have shown that human bile (284) or pure bile salts mixtures, simulating the composition of human bile (154), may increase bicarbonate secretion and plasma levels of secretin immumoreactivity and, in the cat, where STC is the major bile salt, an effect on bicarbonate secretion was observed (153). Although the results presented here do not support the idea that STC on its own stimulates bicarbonate secretion in man, it may act additively with

the caerulein/secretin stimulated bicarbonate secretion, a concept which has support from previous workers (125, 161, 410).

4.5 SUMMARY

(1) A standard duodenal perfusion technique was used to study the effects of luminally perfused sodium taurocholate on basal and caerulein/secretin stimulated biliary and pancreatic secretion and GIH release in man.

(2) During duodenal perfusion with STC alone, using bilirubin as a biliary marker, both basal and caerulein/secretin stimulated bilirubin secretion were suppressed. A successive perfusion with a mixture of the bile salt and essential amino acids in combination, overcame the biliary suppression and biliary secretion rose above basal levels. A further increase in bilirubin secretion was not observed in a subsequent perfusion with essential amino acids alone in these studies.

(3) In studies using ICG as a biliary marker, and an increasing luminal concentration of STC, results indicated that the biliary excretion of ICG was suppressed in healthy subjects with gall bladders. In cholecystectomised subjects, the change in ICG excretion was quantitatively less than in those with intact gall bladders.

(4) No inhibitory effect on basal or caerulein/secretin stimulated trypsin secretion was observed during the STC perfusion: basal trypsin secretion was in fact slightly increased during a prolonged (4 h) perfusion of the bile salt, and caerulein/secretin stimulated trypsin secretion rose during perfusion of STC of the highest concentration (30 mmol/l).

(5) During STC perfusion, basal bicarbonate secretion remained unchanged but caerulein/secretin stimulated bicarbonate secretion was slightly increased.

(6) Plasma levels of PP, GIP and gastrin did not change significantly during duodenal perfusion with STC, but plasma levels of motilin were suppressed.

(7) These results support the view that STC in the duodenum may activate a mechanism which selectively suppresses biliary excretion in man. This is probably due in part to relaxation of the gall bladder and an increase in gall bladder storage of bile. Motilin in plasma may be involved in this mechanism.

CHAPTER V

EFFECTS OF BOVINE PANCREATIC POLYPEPTIDE ON BILIARY AND PANCREATIC SECRETION IN MAN

5.1 INTRODUCTION

Pancreatic polypeptide (PP) (mol. wt. 4200) is a straight linear polypeptide of 36 amino acid residues, which has been isolated and sequenced from many mammalian species, including chickens, pigs, cattle and man (231). It is found to differ only by 1 or 2 amino acid residues in these species (Fig. 37) (231).

In man PP is normally secreted only from the pancreas, the PP-producing cells being located principally in the peripheral area of the pancreatic islets but are also found scattered in the exocrine parenchyma (3, 217, 298). After a meal the blood levels of PP rise substantially (3, 4, 122, 144, 329, 338, 377, 388). The particular nutrients in the duodenum known to stimulate PP release are the same as are responsible for the cholecystokinetic effect on the exocrine pancreas and the biliary tract, *i.e.* certain amino acids and hydrolysed fat (234, 342). The mechanism by which PP is released into the blood by nutrients is not clear, but it seems to involve the vagal cholinergic nervous system, as the release of PP is stimulated by sham feeding (341,378) and diminished or abolished by truncal vagotomy (338, 340, 377) and parenteral administration of atropine (117, 339, 377). PP release, induced by insulin hypoglycaemia, is totally abolished by vagotomy (339). Nevertheless, circulating peptides may add to the response (6) as CCK, administered parenterally, induces PP release, although this seems also to be influenced by the cholinergic tone (149, 233, 257). Like CCK, the biologically active PP may be derived from larger inactive precursors, and these may recently have been identified in the PP producing cell (343).

The physiological role of PP, if any, is not known. Before the following studies were carried out, studies with pharmacological doses in dogs had shown that BPP inhibits pancreatic secretion, relaxed the gall bladder and increased the tone of the common bile duct (231). Pharmacological studies in man had also indicated that i.v. infusion of BPP might reduce biliary and pancreatic secretion (7, 145).

The present investigation was carried out to study the effects of parenteral infusion of BPP on biliary and pancreatic secretion in man at plasma levels of PP which might be expected after a meal. Studies

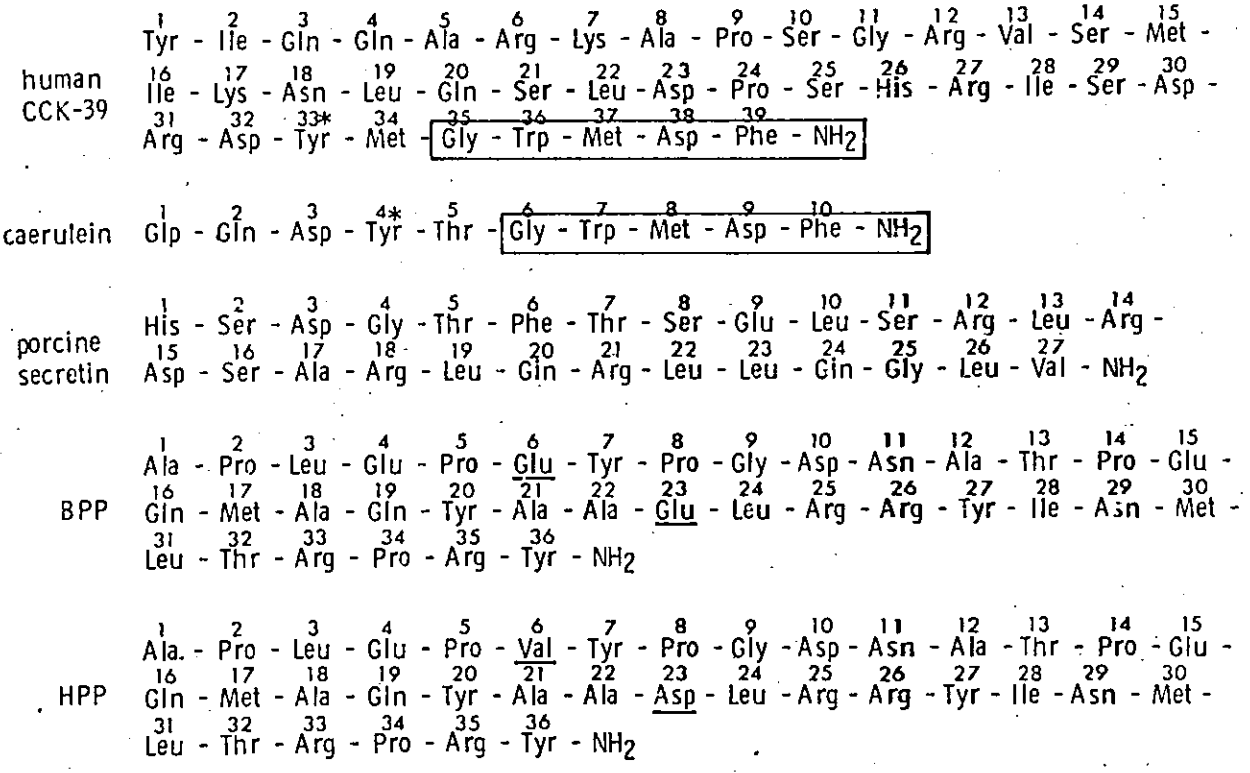


Fig. 37. The aminoacid sequence of human CCK-39, caerulein, porcine secretin, BPP and HPP. *A sulphated group.

were carried out on healthy subjects with gall bladders and on cholecystectomised subjects, as PP was expected also to have an effect on the sphincter of Oddi. Plasma levels of PP and biliary secretion into the duodenum were also monitored in normal subjects in the fasting state as correlation was thought possible between plasma PP and fasting gall bladder storage patterns. In all studies ICG was used as a biliary excretory marker.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

5.2.1.1 Effects of the procedure of intubation on plasma levels of HPP. The possible effects (vagal) of the intubation procedure on plasma levels of HPP were studied in 13 individuals. Plasma HPP was measured immediately before, during and immediately after the placement of the perfusion tube in the duodenum.

5.2.1.2 Fasting gall bladder storage patterns and HPP. Studies on the relationship between fasting gall bladder storage patterns and HPP were carried out in 5 normal subjects (mean age 27 years, range 21-34). Biliary excretion of ICG, serum levels of ICG and plasma levels of HPP were monitored for either 5 h (2 subjects) or 8 h (3 subjects). At the end of each study, 5 µg of caerulein was infused for 40 min in an attempt to obtain a full contraction of the gall bladder and maximal recovery of ICG.

5.2.1.3 Effects of BPP on biliary and pancreatic secretion.
(i) Healthy subjects with gall bladder. The effect of BPP was studied on caerulein and secretin stimulated biliary and pancreatic secretion in 6 healthy subjects (mean age 24 years, range 21-30). Caerulein and secretin were administered by a continuous i.v. infusion for 6 h at the doses of 10 ng $kg^{-1}h^{-1}$ and 0.1 CU $kg^{-1}h^{-1}$, respectively. Between the fourth and fifth hours of the study, an i.v. infusion of BPP (570 ± 28 ng $kg^{-1}h^{-1}$) was superimposed on this background hormone stimulation. Duodenal recovery of ICG, total bile salts and trypsin were monitored throughout the studies to assess the effects of these peptides in combination on the biliary and pancreatic secretion. ICG was measured in serum. The dose of BPP infused in this study and the study on cholecystectomised subjects, described in the next Section (*5.2.1.3.ii*) was determined by measuring BPP in the infusion fluid from the end of the infusion catheter.

(ii) Cholecystectomised subjects. The effect of BPP on basal biliary and pancreatic excretion was studied in 6 cholecystectomised subjects (4 females, 2 males, mean age 51 years, range 34-62). Biliary excretion of ICG, total bile salts and trypsin was monitored for 5 h. Between the third and fourth hour, a parenteral infusion of BPP (1443 ± 200 ng $kg^{-1}h^{-1}$, so from the end of the infusion catheter) was carried out. ICG was measured in serum.

5.2.2 Chemicals and instruments

BPP was obtained from Dr. Ronald E. Chance, Lilly Research Laboratories, Eli Lilly Co. Ltd., Indianapolis, Indiana, U.S.A. For information on secretin, caerulein, ICG and human albumin, see *Sections 4.2.4* and *3.2.1*.

5.2.3 Preparation of infusion and perfusion solutions

For detailed description of the preparation of caerulein, secretin and ICG infusions, see *Section 4.2.5*. BPP was prepared immediately before use in sterile 145 mmol/l saline containing human serum albumin (Lister Institute, Elstree, Herts.). ICG was infused in these studies at the dose of 0.2 mg $kg^{-1}h^{-1}$. The duodenal perfusion solution (145 mmol/l NaCl) contained PEG 4000 (5 g/l).

5.2.4 Perfusion technique

The perfusion technique has been described in detail before, see *Sections 2.2.9* and *2.2.10*.

Jejunal re-infusion of aspirated duodenal fluid was not successfully carried out in the studies described in this chapter.

5.2.5 Sample processing

Blood samples for hormone measurements were collected at 10-30 min intervals into tubes containing aprotinin (Trasylol®, Bayer Ltd., Germany) (400 Kallikrein Inactivator Units (KIU) per ml of blood) and heparin (10 units per ml of blood), centrifuged immediately (1500 *g* for 10 min) and the plasma stored at -20°C until analysed. Blood samples for measurement of ICG in serum were collected at 30 min intervals, centrifuged, and analysed within 8 h from collection (36). Duodenal aspirates were pooled for 10 min on ice, and stored at -20°C until analysed for PEG 4000, total bile salts and trypsin. ICG was measured on fresh samples (<8 h).

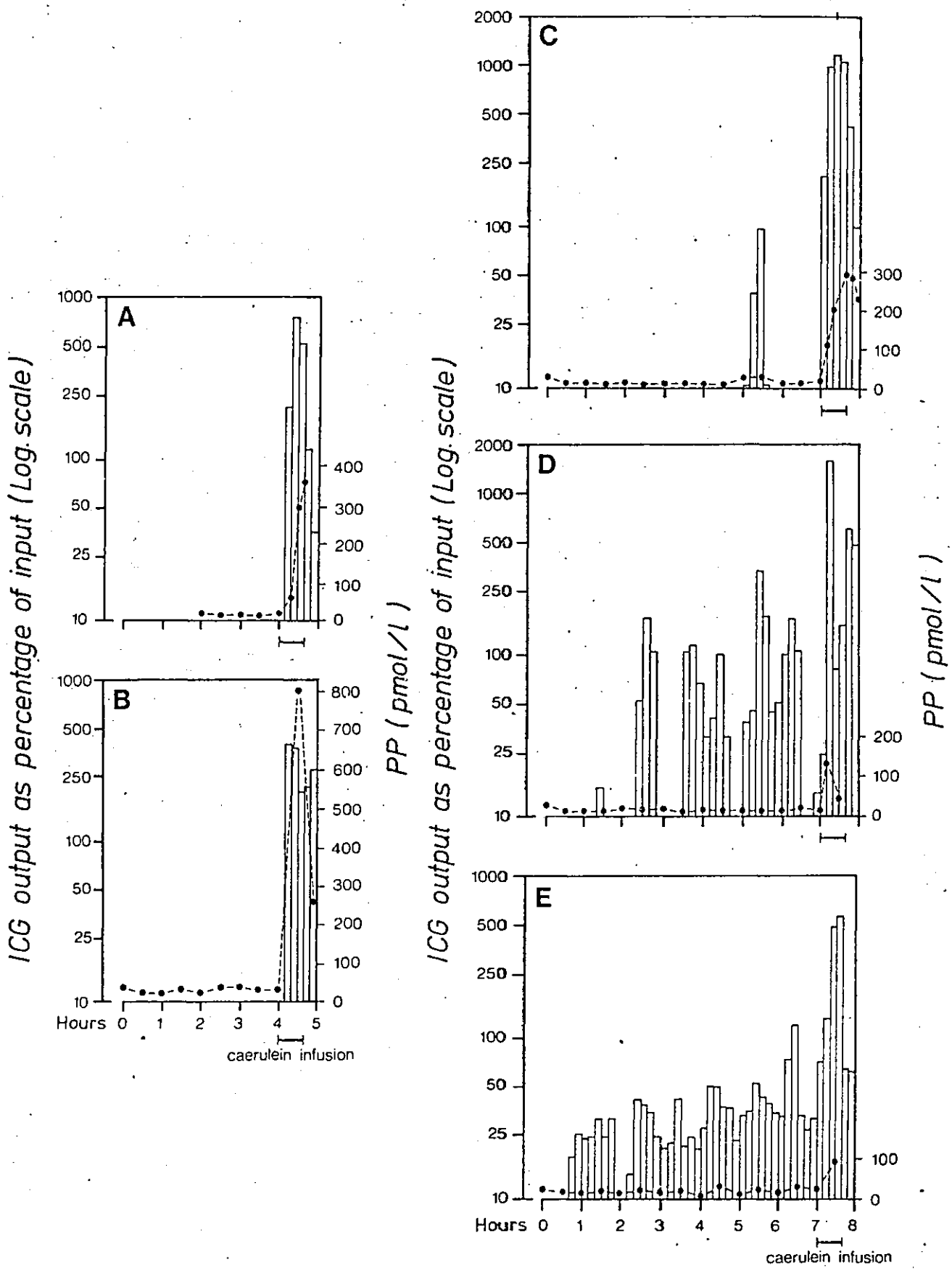


Fig. 38. Biliary output of ICG during either 5 h (A,B) or 8 h (C,D,E) of observation in five healthy fasting males. ICG output is expressed on log scale as percent of intravenous input every 10 min. Plasma levels of PP during the period of study are plotted on the graphs. During the first 40 min of the last hour of every study (bar), 5 µg of caerulein were infused intravenously to empty the gall bladder.

5.2.6 Analytical methods

For measurement of PEG 4000, see *Section 2.2.7*, and for measurements of total bile salts and trypsin, see *Section 4.2.8*. For measurement of ICG, see *Section 3.2.9* and (36). The radioimmunoassay of pancreatic polypeptide was carried out by Dr. T.E. Adrian, see *Section 4.2.8*.

5.2.7 Statistics

Duodenal excretion of ICG, total bile salts and trypsin were calculated by using standard equations for PEG 4000 recovery (138). Duodenal excretion of ICG was expressed as per cent of intravenous ICG input per 10 min. Plasma PP levels were expressed as pmol/l of endogenous and exogenous PP in plasma. Because the secretory data were not normally distributed but had an approximately log normal distribution, a log transformation was taken to normalise the data before statistical evaluation. The data were analysed by two-way analysis of variance (300).

5.3 RESULTS

5.3.1 Effects of duodenal intubation on plasma levels of HPP

Plasma levels of HPP were estimated at 23.7 pmol/l ± 2.9 (SEM, n = 13) immediately before the intubation; 17.3 pmol/l ± 1.2 (SEM) during the intubation; and 19.0 pmol/l ± 3.6 (SEM) immediately after the intubation. A significant difference was not found between these figures.

5.3.2 Fasting gall bladder storage patterns and HPP. Figure 38 shows the biliary excretion of ICG into the duodenum in 5 normal fasting subjects during 5 to 8 h of observation. Plasma levels of HPP during the observation period are plotted on the Figure. Patterns of very variable duodenal recovery of ICG were observed. In 3 subjects (Figs. 38 A,B,C) ICG was virtually not recovered from the duodenum during the whole observation period (4-7 h). In the other 2 subjects (Fig. 38 D,E) the excretion of ICG was fluctuating with even short periods (10-20 min) of complete storage. Prompt gall bladder emptying was achieved in all subjects in the last 40 min of the experiments in response to i.v. dose of caerulein (5 µg/40 min). This dose of caerulein induced an endogenous release of PP of 90-800 pmol/l (Fig. 38). Plasma levels of ICG remained at 574 ± 10 µg/l (SEM, n = 5) in these studies after a steady state had been reached (Fig. 39C).

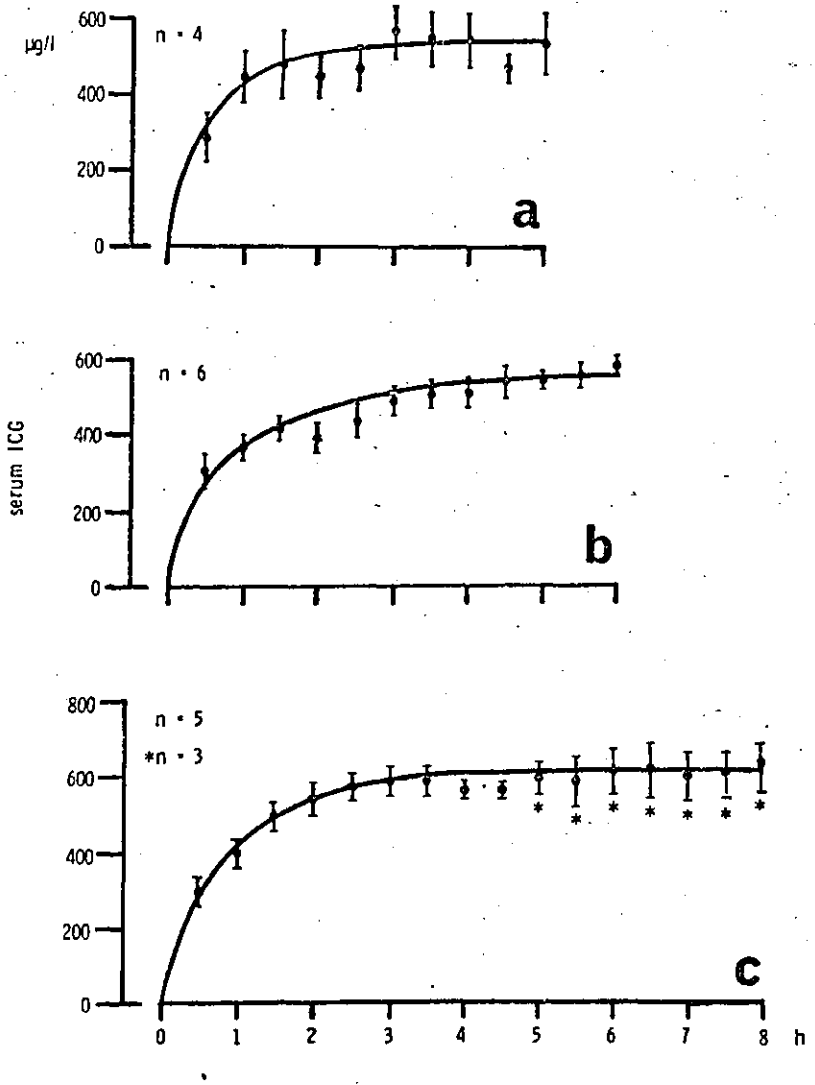


Fig. 39. Serum levels of ICG (µg/l) in: A, cholecystectomised subjects, see *Section 5.3.3.2*; B, studies on healthy subjects with gall bladders, see *Section 5.3.3.1*; C, studies on the fasting gall bladder storage patterns, see *Section 5.3.2*.

5.3.3 Effects of BPP on biliary and pancreatic excretion in man

5.3.3.1 Healthy subjects with gall bladders. Figure 40 shows the biliary excretion of ICG stimulated by caerulein and secretin in healthy subjects with gall bladders. The observation period between the second and sixth hours is shown, where i.v. infusion of BPP was superimposed on the caerulein/secretin stimulation between the fourth and fifth hour. On average, ICG excretion was 61% of i.v. infusion (range 51-76%) during the 2 h prior to the BPP infusion. During the BPP infusion the excretion of ICG fell to 42% (range 37-46%) (p = 0.02). Mean plasma levels of PP were 275 pmol/l (SEM 25.3, n = 6) at the plateau during that time, which is similar to plasma levels observed after a meal. Following the BPP infusion, ICG excretion returned slowly to the levels observed before the BPP infusion. Serum levels of ICG remained relatively stable throughout this study after a steady state had been reached (533 ± 13 µg/l, SEM, n = 6) (Fig. 39B).

Figure 40 also shows the secretion of bile salts and trypsin accompanying the changes in ICG excretion described before. Total bile salt secretion fell significantly during the infusion of BPP and rose significantly during the 1 h recovery period after BPP infusion (p <0.03). However, during the study there was a continuous gradual fall in bile salt secretion, indicating a decreased flux of bile salts through the liver, either due to depletion or misdistribution of the bile salt pool.

The average trypsin secretion fell during BPP infusion to 46% of the average secretion of trypsin during the 2 h prior to the BPP infusion (p <0.01) (Fig. 40), which suggested that PP either had a separate effect on the pancreatic acinar cells or that it promoted gall bladder storage by increasing the tone of the sphincter of Oddi and thereby reduced the pancreatic secretion into the duodenum as well.

5.3.3.2 Cholecystectomised subjects. Figure 41 shows the basal biliary excretion of ICG in cholecystectomised subjects during the second and fifth hours of observation. BPP was infused during the third to fourth hour. Prior to the BPP infusion, ICG had reached on average the 80% excretory level. During BPP infusion no reduction in ICG excretion was observed although plasma PP levels were 2-3 times higher than in the previous study. A gradual fall in bile salt secretion was observed in these subjects, but in contrast to the subjects with gall bladders, no increase in bile salt secretion was observed in the recovery period after the BPP infusion. Trypsin

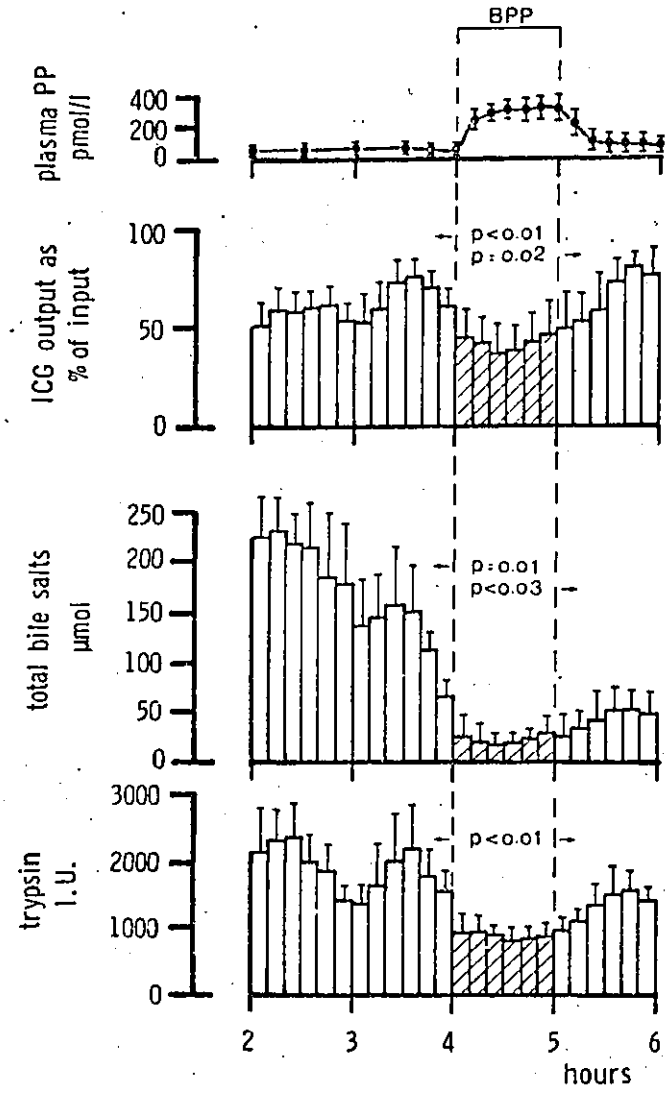


Fig. 40. Plasma levels of PP and duodenal recovery of ICG, total bile salts and trypsin in fasting normal subjects for 2 h before, 1 h during and 1 h after i.v. infusion of 40 ± 2 µg of BPP. The BPP infusion was superimposed on a background infusion of secretin (0.1 CU $kg^{-1}h^{-1}$) and caerulein (10 ng $kg^{-1}h^{-1}$). Results are shown as mean values + SEM (n = 6) for every 10 min period. P values indicate significance of changes of biliary and pancreatic output during the whole 1 h period of BPP infusion in comparison with the immediate 2 h period before BPP infusion ←, or the 1 h period following the BPP infusion →.

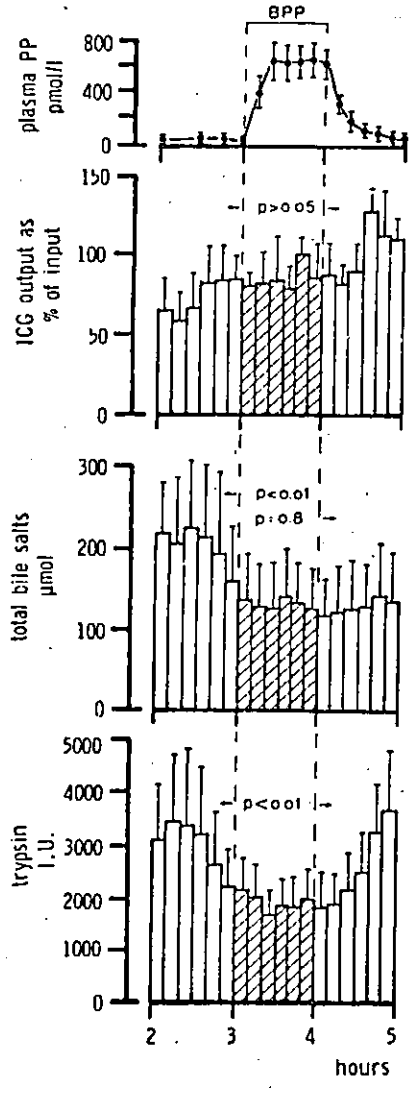


Fig. 41. Plasma levels of PP and the duodenal recovery of ICG, total bile salts and trypsin in fasting subjects who had undergone cholecystectomy. Results represent 1 h before, 1 h during and 1 h after i.v. infusion of 101 ± 14 µg of BPP. Results are shown as mean values +SEM (n = 6) for every 10 min period. P values, see legend to Fig. 40.

secretion, however, was significantly suppressed during the BPP infusion (p <0.01). Blood levels of ICG remained stable in these studies (517 ± 28 µg/l, SEM, n = 6) (Fig. 39A), as in the studies described before (Fig. 39A,B).

5.4 DISCUSSION

The procedure of duodenal intubation and the presence of a perfusion tube in the upper intestinal tract did not affect the plasma levels of PP in these studies, and it is therefore not likely to have interfered with the results of the effects of PP observed here.

The studies on biliary secretion in healthy subjects in the fasting state show that plasma levels of HPP remained at basal levels in spite of very fluctuating biliary excretion. This suggests that there is not a cause-effect relationship between plasma PP and basal biliary excretion, and that at least in the basal state biliary excretion is not regulated by PP.

However, in the studies on healthy subjects with gall bladders, caerulein/secretin stimulated biliary and pancreatic secretion was, during parenteral infusion of BPP, significantly reduced compared with the biliary and pancreatic secretion before and after the infusion. This occurred at plasma levels of PP similar to those measured in man after a protein-rich meal (4). Nonetheless, in cholecystectomised subjects, receiving 2- or 3-fold higher doses of BPP than the gall bladder subjects, no suppression of biliary secretion was observed, although pancreatic secretion was suppressed in these studies to a similar extent as in the studies before.

As an explanation for this, a promotion of gall bladder storage induced by BPP is suggested. There seemed to be no effect on the sphincter of Oddi, even at plasma PP levels 2- or 3-fold higher than expected after a meal. However, trypsin secretion was suppressed to a similar extent in both series of studies, which suggests that BPP had a separate effect on the pancreatic acinar cells or the pancreatic duct.

There are, however, some reservations to these interpretations. Although statistically significant, the suppression of biliary and pancreatic secretion in these studies is weak, and in fact, weaker than has been claimed by others carrying out human studies with BPP (7, 144). Different technique and different background stimulation makes comparison, however, difficult.

The data presented here do not support that the relationship between plasma PP and pancreatic trypsin secretion is dose-dependent, and this further weakens that a causal relationship exists. Animal

studies have also suggested that the relationship between plasma PP and pancreatic enzyme secretion was not dose-dependent at all doses, as Lin *et al.* (231) found no difference in suppression of protein secretion induced by BPP of 1 or 5 μg $kg^{-1}h^{-1}$ in dogs (231, 379).

Although PP may have a weak inhibitory effect on biliary and pancreatic secretion, it still remains uncertain whether this occurs under physiological circumstances and whether the inhibition has any biological importance.

Recent studies have shown that in spite of a rapid clearance of PP from the blood circulation (5), and apparently by the kidneys (44), plasma levels of PP remain high for hours after a meal (3, 329, 338, 388) for a reason as yet unknown. Although of weak effect, PP might therefore act as a long-term inhibitor of secretion in the postprandial state.

BPP was used in the present studies because human PP was not available for parenteral use. The full biological activity of PP is reported to reside in the *C*-terminal hexapeptide of the molecule (231), and BPP may therefore have the same biological activity as the human peptide, as they differ only by two amino acid residues, *i.e.* in positions 6 and 23 (Fig. 37).

Recently studies have been reported which show that PP has an inhibitory effect on basal and endogenously or exogenously stimulated pancreatic secretion in animals (231, 233, 379). These studies suggest that the inhibition is not due to suppression of secretin or CCK release (233, 234), but rather due to a direct effect on the secretory cells of the pancreas. It has been reported that PP did not have an effect on gall bladder muscle *in vitro* (302).

5.5 SUMMARY

(1) Parenteral administration of BPP to man in doses which simulated plasma levels of PP seen during a meal, suppressed caerulein/secretin stimulated biliary and pancreatic enzyme secretion in healthy subjects with gall bladders.

(2) In cholecystectomised subjects, receiving a PP infusion of doses 2- or 3-fold higher than in the healthy subjects described before, no effect on biliary excretion was observed, but pancreatic enzyme secretion was suppressed to a similar extent as before.

(3) No correlation was found between highly variable basal biliary excretion and basal plasma levels of PP, which remained steady at low levels.

(4) These studies in man suggest that BPP may have a weak inhibitory effect on biliary excretion, presumably by relaxing the gall bladder. They also suggest that BPP may have an inhibitory effect on pancreatic enzyme secretion, an effect which is probably direct on the acinar cells. Secretion of bile in the fasting state seems not to be regulated by plasma PP in man.

CHAPTER VI

THE EFFECTS OF JEJUNAL RE-INFUSION OF ASPIRATED DUODENAL FLUID ON BILIARY AND PANCREATIC SECRETION IN MAN

6.1 INTRODUCTION

During diversion of the bile-pancreatic juice from the upper intestinal tract, exocrine pancreatic secretion increases in rats (143, 177), pigs (176) and dogs (15). Re-infusion of the juice into the duodenum, or the jejunum, suppresses the pancreatic secretion in these animals. This inhibition has been attributed to luminal trypsin (143, 176, 177) exerting a negative feedback effect on the pancreas. However, it is not clear whether such a mechanism exists in man. Krawisz *et al.* (210) were unable to find any inhibitory effect on basal pancreatic secretion, but Ihse *et al.* (175) reported that in a patient with a complete obstruction of the common bile duct, due to biliary tract carcinoma, perfusion of the duodenum with trypsin, or the patient's own biliary-pancreatic juice, suppressed the pancreatic secretion. Recent studies in man by Osnes and Hanssen (285) also suggested that duodenal perfusion with pancreatic juice may inhibit pancreatic secretion.

The duodenal perfusion technique has been used extensively to measure biliary and pancreatic secretion in man. It is performed either with jejunal re-infusion of the aspirated fluid (29, 53), retaining a small volume for chemical analysis, or the simpler procedure of discarding the duodenal aspirate is adopted (94, 138, 144, 148, 239, 410, 414). Obviously, using the latter procedure, the loss of bile salts in the duodenal aspirate partially interrupts the enterohepatic circulation and may cause a reduction in hepatic bile salt secretion with consequent reduction in hepatic bile volume (103, 348).

The present study was designed to test whether re-infusion of duodenal aspirate, rich in bile and pancreatic juice, influenced biliary and pancreatic secretion, as measured by the duodenal perfusion technique in man. This was done by comparing the results of perfusion studies with and without jejunal re-infusion. To enrich the duodenal fluid with bile and pancreatic juice, a background stimulation with caerulein and secretin was given throughout both types of studies. The dose of these peptides was adjusted so that they might simulate the biliary and pancreatic responses seen after a meal (60, 417, 418). Similar studies were also carried out in the basal state,

as the mechanism of basal secretion may differ from that of postprandial secretion (357).

6.2 MATERIALS AND METHODS

6.2.1 Experimental protocols

Six volunteers (ages 21-24) each underwent two studies in a random order (series A and B, paired studies), where the secretion of bilirubin, bile salts, trypsin and bicarbonate were monitored for 6 h. In one series of studies (series A), all aspirated duodenal fluid was discarded whilst in series B duodenal fluid was re-infused immediately into the jejunum after aspiration (10 min sampling intervals). Caerulein (2.5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$) were given i.v. throughout these studies as a background stimulation of biliary and pancreatic secretion.

In 6 different subjects (ages 20-25 years), biliary and pancreatic secretion was monitored for 6 h in each individual with (series C) and without (series D) re-infusion of aspirated fluid, using exactly the same experimental design as described before, except that the background stimulation with caerulein and secretin was omitted (basal secretion). In all series (A,B,C,D) 1 h was allowed for equilibration (see *Section 2.2*) before the monitoring of biliary and pancreatic secretion was started.

6.2.2 Instruments and the perfusion technique

The aspiration and perfusion pumps have been described before and the duodenal perfusion technique with gastric aspiration (see *Sections 2.2.9* to *2.2.12*). The perfusion port for the recovery markers was placed opposite the ampulla of Vater and the aspiration holes 15 to 20 cm more distal in the duodenum (15-20 cm duodenal perfusion segment). The jejunal re-infusion segment reached 20 cm into the jejunum. The duodenum was perfused with an iso-osmolar (290 mOsm kg^{-1}) solution of NaCl (145 mmol/l) (pH 6.3), which contained both PEG 4000 (5 g/l) and ^{14}C PEG 4000 (10 µCi/l) as inert volume markers. The perfusion flow rate was about 10 ml/min. The preparation of the infusion solutions of caerulein and secretin have been described elsewhere (see *Section 4.2.5*).

6.2.3 Sample processing and analytical methods

Duodenal aspirates were collected on ice in 10 min samples and a 3 ml aliquot stored in a dark-brown bottle at -20°C until analysed for phenol red. One ml portion of the duodenal fluid was collected separately into a narrow airtight container for analysis of bicarbonate. It was sealed with 1 cm layer of liquid paraffin prior to storage. Gastric aspirates were collected on ice and 10 min pooled samples stored frozen (-20°C) until analysed for phenol red and ^{14}C PEG 4000. Total bile salts in the duodenal fluid were measured by a Sterognost-3α kit (369, 370). The intra- and inter-assay reproducibility of the assay were <10% and <15%, respectively. The assays for measuring ^{14}C PEG 4000 (246), phenol red (23), trypsin (36), bilirubin (305) and bicarbonate (39) have been described before, as have the equations for calculations of total secretory outputs (138).

6.2.4 Statistical evaluation and presentation of data

Statistically the data were evaluated by Student's *t*-test for paired and unpaired data as appropriate on log values (the data were approximately log normally distributed). Before presentation as mean ± SEM in the text, tables and figures, the data were transformed to a linear scale by taking anti-log. Therefore the mean values represent geometric means (300) with the exception of the slope constants ($\bar{\alpha}$) of the least square fit regression lines, where robust analysis was attempted and bisquare means were calculated (see *Appendix V*). Data were also evaluated for trends and changes in trends by least squares fit regression analysis (300), cusum-plot (409), and empirical cumulative distribution function plot (ECDF-plot) (267).

6.3 RESULTS

6.3.1 Estimation of stimulated secretion with and without re-infusion of the duodenal aspirate into the jejunum

6.3.1.1 Bile salt secretion. In the series carried out without re-infusion of the duodenal aspirate (series A), the secretion of total bile salts fell gradually during the observation period (6 h) from 2.6 mmol/h (2.0-3.3, SEM) (2nd h) to 1.4 mmol/h (0.9-1.9) (7th h) (p <0.05) (Fig. 42). The average loss of bile salts in these studies was 3.56 g (2.92-4.35, SEM) (Table 11). When carried out with

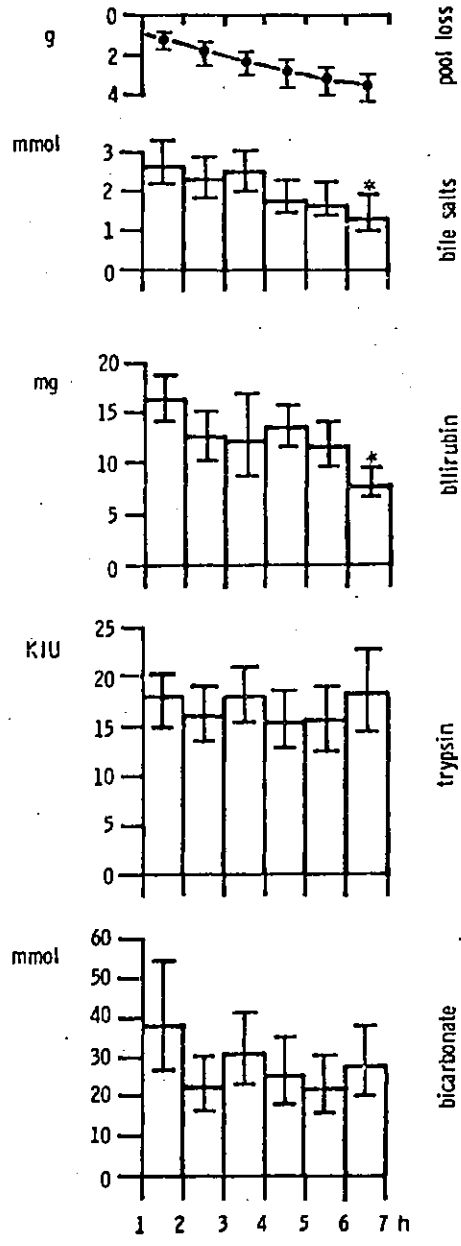


Fig. 42. The cumulative amount of bile salts discarded (top panel), and the stimulated secretion of total bile salts, bilirubin, trypsin and bicarbonate into the duodenum during 6 h (2nd to 7th) of observation in 6 individuals, where the aspirated duodenal fluid was not re-infused into the jejunum (series A). A background stimulation of caerulein (2.5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$) was used throughout these studies. Geometric mean ± SEM (bars). *$p < 0.05$

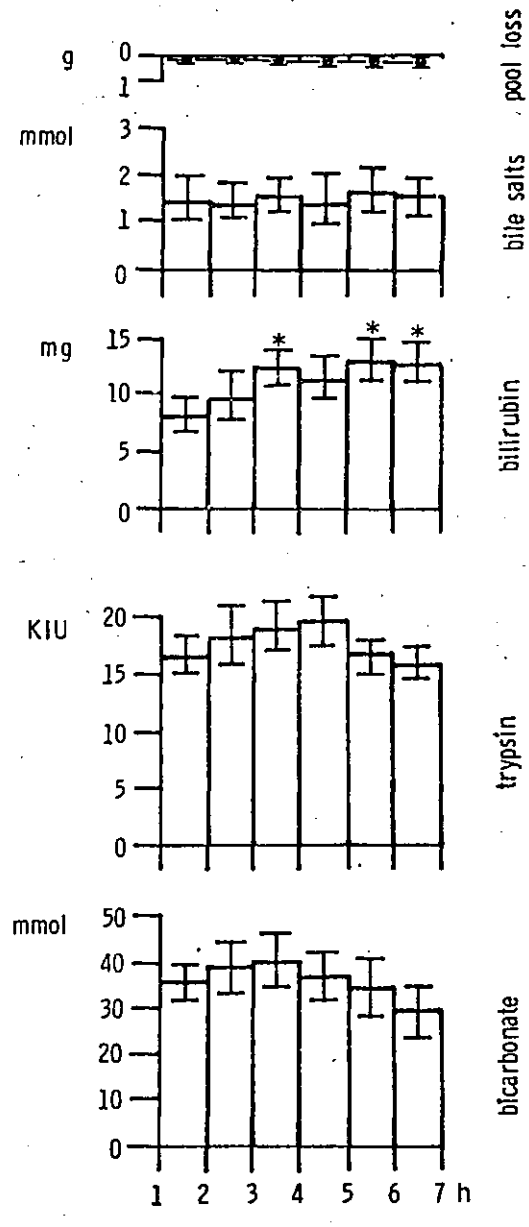


Fig. 43. The cumulative amount of bile salts retained for analysis, and the stimulated secretion of total bile salts, bilirubin, trypsin and bicarbonate into the duodenum during 6 h of observation in 6 individuals, where the aspirated duodenal fluid was re-infused into the jejunum (series B). See further legend to Fig. 42. *$p < 0.05$

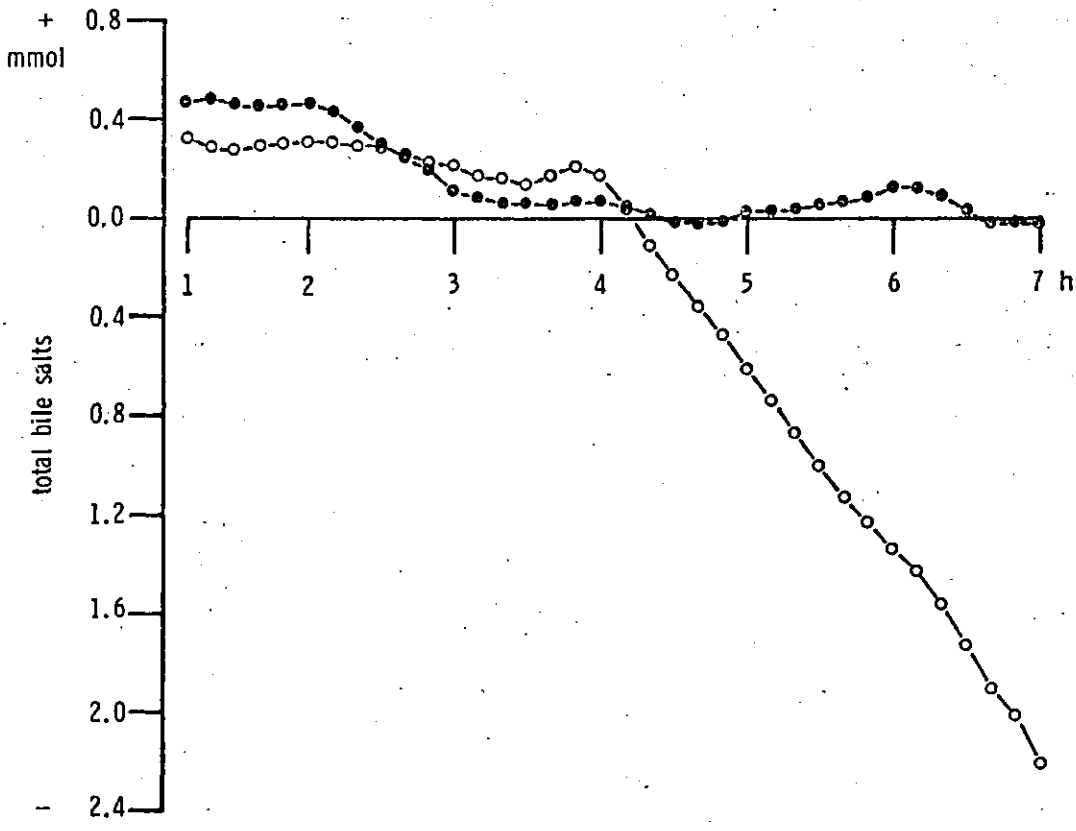


Fig. 44. A cusum-plot of the average secretory output (10 min intervals) of total bile salts from the studies presented in Figs. 42 and 43.
o——o, without jejunal re-infusion (series A);
●——●, with jejunal re-infusion (series B).
See further legends to Figs. 42 and 43.

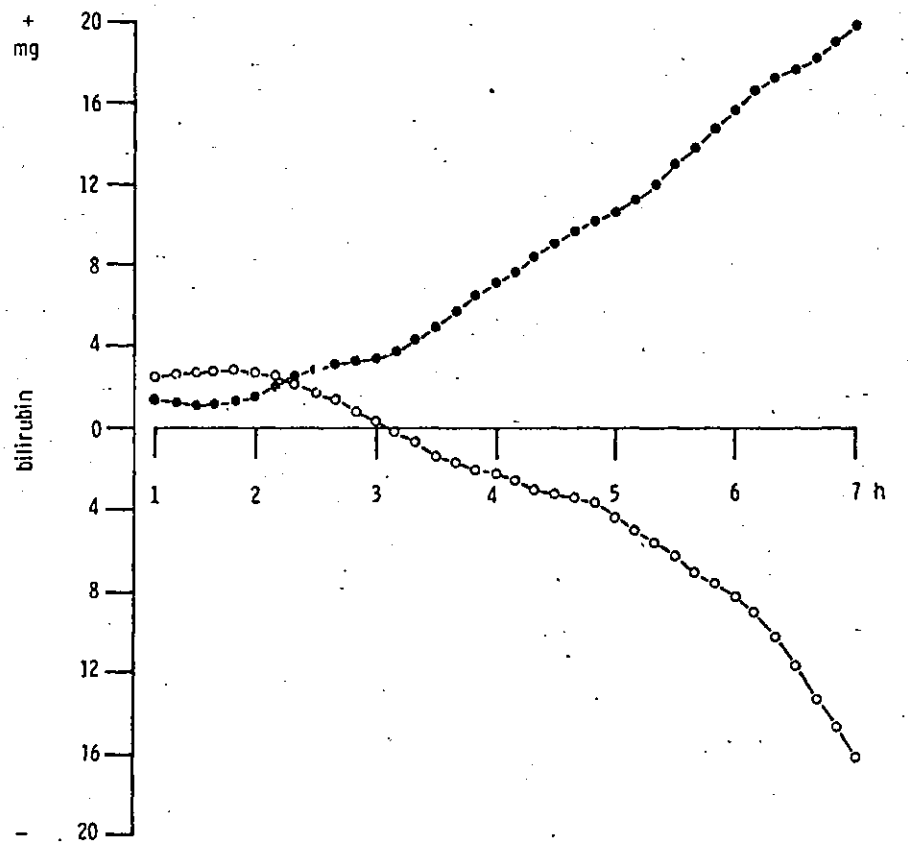


Fig. 45. A cusum-plot of the data on stimulated bilirubin secretion (10 min intervals) from the studies presented in Figs. 42 and 43.
o——o, without jejunal re-infusion (series A);
●——●, with jejunal re-infusion (series B).
See further legends to Figs. 42 and 43.

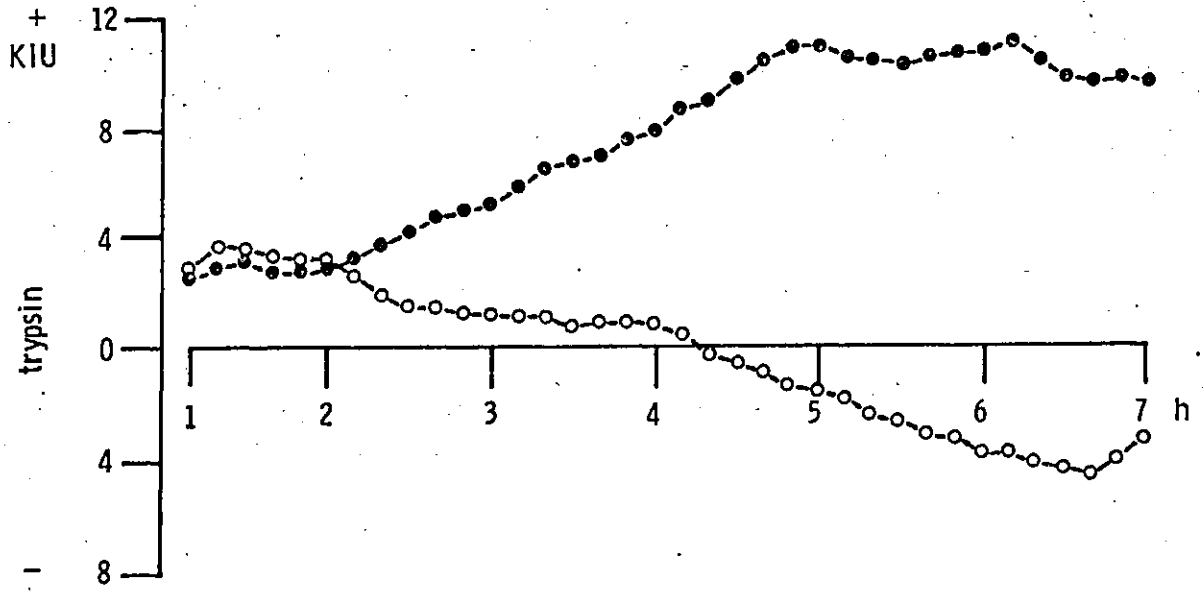


Fig. 46. A cusum-plot of the data on stimulated trypsin secretion from the studies presented in Figs. 42 and 43.

o——o, without jejunal re-infusion (series A);

●——●, with jejunal re-infusion (series B).

See further legends to Figs. 42 and 43.

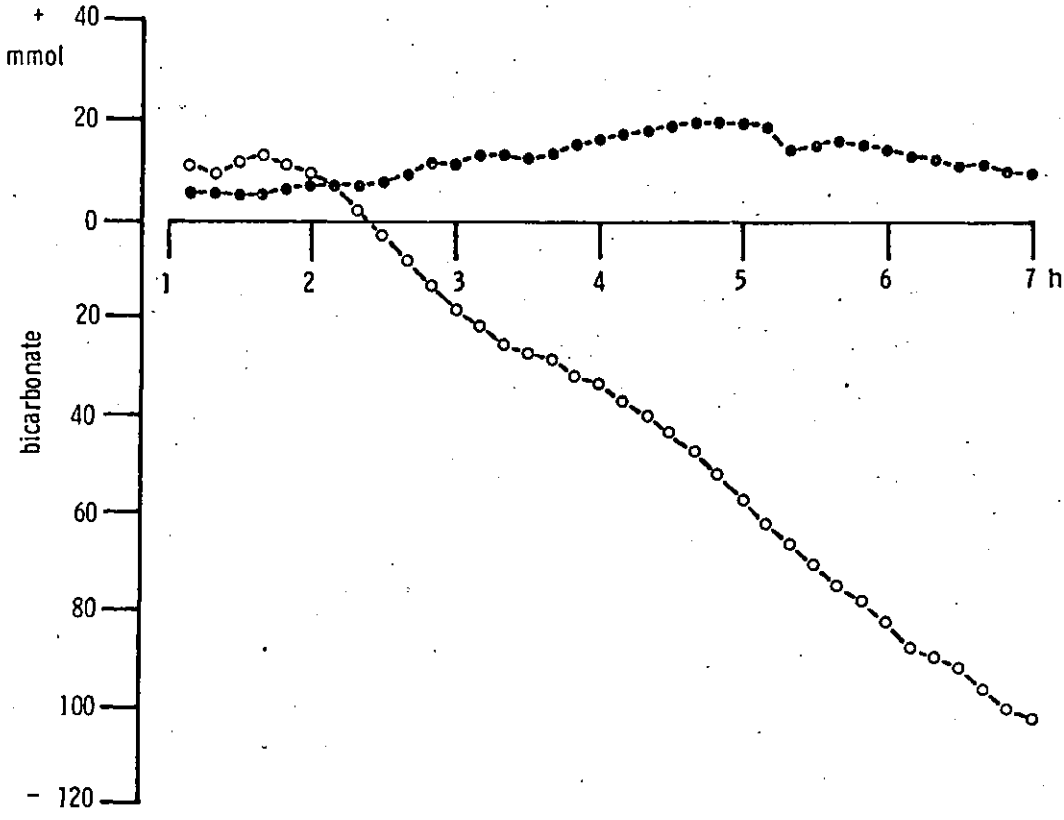


Fig. 47. A cusum-plot of the data on stimulated bicarbonate secretion from the studies presented in Figs. 42 and 43.
○——○, without jejunal re-infusion (series A);
●——●, with jejunal re-infusion (series B).
See further legends to Figs. 42 and 43.

TABLE 11: STIMULATED SECRETION WITHOUT REINFUSION:*

No.	Bile salts discarded (g)	Bilirubin discarded (mg)	Trypsin discarded (KIU)	Aspiration efficiency (%)	Bile salts α	Bilirubin α	Trypsin α	Bicarbonate α
1	1.67	60.81	30.00	82.3	-0.90	-1.05	-1.05	-8.17
2	6.80	153.72	79.36	62.2	-5.40	-2.33	-1.19	+3.68
3	3.26	92.76	56.84	69.3	-3.15	-4.52	-1.28	-1.81
4	5.22	83.80	67.92	48.5	-6.50	-2.60	+0.61	+6.00
5	3.64	151.25	43.29	34.0	+4.80	-2.52	+3.32	-15.10
6	2.91	121.32	27.49	38.4	-5.10	-6.42	+1.77	-32.33
$\bar{x}$	3.56	104.91	47.10	53.1	-4.056	-2.717	+0.242	-4.983
-SEM	2.92	90.41	39.45	46.1	-4.604	-3.076	-0.125	-7.436
+SEM	4.35	121.73	56.23	61.1	-3.508	-2.358	+0.609	-2.530

* For further details, see legend to Fig. 42.

TABLE 12. STIMULATED SECRETION WITH REINFUSION:*

No.	Bile salts reinfused (g)	Bilirubin reinfused (mg)	Trypsin reinfused (KIU)	Aspiration efficiency (%)	Bile salts α	Bilirubin α	Trypsin α	Bicarbonate α
1	4.83	139.29	89.78	78.5	+2.95	+4.57	-2.57	+5.78
2	5.74	66.09	64.19	93.0	-5.10	-0.10	-2.29	-6.98
3	1.45	63.35	74.22	64.7	-0.40	+4.50	+1.01	-3.95
4	5.38	82.08	98.82	94.1	+1.40	+4.90	0.00	-4.45
5	1.48	99.79	66.88	41.5	+0.95	-1.11	-4.60	-6.50
6	2.88	50.85	46.04	29.1	+1.10	+2.29	+1.84	-2.54
$\bar{x}$	3.12	79.00	71.12	61.4	-0.018	+2.947	-1.077	-4.642
-SEM	2.41	68.17	63.68	50.6	-0.536	+2.471	-1.552	-5.108
+SEM	4.04	91.54	79.43	74.5	+0.500	+3.423	-0.602	-4.176

* For further details, see legend to Fig. 43.

jejunal re-infusion of the duodenal aspirate (series B), the bile salt secretion remained stable (1.5 mmol/h, 1.1-2.0, 2nd h, *versus* 1.6 mmol/1, 1.2-2.0, SEM, 7th h, $p < 0.4$) (Fig. 43). The average total amount of bile salts re-infused into the duodenum in these studies was 3.12 g (2.41-4.04, SEM) (Table 12). The amount of bile salts retained for analysis in these studies was 0.22 g (0.17-0.28, SEM) (Fig. 43), or on average 6.6% of the total amount aspirated.

Tables 11 and 12 show, as an estimate of the trend of the bile salt secretion, the slope constants ($\bar{\alpha}$) of the least squares fit regression lines based on calculations of the 6 h secretory data. In the studies without re-infusion, bile salt secretion fell in all individuals except one (Table 11), and was on average (bisquare mean) $\bar{\alpha} = -4.056$ (-3.508 to -4.604, SEM). In these same individuals, the slope constants were positive in 4 out of 6, when the aspirate was re-infused, and on average (bisquare mean) $\bar{\alpha} = -0.018$ (-0.536 to +0.500, SEM, $p < 0.01$) (Table 12).

The cusum-plot was used further to study the average trend of the bile salt secretory data. The average secretion during the first observation hour (2nd h) was used as a reference value. It was subtracted from each data point (the average 10 min output) in succession throughout the studies, and the remainder added algebraically to the previous sum (cumulative sum). Figure 44 shows the weak initial downward trend in bile salt secretion in the series with bile salt re-infusion (series B), and the small upward trend at the end of the study. In contrast, a sharp fall in bile salt secretion was observed by 4 h in the studies without re-infusion (series A), and this trend remained to the end of the study, and was even further increased during the final hour.

6.3.1.2 Bilirubin secretion. In the series without re-infusion of the aspirate (series A), bilirubin secretion fell gradually during the whole observation period (6 h), or from 16.4 mg/h (14.3-18.9, SEM, 2nd h) to 7.9 mg/h (6.5-9.7, SEM, 7th h, $p < 0.05$) (Fig. 42) (slope $\bar{\alpha} = -2.717$) (Table 11). However, in the series where the aspirate was re-infused (series B), an increase in bilirubin secretion from 8.1 mg/h (6.6-9.9, SEM, 2nd h) to 12.9 mg/h (11.2-14.9, SEM, 7th h, $p < 0.01$) (Fig. 43) (slope $\bar{\alpha} = +2.947$) (Table 12) was observed. Figure 45 shows the downward and upward trends, respectively, in bilirubin secretion in the series A and B, expressed by a cusum-plot. The cusum-plot shows that the downward trend did increase in series B (without re-infusion) during the study, especially by 2, 5 and 6 h, respectively.

6.3.1.3 Trypsin secretion. Estimated as hourly outputs, trypsin secretion remained stable in both series of studies, and the total amount of trypsin secreted was not different in the two series (Figs. 42 and 43). However, while there was an overall weak downward trend ($\bar{\alpha}$ = -1.077) in trypsin secretion in the series with re-infusion (series B), there was a slight overall positive trend ($\bar{\alpha}$ = +0.242) in the series without re-infusion (series A) (Tables 11 and 12). This difference was, however, not significant. This is shown further by the cusum-plot in Figure 46.

6.3.1.4 Bicarbonate secretion. The total bicarbonate secretion was marginally greater in the series with jejunal re-infusion (p = 0.05) (series B). In both series a trend to a fall in secretion was observed (Tables 11 and 12), although this was not significant on an hourly basis. In the series without re-infusion, the bicarbonate secretion was less stable and the trend in fall more obvious (Figs. 42,43 and 47).

6.3.2 Estimation of basal secretion with and without re-infusion of the duodenal aspirate

Figures 48 and 49 show the hourly basal secretion of total bile salts, bilirubin, trypsin and bicarbonate during the 6 h (2nd to 7th) of observation in the studies with (series C) and without (series D) re-infusion. At the top of each figure a cumulative plot shows the total amount of bile salts which was retained for analysis in each series of studies. A gradual fall in biliary and pancreatic secretion was observed in both series (Tables 13 and 14) (series C and D), and this may be due to a storage within the gall bladder and the biliary tract, as a large fraction of secreted bile is thought to be stored in the gall bladder during fasting (320).

6.3.3 Comparison between total secretory outputs in stimulated and basal studies

Between the series receiving caerulein/secretin stimulation (series A and B), no significant difference was found in the total (6 h) secretory output of bilirubin, bile salts or trypsin, but bicarbonate secretion was marginally greater (p = 0.05) in the group with re-infusion (series B) (Table 15). In the basal studies, the total

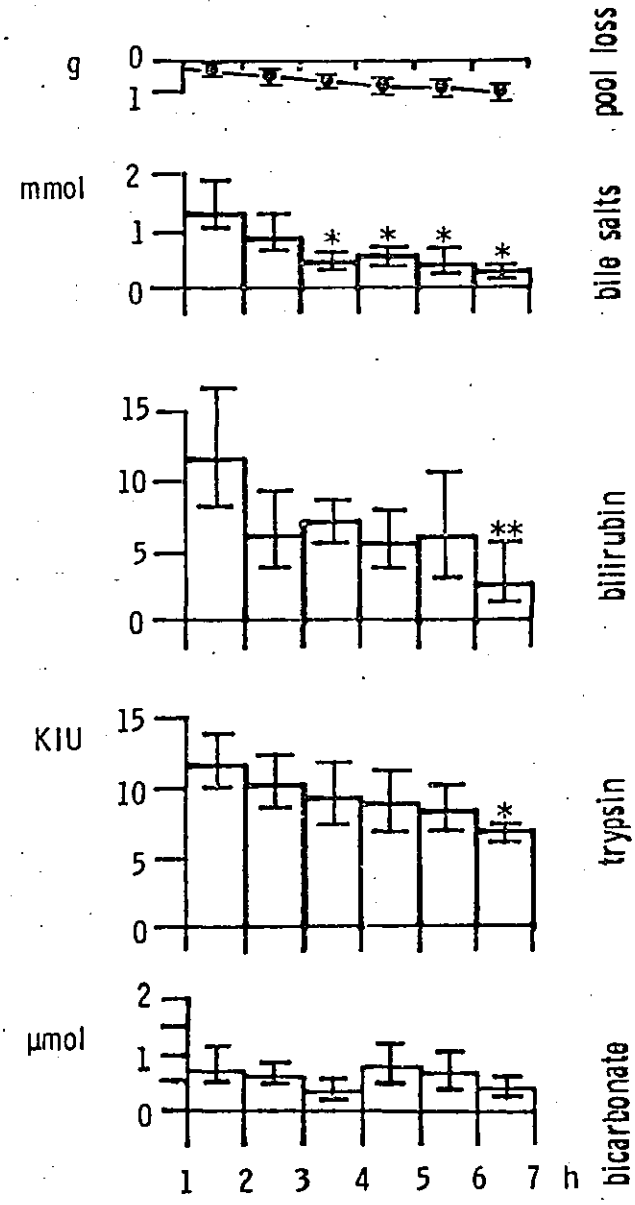


Fig. 48. The cumulative amount of bile salts discarded and basal secretion of total bile salts, bilirubin, trypsin and bicarbonate into the duodenum during 6 h (2nd to 7th h) of observation in 6 individuals, where the aspirated duodenal fluid was not re-infused into the jejunum (series C). Geometric mean ± SEM (bars). $^{*}p < 0.05$ $^{**}p < 0.01$

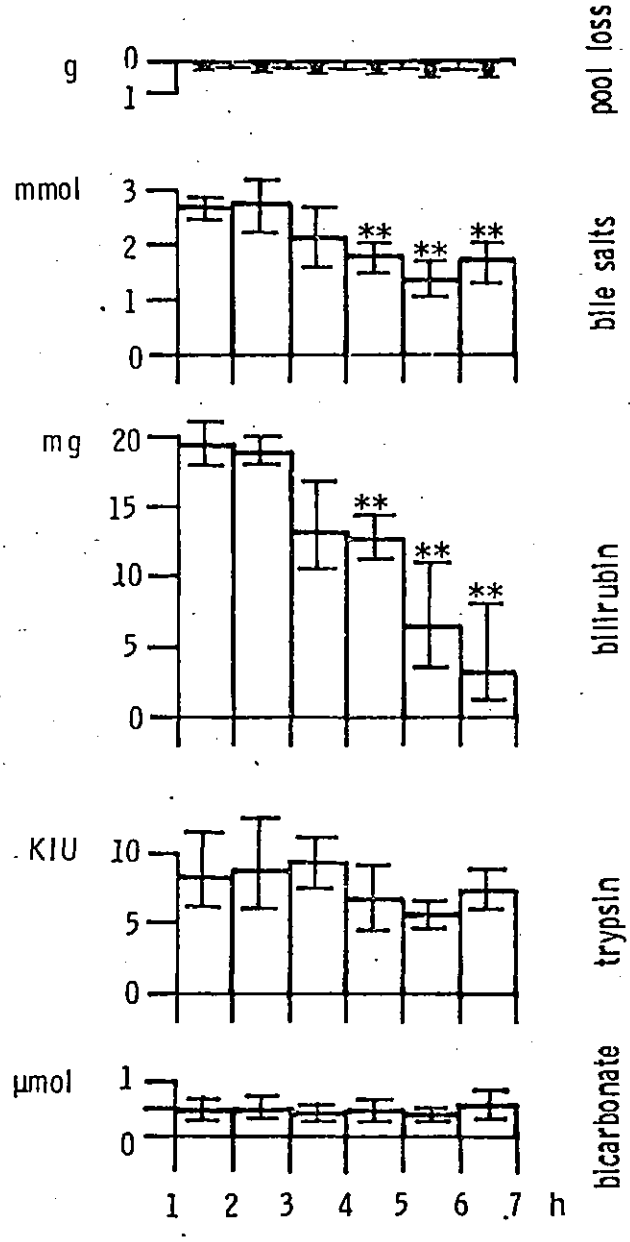


Fig. 49. The cumulative amount of bile salts retained for analysis and basal secretion of total bile salts, bilirubin, trypsin and bicarbonate into the duodenum during 6 h (2nd to 7th h) of observation in 6 individuals, where the aspirated duodenal fluid was re-infused into the jejunum (series D). Geometric mean ± SEM (bars). $^{**}p < 0.01$

Table 13. Basal secretion without reinfusion*

No.	Bile salts discarded (g)	Bilirubin discarded (mg)	Trypsin discarded (KIU)	Aspiration efficiency (%)	Bile salts α	Bilirubin α	Trypsin α	Bicarbonate α
1	0.68	52.48	30.19	46.0	-4.50	-3.40	-7.60	-0.56
2	1.37	94.77	28.33	26.1	-6.05	-3.04	-4.22	+1.13
3	2.94	107.60	19.46	34.2	-15.65	-9.28	-4.63	-0.33
4	0.58	26.96	9.13	27.6	+0.45	+0.46	+0.16	+0.04
5	0.86	38.27	16.25	44.0	-3.35	-5.88	-4.37	-0.34
6	0.67	94.98	22.39	89.7	-2.50	-3.73	-0.57	-0.11
$\bar{x}$	1.01	61.18	19.50	40.55	-3.545	-3.908	-3.741	-0.20
-SEM	0.79	48.50	16.29	33.73	-3.929	-4.429	-4.312	-0.27
+SEM	1.30	77.18	23.33	48.75	-3.161	-3.387	-3.170	-0.13

*For further details, see legend to Fig. 44.

Table 14. Basal secretion with reinfusion*

No.	Bile salts reinfused (g)	Bilirubin reinfused (mg)	Trypsin reinfused (KIU)	Aspiration efficiency (%)	Bile salts α	Bilirubin α	Trypsin α	Bicarbonate α
1	4.74	131.68	17.38	77.4	-0.70	-3.00	+0.50	-0.02
2	2.65	98.64	19.51	46.9	-3.05	-1.10	-0.88	+0.09
3	3.63	114.83	52.20	84.4	+0.35	+4.13	-0.01	-0.01
4	2.11	68.37	26.46	85.1	-3.00	-7.03	-1.89	+0.08
5	1.98	71.54	34.59	49.6	-4.45	-8.59	-6.35	-0.01
6	4.74	125.26	17.38	77.4	-0.70	-3.00	+0.50	-0.03
$\bar{x}$	3.11	98.63	25.59	68.1	-1.885	-3.221	-0.343	+0.02
-SEM	2.65	87.92	21.38	61.0	-2.256	-4.181	-0.526	+0.01
+SEM	3.65	110.64	30.62	76.0	-1.514	-2.261	-0.160	+0.03

*For further details, see legend to Fig. 45.

TABLE 15

Comparison of total secretory output (6 h) in caerulein (2.5 ng $^{-1}h^{-1}$)/secretin (0.1 CU $kg^{-1}h^{-1}$) stimulated studies without (series A) and with (series B) jejunal re-infusion of aspirated duodenal fluid: geometric mean ± SEM.

	Series A (without re-infusion)	Series B (with re-infusion)	t	P
Bile salts mmol	2.0 (1.5-2.6)	1.5 (1.2-2.0)	1.605	<0.5
Bilirubin mg	12.1 (9.7-15.1)	11.0 (9.2-13.3)	0.784	<0.5
Trypsin KIU	16.6 (13.8-19.9)	17.5 (15.7-19.4)	0.620	<0.5
Bicarbonate mmol	26.8 (19.7-36.5)	34.8 (29.8-40.7)	1.869	≃ 0.05

TABLE 16

Comparison of total secretory output (6 h) in basal studies without (series C) and with (series D) jejunal re-infusion of aspirated duodenal fluid: geometric mean ± SEM

	Series C (without re-infusion)	Series D (with re-infusion)	t	P
Bile salts mmol	0.5 (0.3-0.9)	1.9 (1.2-3.1)	6.132	<0.001
Bilirubin mg	5.9 (3.6-9.6)	13.2 (9.3-18.8)	3.431	<0.01
Trypsin KIU	9.1 (7.5-11.0)	7.6 (5.9-9.8)	1.368	<0.5
Bicarbonate mmol	0.6 (0.3-1.0)	0.5 (0.3-0.7)	1.069	<0.5

TABLE 7

Comparison of total secretory output (6 h) in caerulein (2.5 ng $kg^{-1}h^{-1}$)/secretin (0.1 CU $kg^{-1}h^{-1}$) stimulated studies and basal studies: studies without jejunal re-infusion (series A and C): geometric mean ± SEM.

	Series A (without re-infusion)	Series C (without re-infusion)	t	P
Bile salts mmol	2.0 (1.5-2.6)	0.5 (0.3-0.9)	5.875	<0.001
Bilirubin mg	12.1 (9.7-15.1)	5.9 (3.6-9.6)	3.281	<0.01
Trypsin KIU	16.6 (13.8-19.9)	9.1 (7.5-11.0)	5.508	<0.001
Bicarbonate mmol	26.8 (19.7-36.5)	0.6 (0.3-1.0)	17.270	<0.0001

TABLE 18

Comparison of total secretory output (6 h) in caerulein (2.5 ng $kg^{-1}h^{-1}$)/secretin (0.1 CU $kg^{-1}h^{-1}$) stimulated studies and basal studies: studies with jejunal re-infusion (series B and D): geometric mean ± SEM.

	Series B (with re-infusion)	Series D (with re-infusion)	t	P
Bile salts mmol	1.5 (1.2-2.0)	1.9 (1.2-3.1)	1.697	<0.5
Bilirubin mg	11.0 (9.2-13.3)	13.2 (9.3-18.8)	1.262	<0.5
Trypsin KIU	17.5 (15.7-19.4)	7.6 (5.9-9.8)	7.390	<0.0001
Bicarbonate mmol	34.8 (29.8-40.7)	0.5 (0.3-0.7)	27.261	<0.0001

bile salt and bilirubin outputs were significantly greater ($p < 0.001$ and $p < 0.01$, respectively) in the series receiving re-infusion (series D) but no difference was found for trypsin and bicarbonate (Table 16).

Any attempt to compare the total secretory output of stimulated studies with basal studies must be done with care (Tables 17 and 18) as different subjects volunteered in each series of studies, and the variation in secretory output between individuals can be expected to be great. Table 17 shows as expected that caerulein/secretin stimulated secretion was significantly greater than basal secretion in studies carried out without re-infusion. However, in studies carried out with re-infusion, there is not a significant difference in bile salt and bilirubin secretion in stimulated and basal studies. This is not likely to be due to failure of the parenteral stimulation as there is a highly significant difference between stimulated and basal pancreatic secretion in these studies. The reason seems to be that in the basal studies with re-infusion the biliary secretion is increased.

6.3.4 Analysis of the secretory data using empirical cumulative distribution function plot (ECDF-plot)

Figures 50 to 57 show the ECDF-plot (267) of the slope constants (α) for the secretory output in the stimulated and basal studies. The slope constants of the regression lines for the 6 h secretory output in each individual were plotted in the order of magnitude (quantiles) *versus* P, $P = \frac{i - 0.5}{n}$; n = total number of subjects studied, i = location of the order = 1 ... 6. The difference between the α of studies with (▲——▲) and without (●——●) re-infusion was also plotted (△——△). From these pictures one can see that the α for the stimulated bile salt secretion (Fig. 50) was negative on all occasions except one, in the studies without re-infusion of the aspirate, but had become positive in 4 out of 6 in these same individuals when the aspirate was re-infused. The difference between the α of each group of studies was therefore positive. Figure 52 shows the negative slope constants for stimulated bilirubin secretion in all subjects without re-infusion. When carried out with re-infusion, α remained negative in only one individual, and the difference between the two series (A and B) had become highly positive. In the studies on trypsin (Fig. 54) and bicarbonate (Fig. 56), no clear trend was seen, and the quantiles were intermingled with each other. The quantiles for the unstimulated studies (Figs. 51, 53, 55 and 57) were negative on most occasions in both series of studies (series C and D).

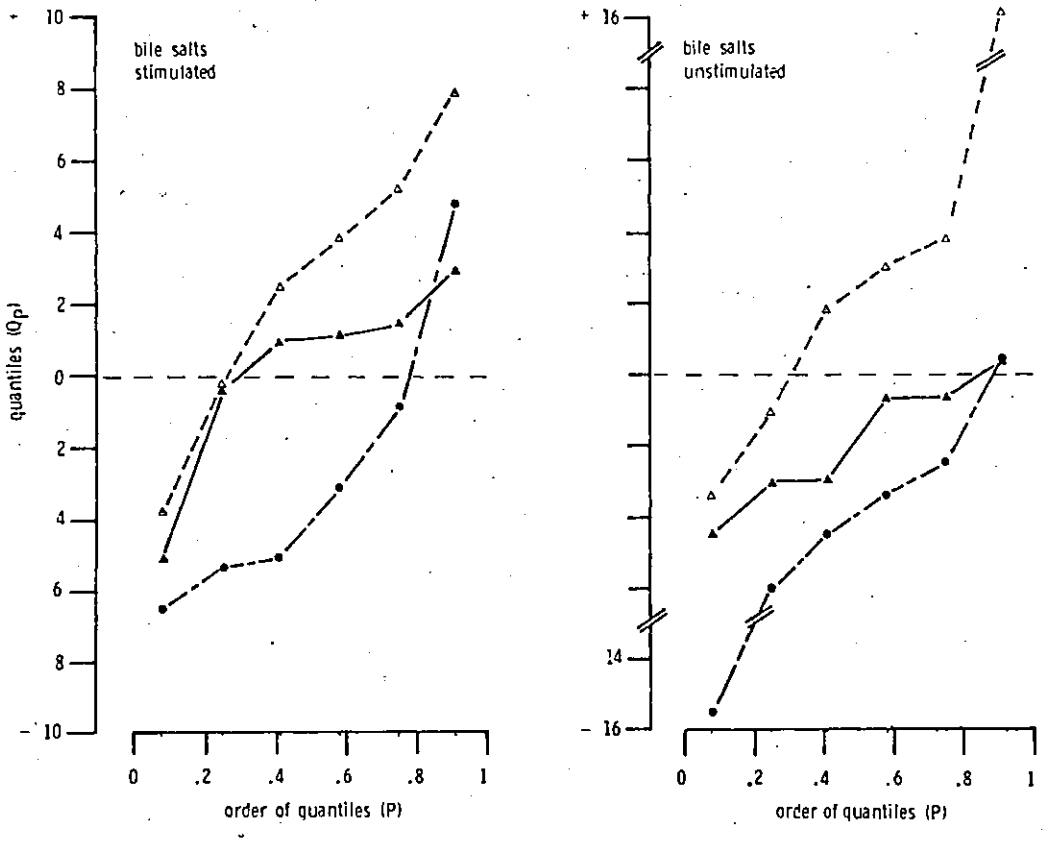


Fig. 50. *(left)* An ECDF-plot of the slope constants (α) of least squares fit regression lines for stimulated secretory output of total bile salts during 6 h of observation.
●——●, without re-infusion (series A);
▲——▲, with re-infusion (series B);
△----△, difference between studies with and without re-infusion.

Fig. 51. *(right)* An ECDF-plot of α of basal bile salt secretion (series C and D). See further legend to Fig. 50.

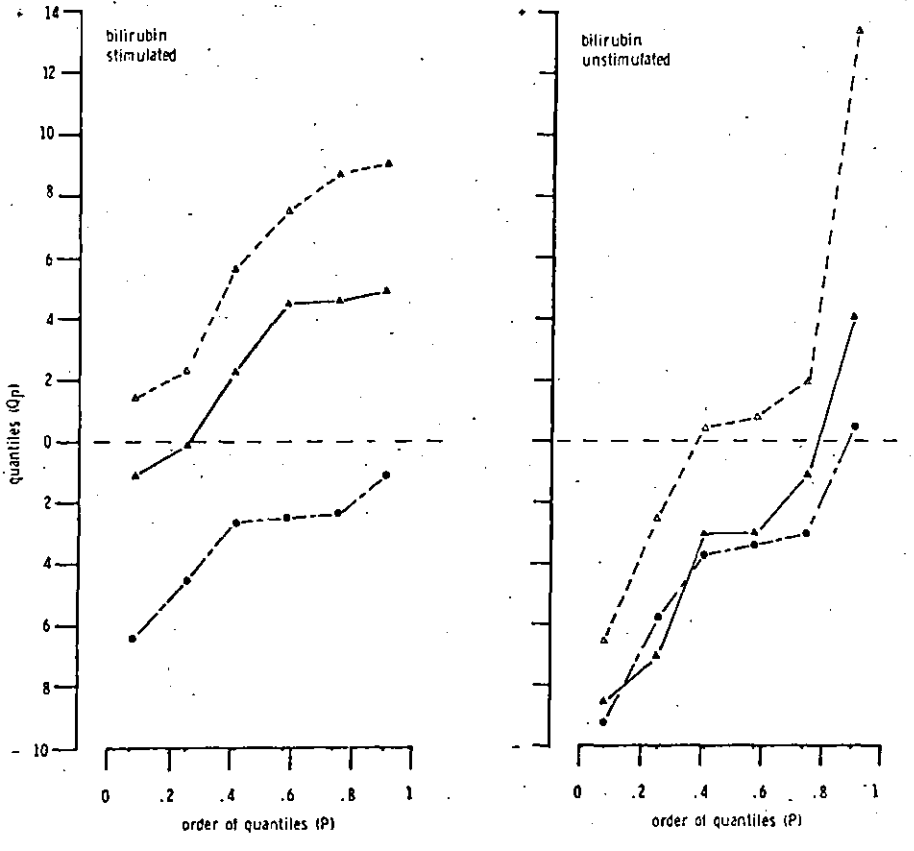


Fig. 52. *(left)* An ECDF-plot of α of stimulation bilirubin secretion (series A and B). See further legend to Fig. 50.

Fig. 53. *(right)* An ECDF-plot of α of basal bilirubin secretion (series C and D). See further legend to Fig. 50.

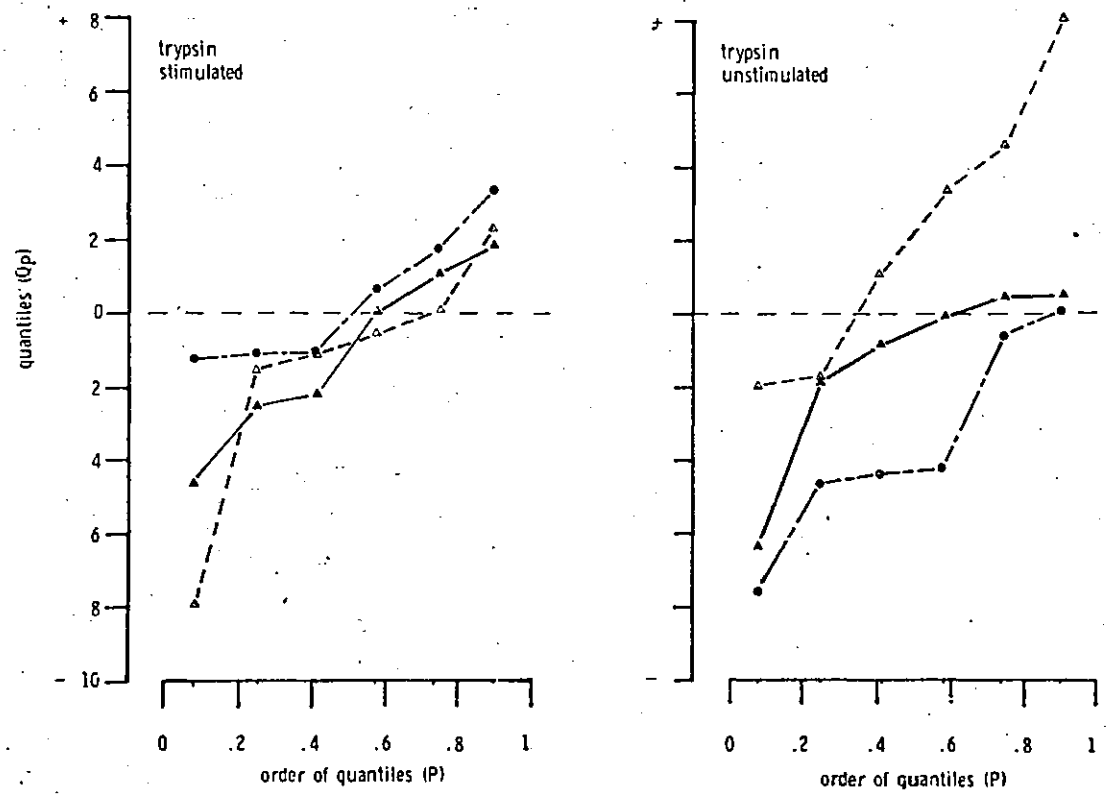


Fig. 54. *(left)* An ECDF-plot of α of stimulated trypsin secretion (series A and B). See further legend to Fig. 50.

Fig. 55. *(right)* An ECDF-plot of α of basal trypsin secretion (series C and D). See further legend to Fig. 50.

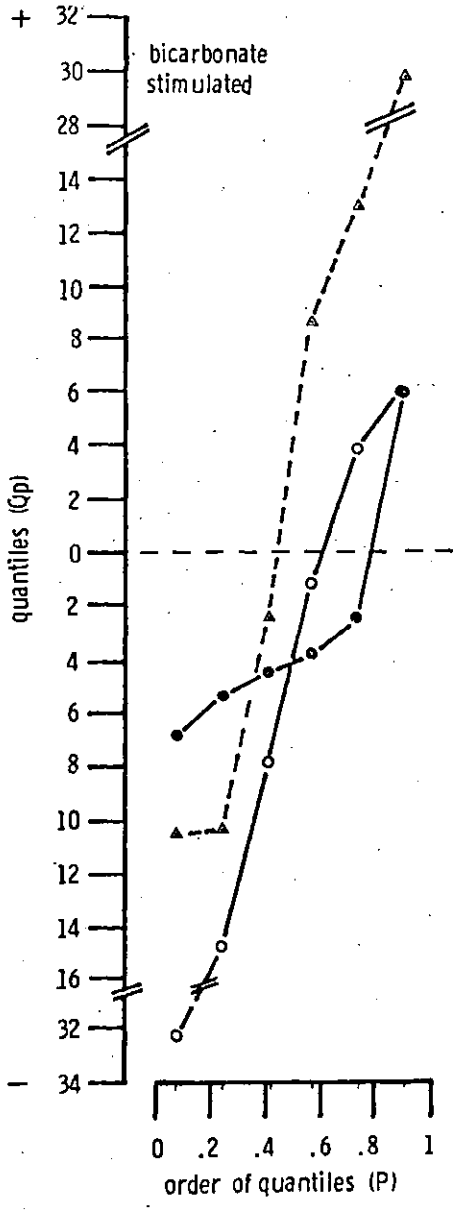


Fig. 56. An ECDF-plot of $\bar{\alpha}$ of stimulated bicarbonate secretion (series A and B). See further legend to Fig. 50.

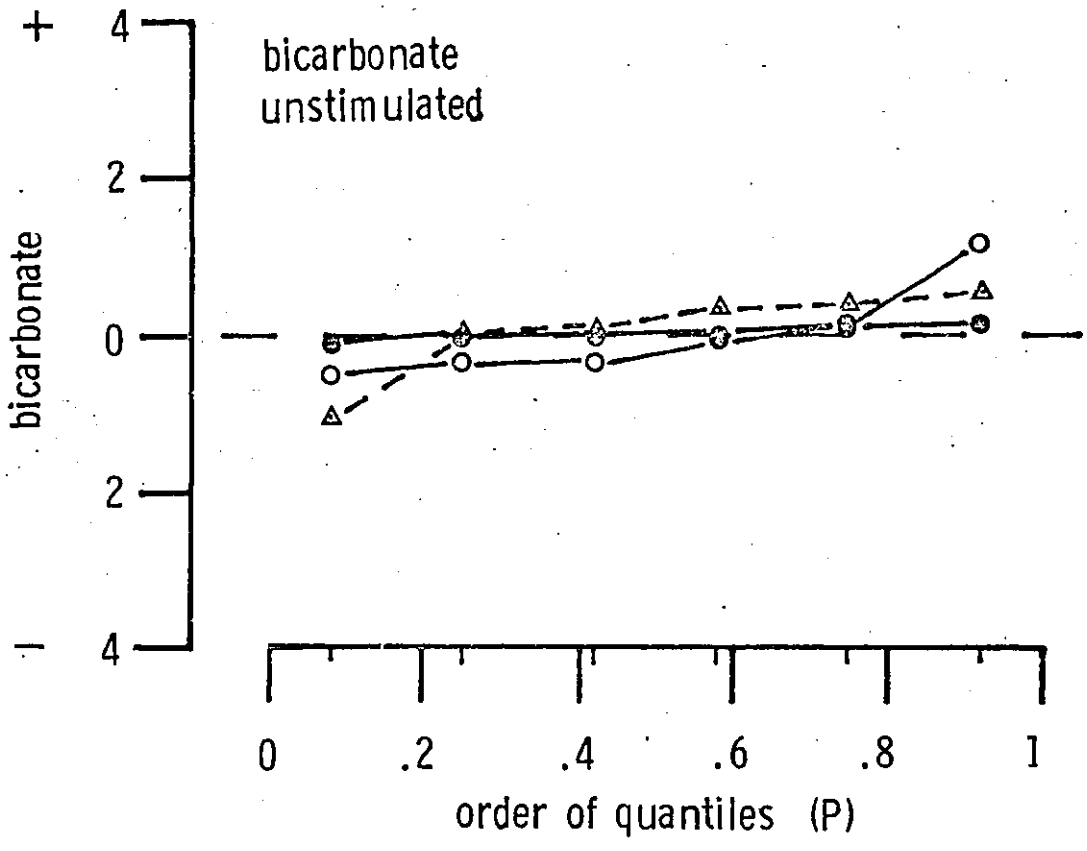


Fig. 57. An ECDF-plot of α of basal bicarbonate secretion (series C and D). See further legend to Fig. 50.

6.4 DISCUSSION

The data presented here show that in studies on humans, carried out with the standard duodenal perfusion technique without re-infusion of the aspirated duodenal fluid into the jejunum, a gradual fall in caerulein/secretin stimulated bile salt and bilirubin secretion was observed during the 6 h of observation. In the same individuals, under exactly the same experimental conditions, when the duodenal aspirate was re-infused into the jejunum, the bile salt secretion remained stable, and the secretion of bilirubin increased. In neither series of studies were there any definite changes in the secretion of trypsin, but the total output of bicarbonate was marginally greater in the studies carried out with re-infusion of the aspirate than in the group where the aspirate was discarded.

The most likely explanation for these findings is that the observed fall in bile salt secretion in the studies without re-infusion was due to depletion of the bile salt pool. Interruption of the enterohepatic circulation apparently influenced the hepatic secretion of bilirubin. Bilirubin, like bromsulphthalein and other anions, is usually considered to have a secretory mechanism independent of that of the bile salts (136). This is supported by the studies of Billing *et al.* (34) in rats, who showed that hepatic secretion of conjugated bilirubin was not associated with hepatic bile salt secretion. Recent studies in man by Mok *et al.* (264) also showed no changes in bilirubin secretion over 24 h in cholecystectomised subjects, who were fed several times during that period, and who responded to feeding with a variable bile salt output. However, Goresky *et al.* (140) have demonstrated in anaesthetised dogs that the biliary secretory maximum (Tm) for bilirubin can be increased by increased hepatic secretion of bile salts, and Shull *et al.* (348) came to the conclusion in their studies on gallstone patients, that secretion of bilirubin into bile was partly associated with the secretion of bile salts. The results presented here support that there is an association between bile salt and bilirubin secretion, although it has to be admitted that the effects of changes in gall bladder storage during these studies cannot be entirely excluded, since they were not carried out on cholecystectomised subjects, and fractional gall bladder storage may be influenced by interruption of the enterohepatic bile salt circulation. However, the studies on bile salt perfusion of the duodenum (see *Chapter IV*) are in favour of an increase in gall bladder storage with an increase in intestinal bile salt concentration. This makes it more likely that the effect of

the interruption of the enterohepatic circulation of bile salts was on the hepatic secretion itself, rather than on the extrahepatic biliary tract. Storage in the gall bladder and the biliary tract may, however, have been a contributory factor in the studies of basal biliary and pancreatic secretion, as a considerable fraction of hepatic bile secretion is considered to enter the gall bladder in the fasting state (165, 320).

The total bile salt pool was not determined in the present studies, so that it cannot be stated how large a fraction of the total pool was actually removed from each individual. The actual figures measured, 3.56 g (2.92-4.35, SEM) in the stimulated series, and 1.01 g (0.79-1.30, SEM) in the basal series, point to that a large part of the total pool was removed in the stimulated studies, as the total pool is quoted to be 2.5-5.0 g (358,403). That a great part of the bile salt pool was removed is also suggested by the fact that the experiments lasted for 7 h, and during this time the duodenal aspiration efficiency was around 50% on average (Tables 11 to 14). The bile salt pool is thought to circulate 6 to 12 times during 24 h (358), and might therefore have circulated 2 to 3 times during the 7 h study period, especially as the cholecystokinin analogue caerulein was administered during the studies, and cholecystokinin is expected to stimulate the intestinal peristalsis (197) and this increases enterohepatic cycling (155). According to these calculations, 70-80% of the pool might have been removed. It was therefore surprising that the fall in bile salt secretion in the stimulated studies without jejunal re-infusion was not greater than observed. Compensation of "*de novo*" synthesis of bile salts above the basal bile salt synthesis rate is not likely, as experimental works in animals show that bile salt synthesis is not increased within 24 h of bile depletion (271).

An error in the estimation of the amount of bile salts removed from the enterohepatic circulation is not expected, since it was not subject to recovery marker estimation, but based only on a direct measurement of the total volume of the duodenal fluid aspirated every 10 min, and the concentration of total bile salts in this fluid. The total bile salts were measured by an established assay and with an acceptable precision and accuracy (369, 370).

The background stimulation by caerulein and secretin was adjusted so that it might simulate the biliary and pancreatic responses seen after a meal (60, 417), and this stimulation resulted in a biliary and pancreatic secretion significantly greater than the basal secretion.

The perfusion technique, however, is often used with a much higher cholecystokinetic stimulation, which is likely to cause a greater loss of bile salts than observed here, when performed without jejunal re-infusion.

It has previously been shown that a bile salt perfusion of the duodenum augmented caerulein/secretin stimulated bicarbonate secretion in man (*Chapter IV*), and similar findings have been observed by others (125, 410). This effect may explain why the stimulated secretory output of bicarbonate was greater when the duodenal aspirate was re-infused than when it was discarded. No such effect was found, however, on basal bicarbonate secretion, which is in agreement with previous results that bile salts in the intestine do not stimulate basal bicarbonate secretion (*Chapter IV*). The results presented here do not support the findings of others that bile or pancreatic juice in the upper intestinal tract affect pancreatic enzyme secretion (175, 239, 285, 286). They do, however, agree with the findings of Krawisz *et al.* (210) that there is no such effect, and this is also in agreement with results reported elsewhere (see *Chapter IV*) that bile salts in the upper intestinal tract do not affect pancreatic enzyme secretion.

In conclusion, these results suggest that duodenal perfusion studies of pancreatic and biliary secretion should be carried out with jejunal re-infusion of the duodenal aspirate, especially in prolonged studies and when using either bile salts or bilirubin as endogenous biliary markers.

6.5 SUMMARY

(1) The standard duodenal perfusion technique was used to measure biliary and pancreatic secretion in normal subjects with and without jejunal re-infusion of the aspirated duodenal fluid.

(2) In studies carried out without re-infusion of the duodenal aspirate, and where a continuous stimulation of caerulein and secretin was used to prevent extrahepatic biliary storage, a gradual fall in bile salt and bilirubin secretion was observed. In contrast, when these experiments were carried out in these same individuals, and under exactly the same experimental conditions, except that the duodenal aspirate, rich in bile and pancreatic juice, was in every successive collection period re-infused into the jejunum, the bile salt secretion remained stable and the bilirubin secretion increased.

(3) In neither series of studies were there any definite changes in the secretion of trypsin.

(4) The total secretory output of bicarbonate was significantly greater in the studies carried out with jejunal re-infusion of aspirated duodenal fluid than in the studies where the aspirate was discarded. However, in both series a fall in bicarbonate secretion was observed during the 6 h observation period.

(5) In the studies carried out under basal conditions, a gradual fall in both biliary and pancreatic secretion was observed in both series of studies.

(6) These results indicate that partial diversion of bile and pancreatic juice from the jejunum may affect hepato-biliary secretion of bile salts and bilirubin in man and the secretion of bicarbonate.

CHAPTER VII

POWER SPECTRAL ANALYSIS OF BASAL AND CAERULEIN/SECRETIN STIMULATED BILIARY AND PANCREATIC SECRETION IN MAN: STUDIES WITH AND WITHOUT JEJUNAL RE-INFUSION OF ASPIRATED DUODENAL FLUID

7.1 INTRODUCTION

It has been shown both in animal and human studies that there are great fluctuations in the secretion of bile into the duodenum (36, 281, 282). This is thought to be due to the motor activity of the gall bladder and the extrahepatic biliary tract, as electrical and pressure recordings from the gall bladder and the sphincter of Oddi reveal rhythmic motor activity of these organs (135, 281, 282, 344, 387).

There is some evidence that these fluctuations have a periodic pattern (135, 192). It was therefore of interest in a work on the secretion of bile and pancreatic juice in man to use a more advanced analytical technique than hitherto to study the possible periodicity of the secretion.

For this purpose, methods for digital frequency analysis (67) were applied to the secretory data which had been obtained by the duodenal perfusion technique, which simultaneously measured the secretion of bile and pancreatic juice into the duodenum. The experimental studies were carried out either during a known stimulation by secretin and the cholecystokinin analogue caerulein, or under basal conditions, where the regulatory mechanism is unknown (357). The effect on the secretory pattern of partial diversion from the jejunum of bile and pancreatic juice was also studied as such a diversion is a common practice when the perfusion technique is carried out.

7.2 MATERIALS AND METHODS

7.2.1 Experimental protocols

The experimental protocols have been described in *Section 6.2.1.* Six volunteers underwent each two studies in a random order, where the secretion of bilirubin, total bile salts and trypsin were monitored for 6 h. In one series of studies, all aspirated duodenal fluid was discarded whilst in the other duodenal fluid was re-infused immediately into the jejunum after aspiration (10 min sampling intervals). Caerulein (2.5 ng $kg^{-1}h^{-1}$) and secretin (0.1 CU $kg^{-1}h^{-1}$) were administered i.v. throughout the studies as a background stimulation of biliary and pancreatic secretion.

In 6 different subjects, biliary and pancreatic secretion was monitored for 6 h in each individual with or without re-infusion of aspirated fluid (random order), using exactly the same experimental design as described before, except that the background stimulation with caerulein and secretin was omitted (basal secretion). In all studies, 1 h was allowed for equilibration of the recovery marker before the monitoring of biliary and pancreatic secretion was started. For chemical analysis of biliary and pancreatic secretory markers and the fluid volume marker ^{14}C PEG 4000, see *Sections 2.2.7* and *4.2.8*.

7.2.2 The recording technique

The standard duodenal perfusion technique used to monitor biliary and pancreatic secretion into the duodenum is described in detail in *Sections 2.2.9* to *2.2.12*. ^{14}C PEG 4000 was used as an inert recovery marker to estimate the volume of fluid in the duodenal test segment at any sampling interval (10 min), according to already established equations (138). The duodenal fluid, continuously aspirated, was sampled every 10 min and the corrected total amount of bilirubin, total bile salts and trypsin in the test segment estimated. In this way discrete values of bilirubin, total bile salts and trypsin secretion were obtained for each individual over the 6 h the studies lasted providing 36 measured data points for each secretory parameter as shown in Figure 58.

7.2.3 The power spectral estimation

The power spectral estimation of the biliary and pancreatic secretory patterns was based on the method of smoothed periodograms (67, Appendix VII) after necessary pretreatment of the data. A cubic spline interpolation was applied to the data in order to obtain a better graphical presentation (Figs. 59 to 60, Appendix VI). A regression line was fitted to the 36 data values (Figs. 58-60), and prior to Fast Fourier Transformation this trend was subtracted from the data together with the arithmetic mean (the dc-factor). A cosine taper was applied to the first and last 10% of the time series in order to reduce their leakage (67), and 28 zeros were attached to the block of 36 data values before spectral estimation in order to satisfy the FFT algorithm used.

The power spectral components for each secretory parameter showed in "frequency of occurrence" plots a positively skewed distribution resembling a chi-square distribution of a low (<6) degree of freedom

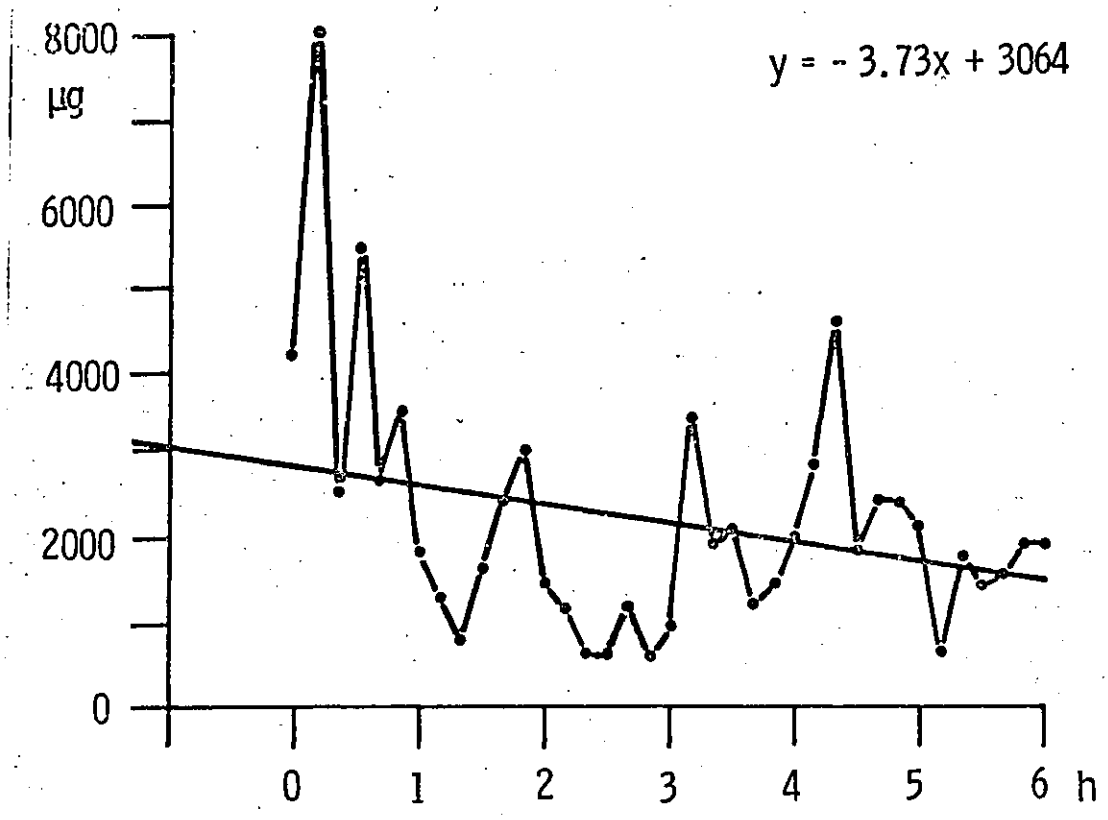


Fig. 58. Six hours basal bilirubin secretion in one individual. Straight lines were drawn between the 10 min sampling data points. A best fit regression line was inserted on the graphs as an estimator of the average linear trend of the 6 h secretory output.

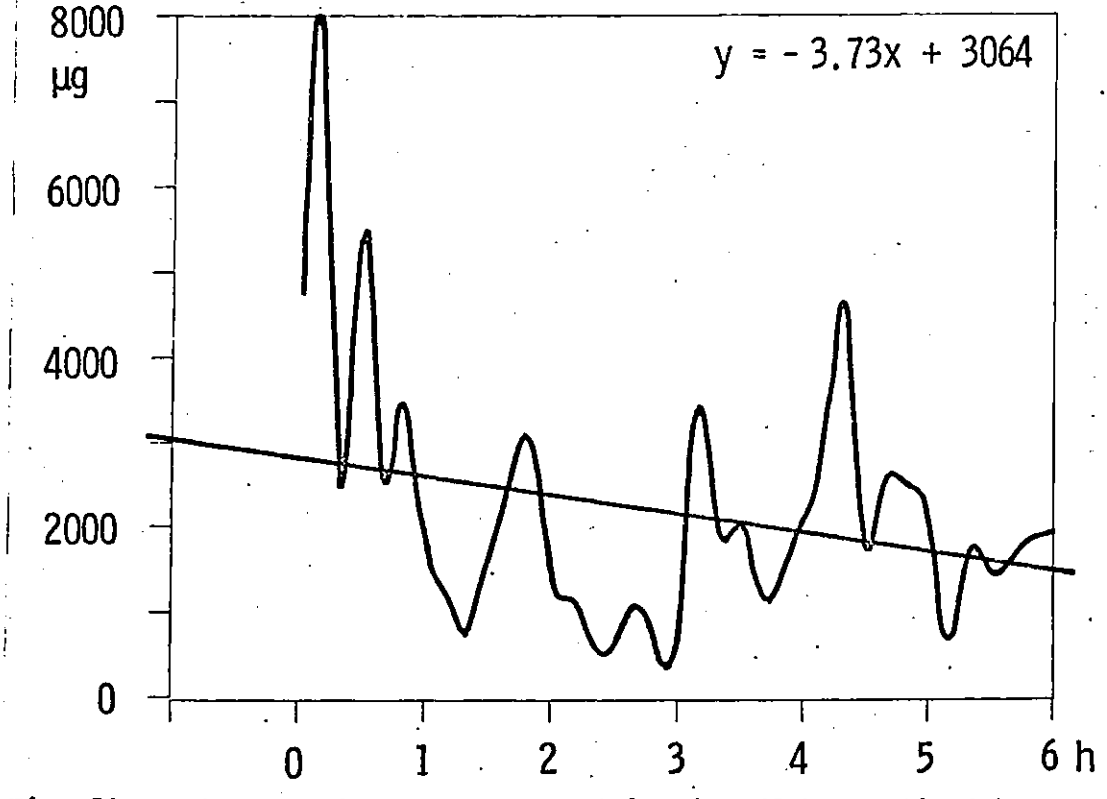


Fig. 59. The same data as presented in Fig. 58, but splined by cubic spline interpolation.

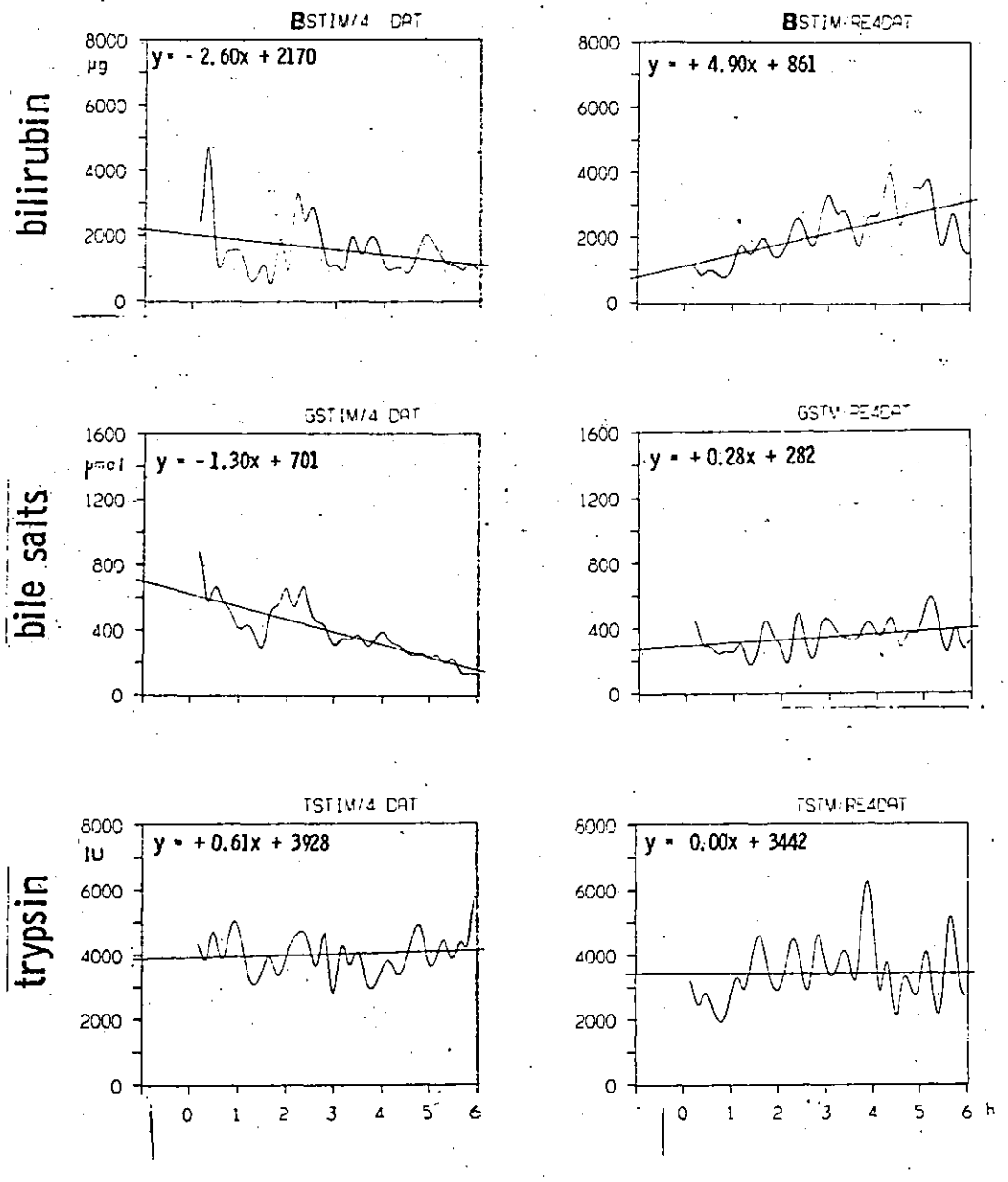
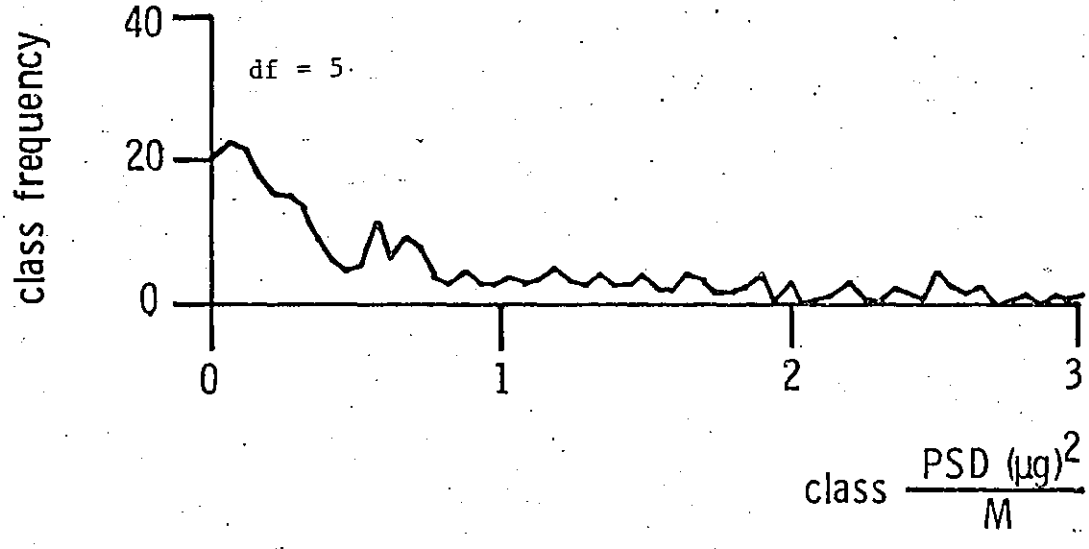


Fig. 60. Six hours secretory output of caerulein/secretin stimulated bilirubin, bile salt and trypsin in one individual without (BSTIM, GSTIM, TSTIM) or with (BSTIM/RE, GSTIM/RE, TSTIM/RE) re-infusion of aspirated duodenal fluid. Splined data. A least squares fit regression line has been inserted on the graphs.



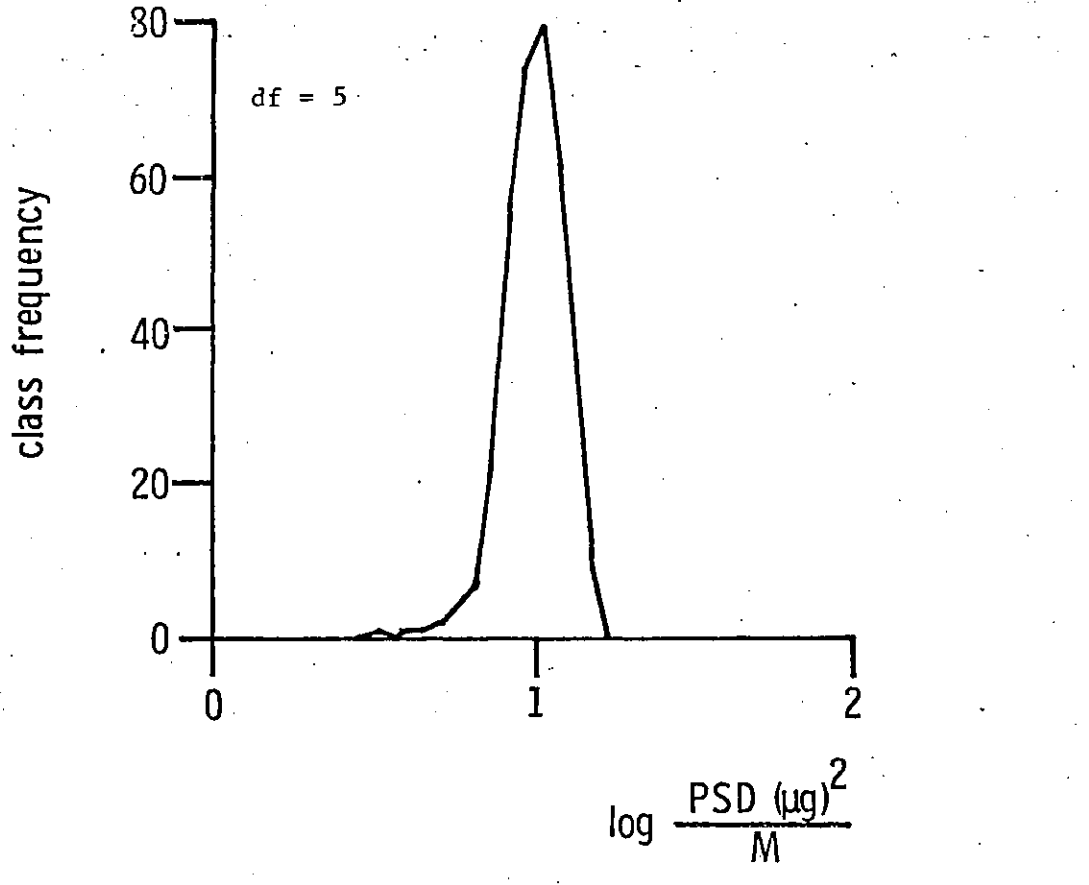


Fig. 61. "Frequency of occurrence" plot of power spectral density (PSD) components before (A) and after (B) log-transformation in a series of 6 subjects (M = mean).
Degrees of freedom (df) = n-1 = 5.

(Fig. 61A). This suggested that a log-transformation of the power spectral density values would symmetrice the amplitude distribution sufficiently to allow parametric statistical methods to be used for significance analysis of the data, and this turned out to be true (Fig. 61B).

In order to detect regions reflecting periodic patterns in the spectra and to estimate their significance in height as well as in location, those regions were considered where the log-spectral density surpassed in height the mean log-spectral intensity in the corresponding graph. A random noise level was defined as the average log-spectral density outside these regions. The significance of the density within the regions was tested against the noise level by Student's t-test.

To estimate changes in distribution and magnitude of the power spectrum in basal and caerulein/secretin stimulated studies, two concepts were defined: specific width and specific area. Specific width was defined as the ratio of the width of the significant spectral region in the periodicity domain and the maximum density within the region. Specific area was defined as the ratio of the area of the significant spectral region as it was defined above and the total area under the average spectral curve over all periodicities. A further comparison between size of the area of the significant regions in basal and stimulated studies was carried out by applying either Student's t-test or F-ratio test or Wilcoxon rank sum test to the data to estimate the level of significance (300).

7.3 RESULTS

Figures 62, 63 and 64 show the power spectral density of basal (A) and stimulated (B) bilirubin, bile salts and trypsin secretion, respectively, in studies without re-infusion of aspirated fluid. The power spectra are expressed as geometric mean ± SD for the group of 6 subjects in each series of studies. The regions significantly above the mean random noise level are demarcated by solid vertical lines together with the appropriate significance values. Figures 65, 66 and 67 show the corresponding results for the basal and caerulein/secretin stimulated studies carried out with re-infusion of aspirated duodenal fluid. Tables 19 to 22 summarise the characteristics of these studies as far as periodicity, specific width and specific area are concerned. A further comparison

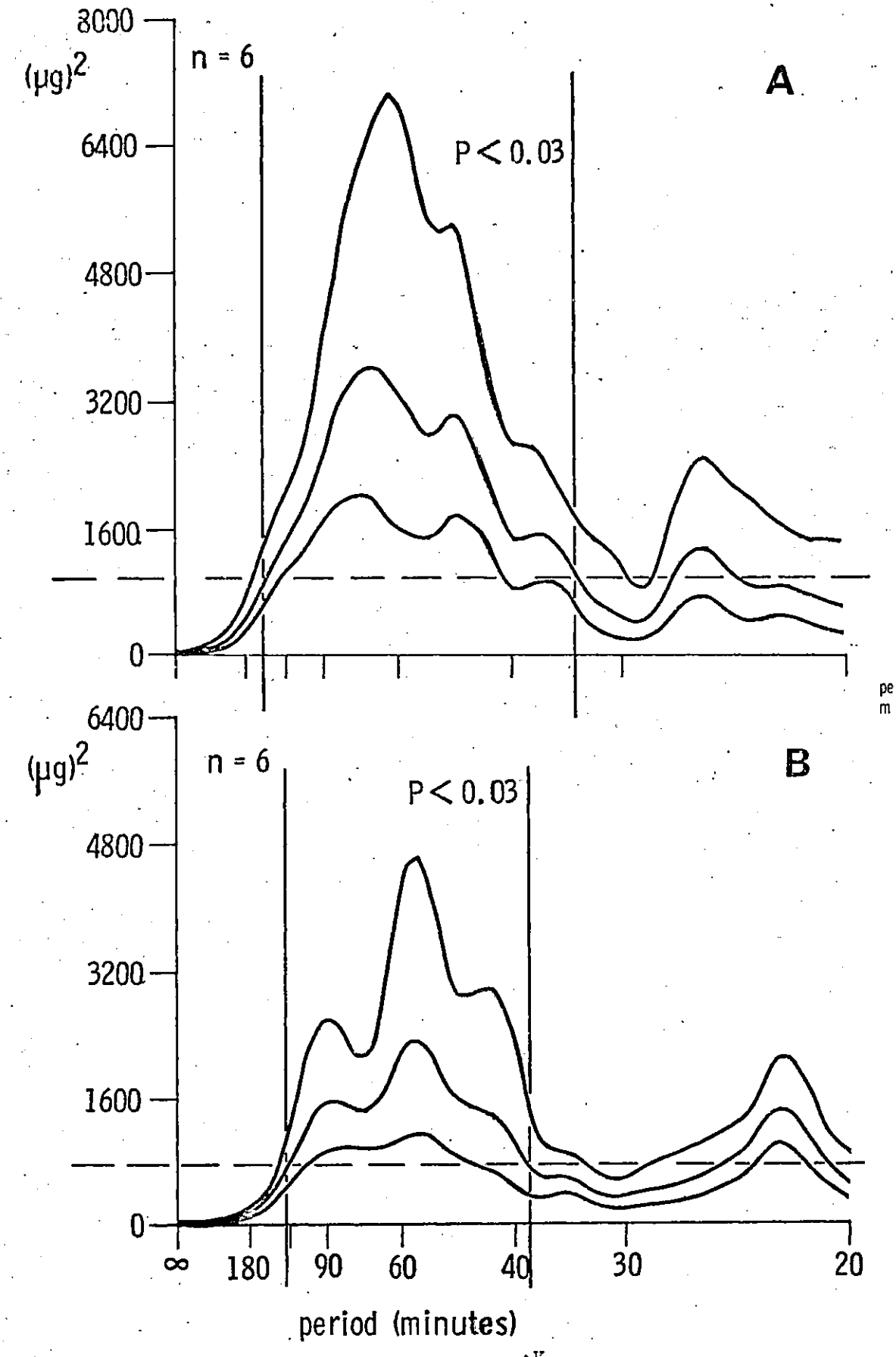


Fig. 62. A. The power spectral density ($\hat{S}_N^K$) of basal bilirubin secretion without re-infusion of duodenal aspirate, expressed as geometric mean ± SD for the group of 6 subjects. The vertical lines demarcate the spectral region which was significantly ($p<0.03$) above the mean random noise level as defined on p. 212.

B. The power spectral density ($\hat{S}_N^K$) of caerulein/secretin stimulated bilirubin secretion without re-infusion of duodenal aspirate, geometric mean ± SD. See further legend to Fig. 62A.

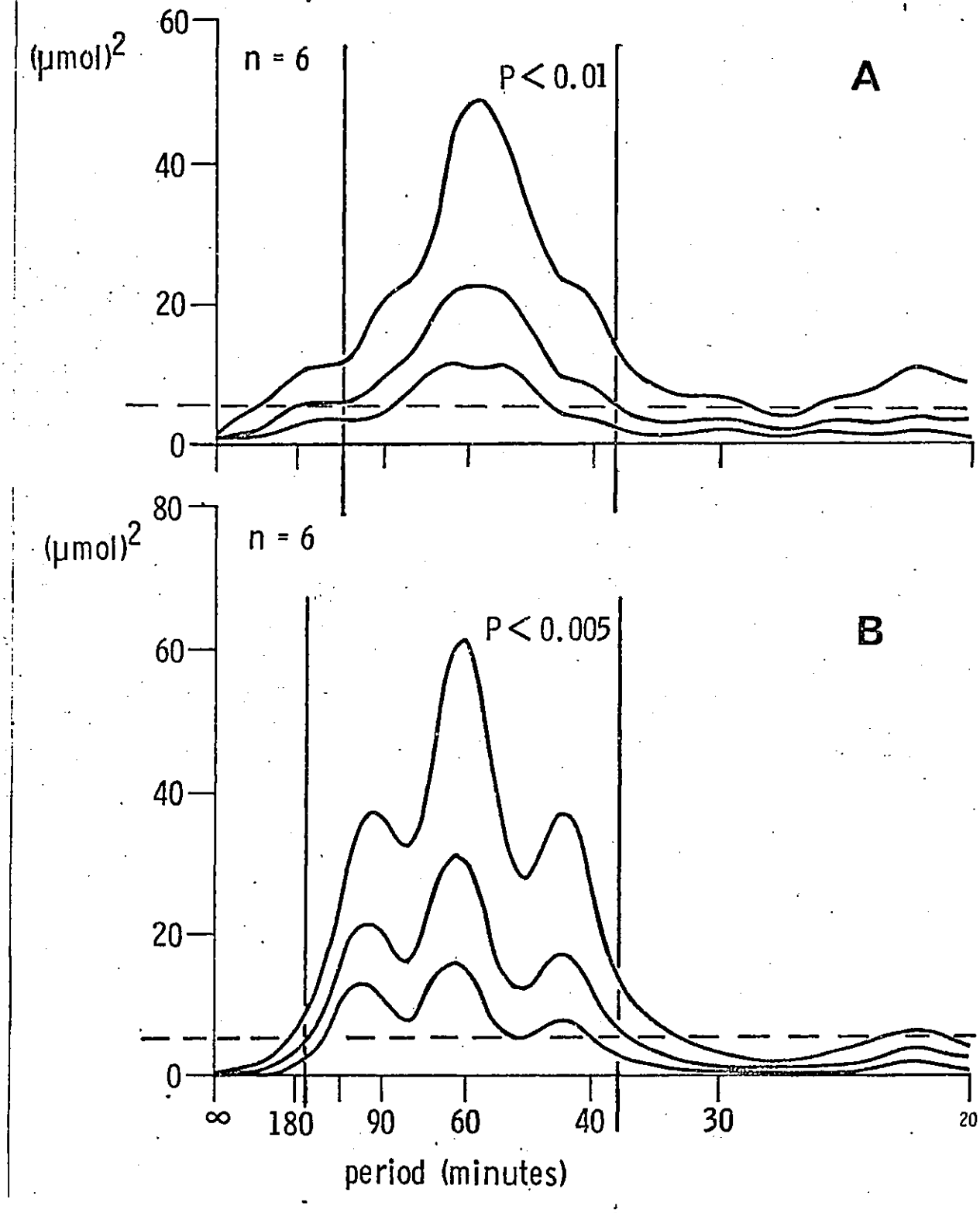


Fig. 63. The power spectral density ($\hat{S}_N^K$) of bile salt secretion in studies without re-infusion of duodenal aspirate, geometric mean ± SD.

A. Basal secretion;

B. Caerulein/secretin stimulated secretion.

See further legend to Fig. 62.

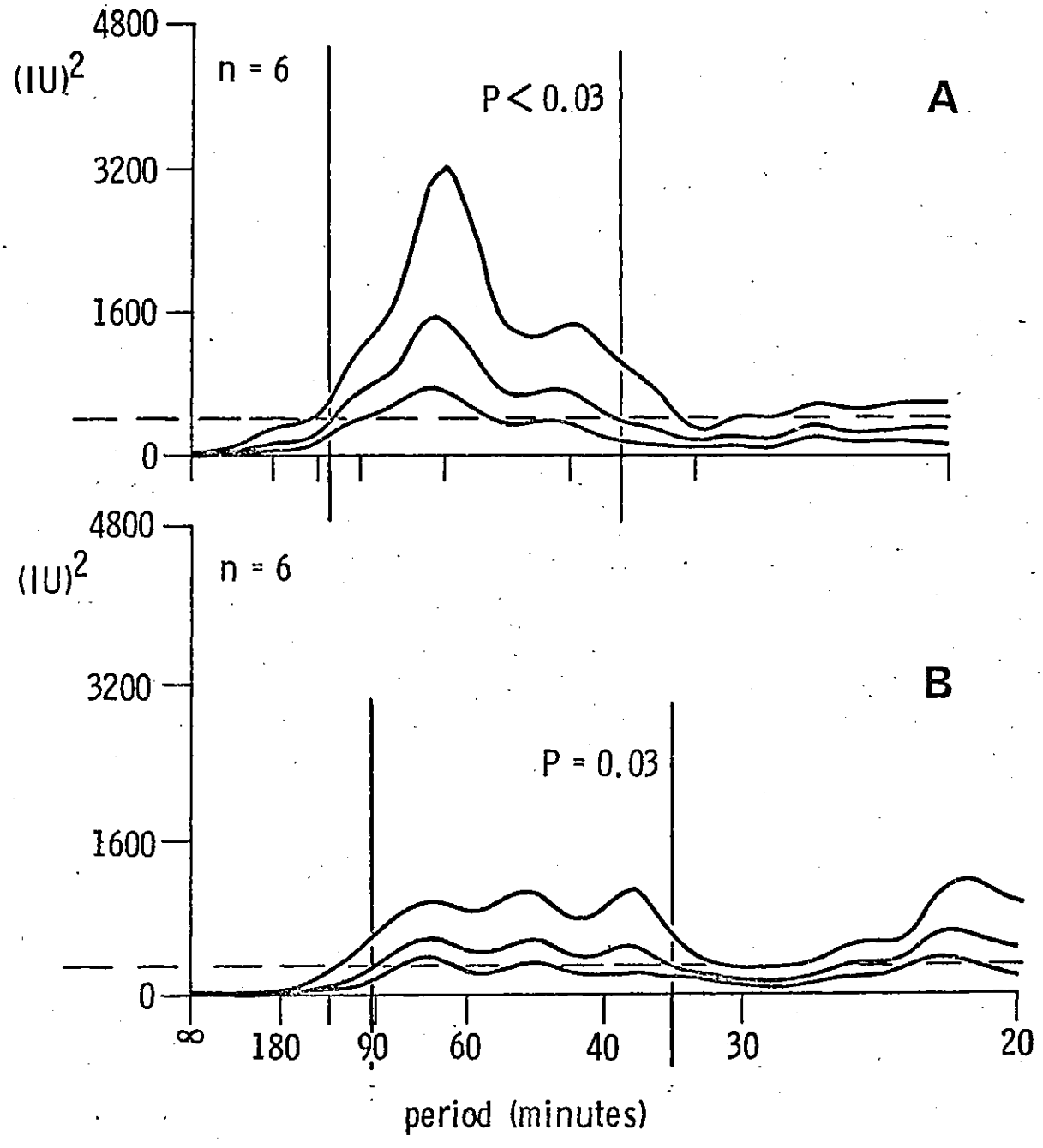


Fig. 64. The power spectral density ($\hat{S}_N^K$) of trypsin secretion in studies without re-infusion of duodenal aspirate, geometric mean ± SD.

A. Basal secretion;

B. Caerulein/secretin stimulated secretion.

See further legend to Fig. 62.

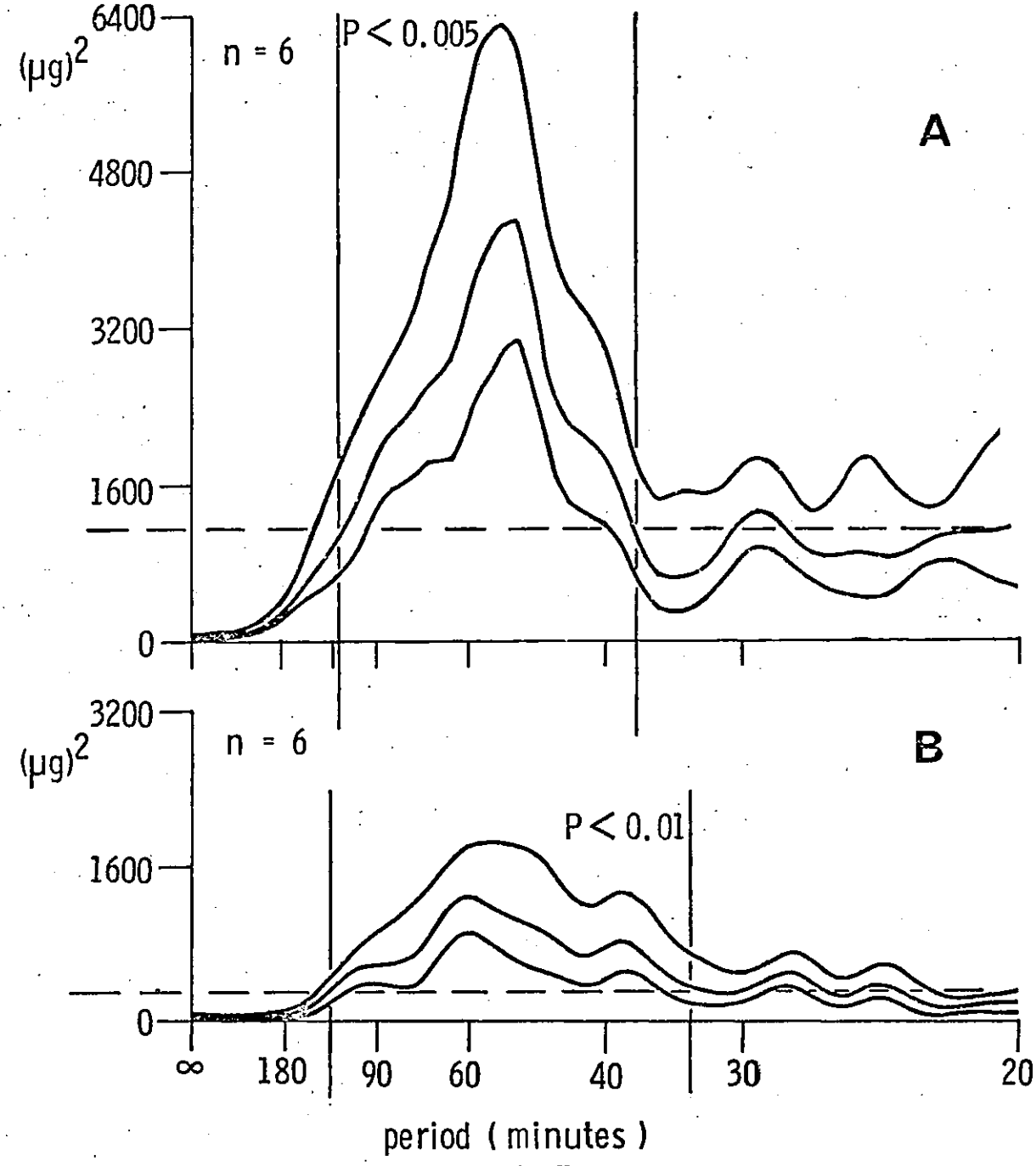


Fig. 65. The power spectral density ($\hat{S}_N^K$) of bilirubin secretion in studies with re-infusion of duodenal aspirate, geometric mean ± SD.

A. Basal secretion;

B. Caerulein/secretin stimulated secretion.

See further legend to Fig. 62.

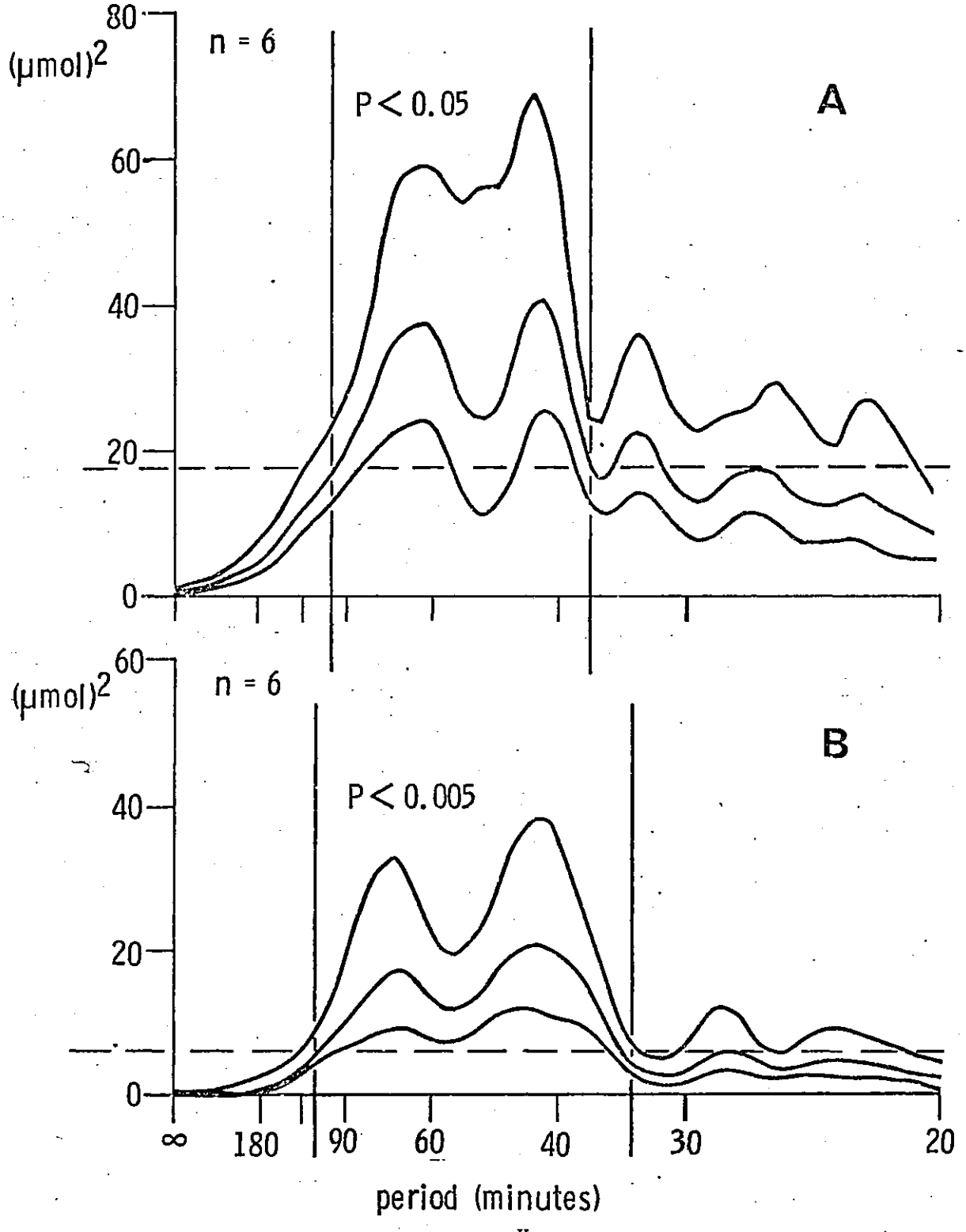


Fig. 66. The power spectral density ($\hat{S}_N^K$) of bile salt secretion in studies with re-infusion of duodenal aspirate, geometric mean ± SD.

A. Basal secretion;

B. Caerulein/secretin stimulated secretion.

See further legend to Fig. 62.

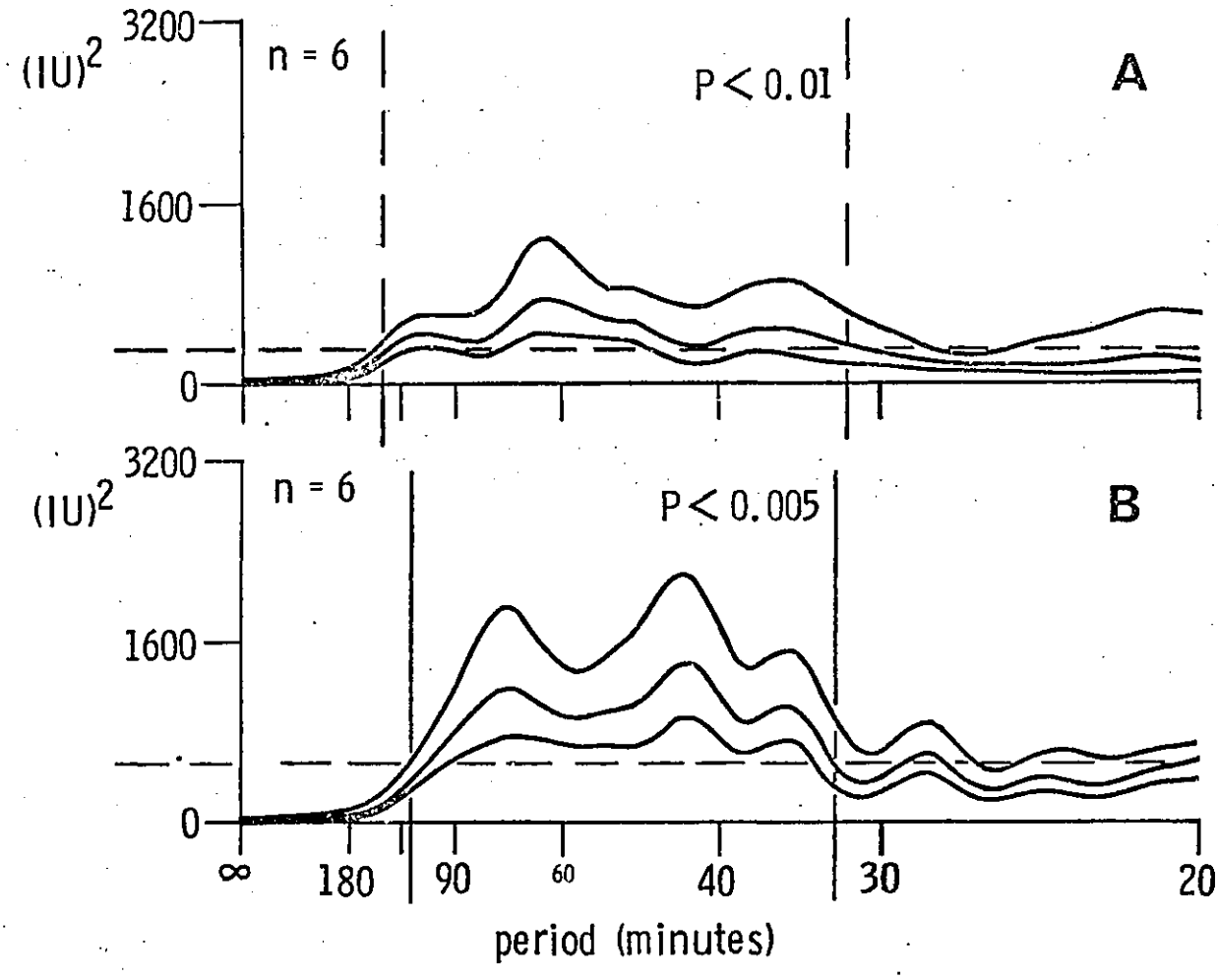


Fig. 67. The power spectral density ($\hat{S}_N^K$) of trypsin secretion in studies with re-infusion of duodenal aspirate, geometric mean ± SD.

A. Basal secretion;

B. Caerulein/secretin stimulated secretion.

See further legend to Fig. 62.

Table 19

Dominant periods in minutes within the regions significantly ($p < 0.05$) above the estimated random noise level in studies without re-infusion of aspirated duodenal fluid. Figures in square brackets on the bottom lines refer to periodicities at the borders of the regions. Figures in round single brackets ( ) and double brackets (( )) refer to spectral peaks of less spectral density.

	Bilirubin	Bile Salts	Trypsin
Basal	69		
(n = 6)	(49)	60	63
	((37))		(41)
	[150 - 33]	[120 - 38]	[111 - 35]
Stimulated	(89)	(100)	69
(n = 6)	55	63	(48)
	(42)	((44))	((38))
	[124 - 38]	[165 - 37]	[91 - 34]

Table 20

Dominant periods in minutes within the regions significantly (p <0.05) above the estimated random noise level in studies with re-infusion of aspirated duodenal fluid. See further legend to Table 19.

	Bilirubin	Bile Salts	Trypsin
Basal	51	(61)	(106)
(n = 6)		42	63
		((33))	(36)
	[114 - 37]	[95 - 31]	[131 - 31]
Stimulated	60	(70)	(71)
(n = 6)	(39)	42	43
			((35))
	[120 - 32]	[109 - 34]	[105 - 32]

Table 21

Centre value (To, min) of periodicity of the significant regions, and specific width (Wsp) and specific area (Asp) for the secretory parameters bilirubin, bile salts and trypsin, respectively, in studies carried out without re-infusion of aspirated duodenal fluid.

		Bilirubin	Total Bile Salts	Trypsin
Basal	To	54	57	53
(n = 6)	Wsp	1.09	1.71	2.16
	Asp	0.76	0.76	0.70
Stimulated	To	58	60	50
(n = 6)	Wsp	1.31	1.44	5.13
	Asp	0.61	0.89	0.51

N.B. The centre values (To) are all within the range ≤10 min (50-60), *i.e.* within or equal to the sample rate of the studies.

Table 22

Centre value (To, min) of periodicity of the significant regions, and specific width (Wsp) and specific area (Asp) for the secretory parameters bilirubin, bile salts and trypsin, respectively, in studies carried out with re-infusion of aspirated duodenal fluid.

		Bilirubin	Total Bile Salts	Trypsin
Basal	To	56	47	51
(n = 6)	Wsp	0.70	1.11	5.37
	Asp	0.64	0.58	0.74
Stimulated	To	51	52	49
(n = 6)	Wsp	2.97	2.05	2.57
	Asp	0.76	0.77	0.72

N.B. The centre values (To) are all within the range of <10 min (49-56), *i.e.* within the sampling rate of the studies.

Table 23

Power spectral density of the significant regions (peak power density) and total power spectral density in basal and stimulated studies without jejunal re-infusion, geometric mean ± SEM.

	Bilirubin $(\mu g)^2$		Total Bile Salts $(\mu mol)^2$		Trypsin $(IU)^2$	
	Peak power density	Total power density	Peak power density	Total power density	Peak power density	Total power density
Basal	0.57×10^4	0.82×10^4	0.40×10^2	0.54×10^2	1.25×10^3	1.67×10^3
($n = 6$)	2.02×10^4	2.67×10^4	0.95×10^2	1.30×10^2	4.49×10^3	5.78×10^3
	0.33×10^4	0.62×10^4	0.19×10^2	0.29×10^2	0.98×10^3	1.18×10^3
Stimulated	0.36×10^4	0.48×10^4	0.56×10^2	0.62×10^2	1.23×10^3	1.67×10^3
($n = 6$)	$^{**}1.05 \times 10^4$	$^{**}1.69 \times 10^4$	1.38×10^2	1.50×10^2	$^{**}3.14 \times 10^3$ †	5.82×10^3
	0.19×10^4	0.30×10^4	0.28×10^2	0.32×10^2	0.58×10^3	1.28×10^3

* $p<0.05$)
** $p<0.03$) compared with basal studies
*** $p<0.01$)

†According to F-ratio test only.

Table 24

Power spectral density of the significant regions(peak power density) and total power spectral density in basal and stimulated studies with jejunal re-infusion, geometric mean ± SEM.

	Bilirubin $(\mu g)^2$		Total Bile Salts $(\mu mol)^2$		Trypsin $(IU)^2$	
	Peak power density	Total power density	Peak power density	Total power density	Peak power density	Total power density
Basal	0.29×10^4	0.60×10^4	0.53×10^2	0.89×10^2	2.67×10^3	3.30×10^3
(n = 6)	1.74×10^4	2.53×10^4	1.98×10^2	3.35×10^2	5.98×10^3	8.21×10^3
	0.56×10^4	0.35×10^4	0.32×10^2	0.53×10^2	1.15×10^3	1.77×10^3
Stimulated	0.15×10^4	0.21×10^4	0.33×10^2	0.47×10^2	1.72×10^3	2.03×10^3
(n = 6)	***0.64×10^4	***0.84×10^4	**1.11×10^2	**1.31×10^2	*8.20×10^3†	*11.32×10^3†
	0.10×10^4	0.13×10^4	0.18×10^2	0.20×10^2	1.14×10^3	1.78×10^3

*$p \leq 0.05$)
**$p < 0.03$) compared with basal studies
***$p < 0.01$)

†According to F-ratio test only.

Table 25

Total secretory output (6 h) in basal and caerulein/secretin stimulated studies without jejunal re-infusion of aspirated duodenal fluid, geometric mean ± SEM.

	Bilirubin (mg)	Total Bile Salts (mmol)	Trypsin (KIU)
Basal	45.3	4.3	55.2
(n = 6)	(35.3 - 58.1)	(3.3 - 5.6)	(47.1 - 64.7)
Stimulated	*80.5	*12.4	*104.0
(n = 6)	(70.5 - 92.0)	(9.8 - 15.7)	(89.7 - 120.5)

*$p \leq 0.05$ compared with the basal studies.

Table 26

Total secretory output (6 h) in basal and caerulein/secretin stimulated studies with jejunal re-infusion of aspirated duodenal fluid, geometric mean ± SEM.

	Bilirubin (mg)	Total Bile Salts (mmol)	Trypsin (KIU)
Basal	56.1	8.4	47.4
(n = 6)	(37.0 - 85.1)	(5.7 - 12.4)	(37.4 - 60.1)
Stimulated	69.2	9.2	*104.7
(n = 6)	(59.7 - 80.2)	(6.9 - 12.2)	(95.5 - 114.8)

*$p \leq 0.05$ compared with the basal studies.

of changes in total power between basal and stimulated studies is shown in Tables 23 and 24. Data on the total secretory output in these studies is shown in Tables 25 and 26.

7.4 DISCUSSION

Power spectral analysis has been used on many biological signals in the past, including electroencephalographs, electromyographs, blood pressure changes and heart rate. Nonetheless, it does not seem to have been used on data on biliary and pancreatic secretion.

The power spectral analysis presented here shows that there were no obvious shifts in peak values (Tables 19 and 20) or centre values (Tables 21 and 22) of periodicity between basal and stimulated studies. For bilirubin and total bile salts these values were between 50-70 min, indicating that the main fluctuations in biliary secretion occurred at that periodicity. However, subsidiary periods around 40 min and 90-110 min were observed in the stimulated studies (Figs. 62 to 67). Attenuation of the power spectrum made it difficult to detect definite density peaks for trypsin secretion.

Peak power density and total power density of biliary secretion were significantly reduced in caerulein/secretin stimulated studies, compared with basal studies (Tables 23 and 24), and at least relatively to density / the power spectrum was more distributed in the periodicity domain in these studies, as shown by an increase in specific width. There were, however, little changes in specific area, as fall in peak power density was usually followed by a fall in total power with little or no change in the ratio of these two parameters.

The present results show that the combined background stimulation of the secretory output by caerulein and secretin was effective (Tables 25 and 26) and that it stabilised the biliary secretion. Probably this was caused rather by an effect on the gall bladder than on the sphincter of Oddi, as the secretion of trypsin, modulated by the sphincter of Oddi but not the gall bladder, showed contradictory results.

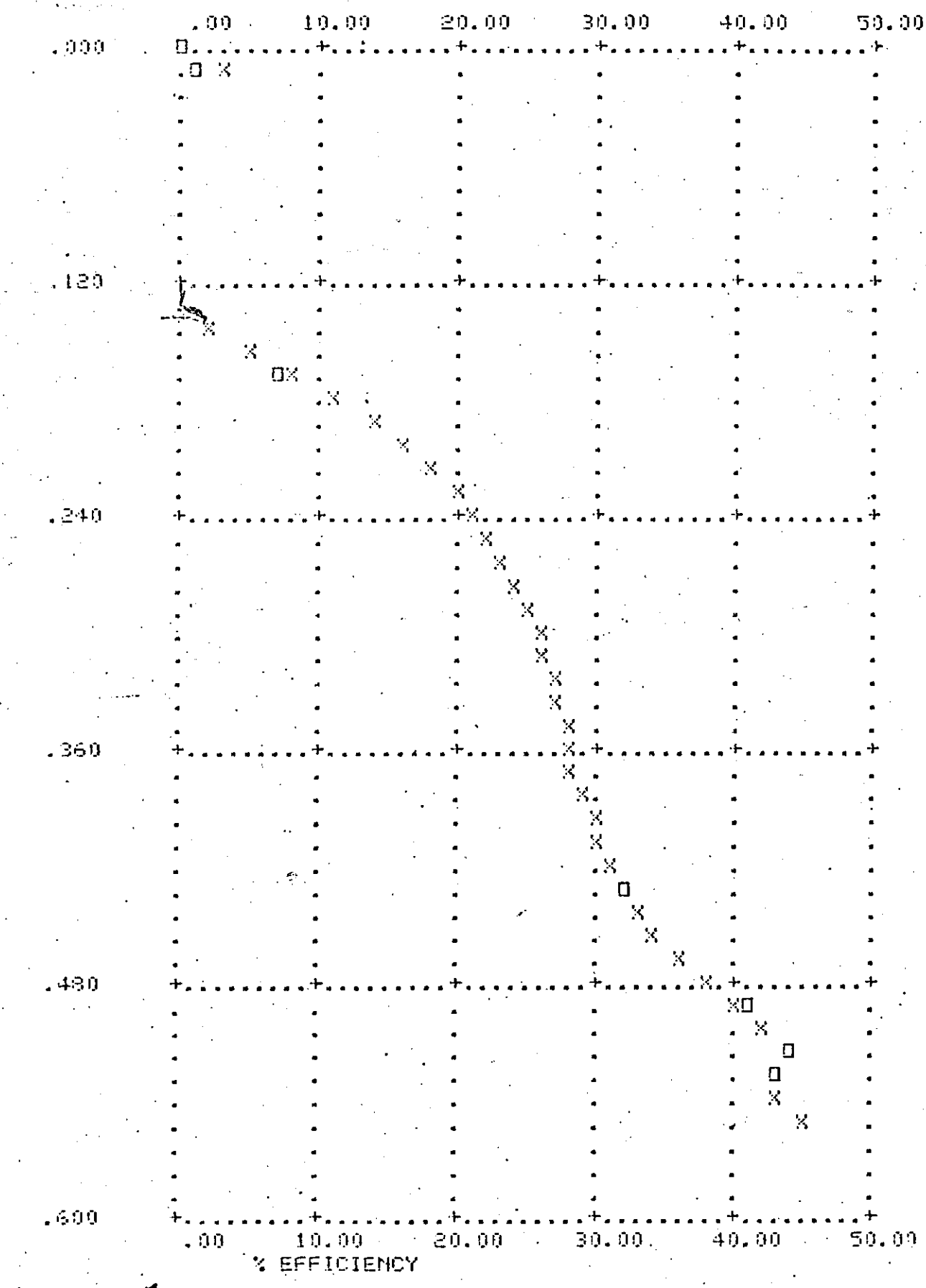
7.5 SUMMARY

(1) Power spectral analysis was carried out on data on biliary and pancreatic secretion into the duodenum in man.

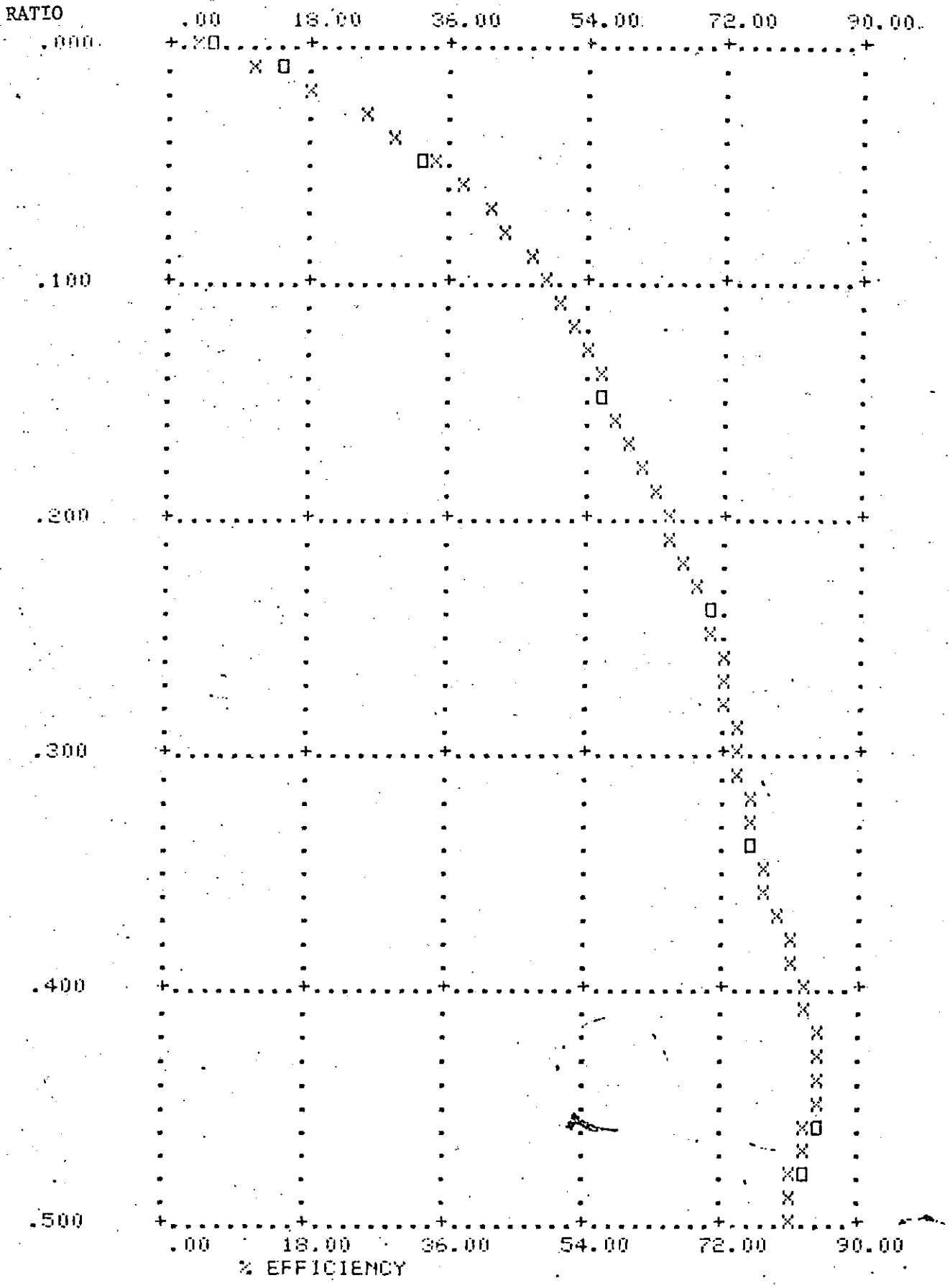
(2) In the basal state, marked fluctuations were observed in biliary and pancreatic secretion with a dominant periodicity of 50-70 min.

(3) During caerulein/secretin stimulation, total biliary and pancreatic secretion was increased compared with basal studies. There was a dominant periodicity of 50-70 min in the stimulated state similar to the basal studies, but subsidiary periods of around 40 min and 90 - 110 min were also observed. Peak power density as well as total power density were reduced in the stimulated studies compared with the basal studies, indicating stabilisation of the secretion.

APPENDIX



Appendix I A computer plot of the counting efficiency of the low energy quenched standard of ^{3}H PEG 4000 prepared in varying amounts of picric acid. Note full scale efficiency 50.0%.



Appendix II A computer plot of the counting efficiency of the high energy quenched standard of ^{14}C PEG 4000 prepared in varying amounts picric acid. Note full scale efficiency 90.0%:

APPENDIX III Percentage recovery of phenol red and ^{14}C PEG 4000 from the stomach and duodenum, perfusion flow rate and steady state of ^{14}C PEG 4000, during the 2 h equilibration period in 66 normal subjects.

No.	Efficiency of gastric aspiration %	Gastro-duodenal flux % PR	Efficiency of duodenal aspiration %	Duodeno-gastric reflux % ^{14}C PEG 4000	Perfusion flow rate ml min^{-1}	'Steady state' CV of ^{14}C PEG 4000
1	89.4 ± 4.0	14.2 ± 1.6	59.0 ± 9.4	29.7 ± 1.7	7.5	10.7
2	97.6 ± 9.8	7.7 ± 0.7	31.1 ± 3.4	1.2 ± 0.8	9.8	5.9
3	96.2 ± 15.2	8.5 ± 0.3	54.9 ± 5.1	5.3 ± 1.4	10.1	2.9
4	84.2 ± 6.0	4.9 ± 0.2	79.4 ± 5.8	3.6 ± 0.8	9.0	4.4
5	85.9 ± 3.8	23.2 ± 1.0	50.3 ± 3.7	19.2 ± 2.2	10.0	6.3
6	97.4 ± 3.7	8.0 ± 2.0	40.5 ± 3.6	4.7 ± 1.3	7.4	8.4
7	81.5 ± 12.6	6.2 ± 0.1	49.7 ± 6.6	3.9 ± 0.7	9.0	6.9
8	96.2 ± 9.0	10.2 ± 0.7	50.6 ± 2.9	5.9 ± 1.3	8.7	7.5
9	81.0 ± 8.6	13.2 ± 3.8	55.3 ± 3.0	11.9 ± 1.4	8.8	3.9
10	91.8 ± 6.1	-	44.9 ± 3.5	15.5 ± 3.6	8.6	5.4
11	68.7 ± 5.2	27.2 ± 7.9	21.9 ± 2.4	8.7 ± 2.9	7.3	28.0
12	64.7 ± 6.6	29.5 ± 8.5	25.6 ± 6.0	1.3 ± 0.7	7.1	20.9
13	81.6 ± 6.5	21.7 ± 1.0	38.3 ± 4.1	12.9 ± 2.7	7.5	29.3
14	79.7 ± 5.0	4.4 ± 1.3	41.0 ± 3.6	21.9 ± 4.0	7.5	19.0
15	60.1 ± 10.8	3.8 ± 0.2	24.9 ± 3.0	37.8 ± 6.4	6.6	18.2
16	75.6 ± 6.9	4.5 ± 0.3	29.9 ± 3.0	13.9 ± 4.6	6.7	21.6
17	82.6 ± 10.6	13.5 ± 1.6	34.9 ± 3.6	20.6 ± 3.1	6.8	9.9
18	87.4 ± 8.1	8.7 ± 0.6	55.2 ± 7.7	10.5 ± 2.5	6.8	25.1
19	91.9 ± 9.1	11.7 ± 0.5	54.9 ± 4.9	1.4 ± 0.2	7.7	9.6
20	83.8 ± 10.3	17.0 ± 1.5	54.5 ± 5.5	16.5 ± 4.6	8.8	9.8
21	61.6 ± 6.4	42.9 ± 10.4	45.0 ± 8.1	20.3 ± 3.6	7.3	31.0
22	79.5 ± 8.9	29.2 ± 0.9	53.0 ± 6.3	14.3 ± 2.1	7.4	7.7
23	77.6 ± 7.8	32.5 ± 5.5	82.0 ± 5.7	2.0 ± 0.6	8.1	5.0
24	66.6 ± 7.4	12.6 ± 1.3	49.4 ± 3.3	4.9 ± 1.5	7.6	18.4
25	76.6 ± 10.3	11.9 ± 1.6	30.8 ± 5.1	21.5 ± 5.3	7.8	24.1
26	85.0 ± 15.3	5.7 ± 0.3	42.7 ± 3.8	25.2 ± 5.5	7.7	17.5

No.	Efficiency of gastric aspiration %	Gastro-duodenal flux % PR	Efficiency of duodenal aspiration %	Duodeno-gastric reflux % ^{14}C PEG 4000	Perfusion flow rate ml min^{-1}	'Steady state' CV of ^{14}C PEG 4000
27	76.3 ± 12.2	-	70.4 ± 4.0	1.7 ± 0.3	6.0	6.1
28	66.2 ± 10.3	7.9 ± 1.4	31.0 ± 4.0	6.1 ± 1.2	7.0	17.3
29	75.3 ± 10.8	12.7 ± 1.7	36.9 ± 5.2	3.6 ± 1.1	7.0	14.6
30	95.3 ± 16.5	10.7 ± 1.4	77.0 ± 7.3	8.1 ± 3.1	8.4	7.9
31	77.9 ± 11.2	14.4 ± 1.2	52.7 ± 4.7	6.1 ± 1.0	8.2	7.8
32	85.5 ± 7.3	15.2 ± 0.5	76.0 ± 5.3	0.6 ± 0.2	5.6	7.6
33	91.3 ± 11.0	15.2 ± 1.8	85.1 ± 4.5	0.3 ± 0.1	5.4	12.0
34	91.6 ± 5.2	24.3 ± 2.9	63.6 ± 5.4	5.8 ± 1.9	5.5	17.8
35	63.8 ± 8.2	37.5 ± 6.2	44.0 ± 2.5	2.3 ± 0.4	13.5	9.7
36	99.6 ± 15.9	1.4 ± 0.8	57.8 ± 6.7	14.3 ± 2.2	5.4	8.6
37	88.5 ± 7.2	10.2 ± 1.9	76.5 ± 5.1	2.5 ± 0.6	5.6	20.9
38	84.4 ± 13.0	19.3 ± 3.0	66.4 ± 5.3	6.8 ± 3.5	5.4	18.2
39	92.3 ± 8.7	20.6 ± 1.8	62.7 ± 5.0	5.6 ± 1.7	5.1	14.6
40	77.9 ± 4.7	23.0 ± 3.1	57.6 ± 5.7	1.2 ± 1.1	5.2	18.2
41	87.0 ± 9.8	-	70.2 ± 9.6	6.8 ± 1.0	9.0	5.5
42	77.2 ± 4.8	34.5 ± 7.5	37.2 ± 7.3	3.2 ± 0.9	7.9	33.8
43	72.3 ± 10.3	33.3 ± 3.5	60.7 ± 7.4	10.5 ± 1.9	5.5	17.5
44	81.2 ± 11.6	18.1 ± 2.7	63.2 ± 7.2	8.2 ± 1.5	5.6	11.0
45	89.5 ± 4.2	26.7 ± 7.1	37.2 ± 4.5	2.7 ± 2.2	7.9	6.7
46	75.9 ± 5.3	18.0 ± 2.0	39.3 ± 5.7	15.9 ± 2.8	7.7	35.1
47	62.2 ± 3.6	12.1 ± 0.6	26.4 ± 4.0	19.2 ± 2.2	7.7	12.4
48	75.8 ± 4.9	15.0 ± 0.8	58.6 ± 4.5	8.2 ± 2.6	7.5	13.3
49	77.6 ± 7.4	14.4 ± 0.9	70.5 ± 4.4	14.0 ± 1.4	7.6	17.8
50	97.3 ± 5.3	10.6 ± 1.0	70.6 ± 8.2	8.9 ± 1.4	8.9	6.6
51	88.1 ± 8.6	8.4 ± 4.4	61.4 ± 3.5	7.5 ± 1.5	8.9	12.7
52	99.5 ± 9.1	11.2 ± 2.3	54.7 ± 5.9	2.8 ± 1.2	9.0	12.5

No.	Efficiency of gastric aspiration %	Gastro-duodenal flux % PR	Efficiency of duodenal aspiration %	Duodeno-gastric reflux % ^{14}C PEG 4000	Perfusion flow rate ml min^{-1}	'Steady state' CV of ^{14}C PEG 4000
53	95.7 ± 13.6	10.7 ± 0.8	46.6 ± 4.1	5.2 ± 0.9	4.0	7.5
54	63.9 ± 6.9	14.1 ± 2.4	6.26 ± 5.1	1.8 ± 1.1	6.2	9.5
55	75.0 ± 7.7	5.6 ± 1.6	59.4 ± 5.0	3.4 ± 0.8	8.8	6.4
56	89.9 ± 5.0	7.4 ± 0.7	63.1 ± 4.2	12.3 ± 2.7	3.7	12.4
57	91.0 ± 4.3	2.7 ± 0.4	66.7 ± 6.6	1.2 ± 0.6	3.2	31.3
58	96.5 ± 6.3	-	60.7 ± 6.0	0.7 ± 0.1	3.3	4.7
59	93.3 ± 11.7	10.6 ± 0.9	31.9 ± 2.2	0.3 ± 0.1	3.4	7.5
60	92.9 ± 8.4	8.6 ± 0.7	51.0 ± 3.7	4.1 ± 1.2	3.2	11.5
61	72.0 ± 7.0	22.6 ± 2.9	19.7 ± 2.2	16.9 ± 2.3	8.6	10.4
62	84.6 ± 13.0	16.0 ± 1.7	47.4 ± 3.5	3.0 ± 1.7	3.0	9.9
63	69.2 ± 8.2	41.3 ± 8.8	17.9 ± 3.2	12.2 ± 2.3	8.3	11.2
64	90.3 ± 9.1	11.5 ± 0.6	38.7 ± 2.7	5.7 ± 1.0	8.1	5.1
65	96.0 ± 9.2	11.4 ± 0.8	37.2 ± 6.2	14.9 ± 2.6	10.5	2.0
66	65.2 ± 6.7	22.7 ± 9.7	79.6 ± 10.0	2.5 ± 0.8	13.5	11.4
$\bar{x}$ ± SEM	81.5 ± 8.5	15.7 ± 2.4	50.4 ± 5.0	9.0 ± 1.9	7.3 ± 0.3	13.1 ± 0.1

APPENDIX IV Buffers of constant and low ionic strength for spectrophotometric pK determinations (I = 0.01). Figures indicate ml. (Note: all quantities to final volume of 100 ml by water). *I >0.01. See further ref. 81.

pH:	-0.5	0.40	1.00	1.60	2.20	3.00	4.00	5.00	6.00	7.00	8.00	9.00	10.00	11.00	11.60	12.40	13.0
3M HCl	*100																
0.1M HCO_2H							15.03										
0.1M CH_3CO_2H								15.34	20.07								
0.02M KH_2PO_4										13.96							
0.01M K_2HPO_4										24.04							
0.025M $Na_2B_4O_7$												20.00					
0.1M KOH					2.76	8.89	9.98	9.99									
0.05M KOH									14.00								
0.1M Tris											15.84						
0.1M HCl											10.00			9.23	6.94		
0.1M H_3BO_3												6.89					
0.02M $NaHCO_3$													17.41				
0.01M Na_2CO_3													21.89				
0.2M KCl				*34.0												*25	*25
1M HCl		*100															
0.1M $ClCH_2CO_2H$					50.99	16.21											
0.2M Butylaine free base														12.28	33.97		
0.2M HCl			*67.0	*16.0												*16.2	*66.

APPENDIX V Calculation of bisquare mean and variance (robust analysis*).

Step 1: Calculate the median $\hat{\theta}_o$ for α_i ($i = 1 \ldots 6$)

Step 2: Calculate the residual $r_i = \alpha_i - \hat{\theta}_o$

Step 3: Calculate the median for $|r_i| = S_o$

Step 4: Calculate the weight factor $w_i = \left[1 - \left(\frac{r_i}{6S_o}\right)^2\right]^2$

Step 5: Calculate $w_i \cdot \alpha_i$

Step 6: Calculate $\hat{\theta}_1 = \frac{\sum_{i=1}^{n} w_i \alpha_i}{\sum_{i=1}^{n} w_i}$

Step 7: Repeat this process by using $\hat{\theta}_1$ as the new median, *i.e.* $r_i = \alpha_i - \hat{\theta}_1$ and find $\hat{\theta}_2$. If there is a difference between $\hat{\theta}_1$ and $\hat{\theta}_2$, carry on calculating $\hat{\theta}$ until there is no difference between $\hat{\theta}_{x-1}$ and $\hat{\theta}_x$ and the bisquare mean has been found.

Step 8: Calculate the variance for the bisquare mean ($\hat{\theta}_x$)

$$\text{var}\,\hat{\theta}_x = \left(\frac{n}{n-1}\right) \frac{\sum_{i=1}^{n} w_i^2 r_i^2}{\left[\Sigma^1 (1 - \left(\frac{r_i}{6S}\right)^2)(1-5\left(\frac{r_i}{6S}\right)^2)\right]^2}$$

$$SD = \sqrt{\text{var}\,\hat{\theta}_x}$$

S = the median for $|r_i|$ from the calculation of $\hat{\theta}_x$

Robust analysis = analysis based on the property (robustness) that if changing a small part of the body of the data, no matter what part or how substantially, no substantial change is made in the summary of the data. Bisquare mean is a more robust tool of summing up data than arithmetic mean or geometric mean since it gives outliers low weight so their influence on the mean is minimal (267).

APPENDIX VI Cubic spline interpolation.

A cubic spline interpolation (a piecewise-cubic interpolation) is based on constructing a function, $g_3(t)$ (a 3rd degree polynomial), which interpolates between sampled data values $1(t_i)$ at points t_i; $i = 1, \ldots N$. In writing $g_3(t)_i$ for each cubic piece of $g_3(t)$ around t_i as:

$$g_3(t)_i = c_{o,i} + c_{1,i}(t - t_i) + c_{2,i}(t - t_i)^2 + c_{3,i}(t - t_i)^3$$

the $c_{k,i}$; $k = 0, \ldots 3$, are computed with the constraints that $g_3(t)$ goes through the data point at the sample instants, *i.e.* $g_3(t)c = t_i = 1(t_i)$, and that $g_3(t)$ is at sample points twice continuously differentiable ("the spline condition"). This is obviously an extension of a more familiar line approximation $g_1(t) = c_{o,i} + c_{1,i}(t - t_i)$ (*i.e.* if we set $c_{2,i} = c_{3,i} = 0$), and a parabolic approximation $g_2(t) = c_{o,i} + c_{1,i}(t - t_i) + c_{2,i}(t - t_i)^2$ (*i.e.* if $c_{3,i} = 0$).

The spline interpolation takes its name from the draftman's "spline", since it approximates the shape of a flexible ruler when constrained to pass through the points $(t_i, 1_i)$.

APPENDIX VII Equations on which the power spectral analysis was based.[a]

The power spectral analysis (67) was carried out on the secretory patterns (raw data) of bilirubin, total bile salts and trypsin by using the following equations (for further details of computation, see ref. 67, p. 286 and pp. 466-468).

$$\hat{S}_N^K (mf) = \frac{1}{K} \sum_{k=1}^{K} S_{N,k} (mf) \quad (1)$$

$$S_{N,k} (mf) = \frac{N}{U} \cdot X_{N,k} (mf) \cdot X^*_{N,k} (mf) \quad (2)$$

$$X_{N,k} (mf) = \frac{1}{N} \sum_{n=0}^{N-1} w_n x_n (nT) e^{-j2\pi mfnT} \quad (3)$$

$$w_n = \tfrac{1}{2} (1 - \cos \frac{\pi n T}{T_o}); n = \begin{matrix} 1, \ldots 0.1\,N \\ 0.9\,N \ldots N \end{matrix} \quad (4); \quad U = \frac{1}{N} \sum_{n=0}^{N-1} w_n^2 \quad (5)$$

$\hat{S}_N^K$: the average power spectral density (PSD) over K individuals of N number samples.

$S_{N,k}$ (mf): PSD of the k-th individual estimated as a "periodogram" (defined by the equation above).

$X_{N,k}$ (mf): Discrete Fourier-transformation of window's data-samples.

w_n: a time window (cosine window); was applied to taper the 10% leading and tailing ends of the data-series to reduce discontinuities due to leakage (Gibb's phenomena). Further window-smoothing to secure convergence of the transformation was not necessary.

N: total number of samples.

T: the interval between samples in the time domain in sec (10 · 60 sec).

f: 1/NT, the interval between points on the frequency scale in Hz.

m: index for the frequency.

n: index for the samples.

x: the total amount of bilirubin (µg), total bile salts (µmol) or trypsin (IU).

e: 2.72, the base for the natural logarithm.

j: $\sqrt{-1}$.

T_o: 0.1 · N = 3.6 = 10% of the total duration of the experiment expressed as number of points.

U: compensation factor. π: 180°

a) This work was carried out in collaboration with Dr. S. Björnsson and colleagues, Department of Electrical Engineering, Gömlu Loftsskeytastödinni, University of Iceland.

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List of Publications from this Work

The co-authors are thanked for their contribution.

1. Greenberg, G.R., McCloy, R.F., Björnsson, Ó.G., Adrian, T.E., Chadwick, V.S., Baron, J.H. and Bloom, S.R. (1978) Investigation of a physiological role for pancreatic polypeptide (PP) in man. Scand. J. Gastroenterol. *Suppl. 13*, 68 (Abstract)

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4. Björnsson, Ó.G., Christofides, N.D., Adrian, T.E., Rump, S., Fletcher, D., Bloom, S.R. and Chadwick, V.S. (1979) Effects of duodenal perfusion of sodium taurocholate on biliary and pancreatic output and gut hormone release in man. Clin. Sci. *57*, 29P (Abstract)

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6. Björnsson, Ó.G. and Chadwick, V.S. (1979) The use of four-lumen duodenal perfusion tube technique and indocyanine green (ICG) as a biliary marker to quantify gallbladder storage and emptying in man. 1st Biannual Meeting of Icelandic Medical Doctors, September, p. 17 (Abstract)

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