EFFECT OF SECRETIN, CHOLECYSTOKININ OCTAPEPTIDE (CCK-8) AND A SOMATOSTATIN ANALOGUE ON EXPERIMENTAL PANCREATIC CARCINOGENESIS

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DEDICATION

This thesis is dedicated to my loving parents,

Harry and Violet Haddock.

I owe them so much.

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LIST OF ABBREVIATIONS

The following abbreviations are used in this thesis:

AACN	Atypical acinar cell nodule
BAP	N-nitrosobis(2-acetoxypropyl)amine
BHP	N-nitrosobis(2-hydroxypropyl)amine
BOP	N-nitrosobis(2-oxopropyl)amine
BRDU	Bromodeoxyuridine
BW	Body weight
CCK	Cholecystokinin
CCK-8	Cholecystokinin octapeptide
CIS	Carcinona-in-situ
DCA	Deoxycholic acid
DHPN	N-nitroso-2,2'-dihydroxy-di-n-propylamine or Di-hydroxy-di-n-propyl nitrosamine (same chemical)
DMNM	2,6-dimethylnitrosomorpholine
DPN	N-nitroso-dipropylamine
EGF	Epidermal growth factor
GHRIH	Growth hormone release inhibiting hormone
GIP	Gastric inhibitory polypeptide
H&E	Haematoxylin & eosin
HPOP	N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine
2-HPPN	N-nitroso-(2-hydroxypropyl)-n-propylamine
IDDM	Insulin-dependent diabetes mellitus
IP	Intraperitoneal
LCA	Lithocholic acid
MFO	Mixed function oxidase
MPN	N-nitrosomethyl-n-propylamine
NADPH	Nicotinamide adenine dinucleotide phosphate
NIDDM	Non-insulin dependent diabetes mellitus
OPPN	N-nitroso-(2-oxopropyl)-n-propylamine
Q1:Q3	Interquartile range
SC	Subcutaneous
SMR	Standard mortality rate
SRIF	Somatotropin release inhibiting factor
VIP	Vasoactive intestinal polypeptide

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Dr. David Harrison, Senior Lecturer in the University Department of Pathology, University of Edinburgh proved to be a helpful collaborator in some aspects of this work, particularly with respect to general histological guidance and to the analyses, both histological and flow cytometric, outlined in

Chapter 6. Mr. Eric Miller, technician in the University Department of Pathology, University of Edinburgh, carried out the tissue preparation and flow cytometric analysis decribed in the same chapter.

The Imperial Cancer Research Fund (ICRF) provided funds to allow part of this work to be undertaken. Sandoz Pharma Division (Basle, Switzerland) supplied the somatostatin analogue, SMS 201-995 used in Chapters 3, 4 and 5. Both are due my sincere thanks.

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PRESENTATIONS AND PUBLICATIONS

Parts of this work have been presented to learned societies:

1. Haddock, G., Harrison, D.J. & Carter, D.C.
   The effect of SMS 201-995 on experimental pancreatic carcinogenesis in the Syrian golden hamster. February 1990
   Caledonian Society of Gastroenterology, Glasgow.

2. Haddock, G., Harrison, D.J. & Carter, D.C.
   The effect of SMS 201-995 on experimental pancreatic carcinogenesis in the Syrian golden hamster. September 1990
   British Society of Gastroenterology, Southampton.

3. Haddock, G., Miller, E.P., Harrison, D.J. & Carter, D.C.
   Flow cytometric analysis of BOP treated hamster pancreas. September 1990
   British Society of Gastroenterology, Southampton.

4. Haddock, G., Miller, E.P.M., Bishop, D., Carter, D.C. & Harrison, D.J.
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   Pathological Society of Great Britain and Ireland, Cambridge.
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The following papers from this thesis have been published:

1. Haddock, G. & Carter, D.C. (1990)
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2. Haddock, G., Harrison, D.J. & Carter, D.C. (1991)
   The effect of the somatostatin analogue SMS 201-995 on experimental pancreatic carcinogenesis in the Syrian golden hamster.
   Carcinogenesis, 12, 1103-1107.

DECLARATION

These studies were performed between October 1987 and September 1989 when I was a Research Fellow in the University Department of Surgery, Royal Infirmary, Glasgow and subsequently in the University Department of Surgery, Royal Infirmary, Edinburgh.

I declare that I am the sole author of this thesis. All the studies were planned and carried out by myself except as acknowledged above. When assistance has been obtained from others, such help has been freely acknowledged.

SUMMARY OF THESIS

The incidence of pancreatic cancer has increased throughout the world in the last 60 years such that it is now the fourth leading cause of cancer death in the United Kingdom. The tumour usually presents at an advanced stage and cure is rarely possible.

Extensive epidemiological investigations during this century suggest that cigarette smoking is the most consistent aetiological factor in the development of pancreatic cancer. Differences in dietary consumption of meat, fat, fruit and vegetables have also been implicated in altering susceptibility to this tumour; high consumption of fat and protein has been thought to increase risk while consumption of fruit and vegetables has been thought to be protective. It remains unclear how these dietary differences affect the risk of developing pancreatic cancer. One possible mechanism that has been suggested for the effects of protein and fat in this regard has been through their influence on endogenous secretion of gastrointestinal hormones such as secretin and cholecystokinin.

The late presentation of pancreatic cancer in man makes investigation of the early stage of the carcinogenic process impossible. Two animal models of pancreatic carcinogenesis have been developed in the last 20 years which have allowed investigators to increase our knowledge of some of the processes which

go on in this disease. Azaserine has been shown to induce pancreatic acinar cell tumours in rats whereas the nitrosamine family of chemicals, in particular N-nitrosobis(2-oxopropyl)amine (BOP), induces pancreatic tumours with a ductal or ductular morphology in the Syrian golden hamster.

The BOP hamster has generally been accepted as the best model for investigating aspects of pancreatic carcinogenesis. In addition to the fact that the tumour induced in the hamster is morphologically similar to the commonest form of human pancreatic cancer (namely ductal adenocarcinoma), the clinical features in the hamster with advanced disease resemble those seen in man, including a propensity for the tumour to metastasize to the liver and other organs.

The BOP hamster model has been used widely to investigate some of the factors which might predispose to the development of pancreatic cancer in man. Given that secretin and cholecystokinin have been implicated in the role of diet in the aetiology of human pancreatic cancer, the effects of these and other gastrointestinal hormones have been investigated in the BOP-hamster model. Cholecystokinin and a number of its analogues and secretin have been reported to increase the incidence and extent of BOP induced pancreatic cancer in hamsters. Somatostatin, a gastrointestinal hormone with numerous, predominantly inhibitory, actions, (and largely because of these

inhibitory properties,) has been proposed as a possible treatment for pancreatic cancer in man. The aims of this thesis were to investigate the effects of secretin, an octapeptide analogue of cholecystokinin (CCK-8) and a long-acting somatostatin analogue (SMS 201-995) on BOP induced pancreatic carcinogenesis in the Syrian hamster.

The hamster model was successfully established. Intraperitoneal (IP) and subcutaneous (SC) BOP were compared for effect on the pancreas. Lesions seen throughout the 20 weeks study period were as previously reported in the literature for both groups. The degree and extent of pre-malignant and malignant change seemed to be greater after 20 weeks in the SC group compared to the IP group.

In the second experiment, CCK-8 and secretin stimulated pancreatic juice output when infused intravenously. Pancreatic juice protein and bicarbonate output were also increased. SMS 201-995 suppressed pancreatic juice output. When infused simultaneously with either secretin or CCK-8, SMS 201-995 was still able to suppress pancreatic juice output.

Repeated SC injections of secretin, CCK-8 and SMS 201-995 did not have any effect on pancreatic wet weight or DNA content after one or six weeks of treatment.

When administered with the carcinogen BOP, CCK-8 did not seem to influence pancreatic carcinogenesis. Secretin and, surprisingly, SMS 201-995, did seem to promote the development of pre-malignant and malignant pancreatic lesions seen histologically. Given that small doses of SMS 201-995 seem to promote pancreatic carcinogenesis in the hamster model, and given the wide interperson variation seen in man in relation to the pharmacology of many compounds, further investigation into the properties of somatostatin analogues should be undertaken before suggesting that these substances should be used to treat human pancreatic cancer.

In the light of the finding that the hormones could influence pancreatic carcinogenesis in doses which did not seem to influence pancreatic wet weight or DNA content, a further experiment was carried out. Flow cytometry has been used for many years to investigate tumour cell ploidy and cell cycle activity. This technique was used to investigate the effects of BOP on pancreatic cell cycle activity in the hamster. Bromodeoxyuridine (BRDU) immunostaining was used to identify which cells were undergoing proliferation. BOP significantly increased cell cycle activity during the 20 week experiment. Percentage S-phase fraction and G_2/M fraction both increased. BRDU immunostaining demonstrated that the cells which were undergoing proliferation were predominantly ductal and

ductular cells.

In conclusion, the nitrosamine carcinogen BOP causes cellular damage and subsequent proliferation in the pancreas. These proliferating cells from which tumours probably develop might be susceptible to the effects of other substances, including gastrointestinal hormones, which have been shown to act as tumour promotors in animal models. Nitrosamines, which are carcinogenic in the hamster, are present in cigarette smoke and cigarette smoking has been identified as the most likely aetiological factor for pancreatic cancer in man. Dietary factors, which have been implicated in increasing risk for human pancreatic cancer, could act through their effects on various gastrointestinal hormones thus providing a mechanism whereby diet could act as a tumour promotor in man. SMS 201-995 seemed to act as a tumour promotor in the hamster model. Its use to treat pancreatic cancer in man cannot yet be justified.

CHAPTER 1

PANCREATIC CANCER

HISTORICAL REVIEW

1.1 Introduction

1.2 Demographics

1.3 Aetiology/Epidemiology

1.4 Human Pathology

1.5 Animal Models of Pancreatic Cancer

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1.9 Summary

1.1 Introduction

The incidence of pancreatic cancer has increased steadily in the last 60 years throughout the world. Diagnosis is not often made until the disease is at an advanced stage and no reliable screening tests exist to detect early disease. Curative surgical therapy is only applicable to a small minority of patients and, even where possible, carries a high operative mortality and morbidity. Effective palliation is the aim of treatment in most patients but even this is sometimes not attainable. Responses to chemotherapy and radiotherapy have been singularly disappointing.

1.2 Demographics

The incidence of pancreatic cancer has increased in the last 60 years such that it is now the fourth leading cause of cancer death in the United States of America after lung, colorectal and breast cancer (National Center for Health Statistics, 1968). Between 1920 and 1970 the age-adjusted mortality rate for pancreatic cancer in the USA increased from 2.9 per 100,000 to 9 per 100,000 (Gordis and Gold, 1984). In England and Wales, the incidence of pancreatic cancer doubled between 1930 and 1970 (Office of Population Censuses and Surveys, 1975). In 1950, the mortality rate for pancreatic cancer in Japan was 1 per 100,000 population; in 1974 this had risen to nearly 6 per 100,000 population (Aoki and Ogawa, 1978). In recent years, it has been suggested that the annual incidence in the USA is now levelling off at approximately 10 new cases per 100,000 population (Gordis and Gold, 1984).

The incidence of pancreatic cancer varies with age, sex and race. In most countries the incidence is higher in males than in females by a ratio of about two to one (Aoki and Ogawa, 1978). The disease is rare in those under 25 years of age and uncommon under the age of 45 years (Morgan and Wormsley, 1977). Over 80% of cases occur in the 60-80 years age group (Gordis and Gold, 1984). Pancreatic cancer is more common in Western or industrialised countries,

although the highest incidence in men occurs in New Zealand Maoris, native Hawaiians and black Americans and in women, Hawaiians, Latins in El Paso and Maoris. The lowest rates are reported in Nigeria and India. In the USA, in both sexes, blacks have a higher rate of pancreatic cancer than whites (Fraumeni, 1975).

It is unclear whether geographic and racial demographic differences represent differences in genetic susceptibility to developing pancreatic cancer, or differences in exposure to environmental influences. Studies of migrating populations, which have been useful in investigating the aetiology of other tumours, have largely failed to shed light on the aetiology of pancreatic cancer. In 1957, Smith reported on the incidence of pancreatic cancer among Japanese immigrants to the United States of America. Migrants from Japan to America had a higher incidence of pancreatic cancer than native Americans or Japanese living in Japan. The pancreatic cancer risk to American-born offspring of such Japanese migrants to the USA was at a level between those of the native populations of the two countries in subsequent studies (Buell and Dunn, 1968; Haenszel and Kurihara, 1968). It has been suggested that this discrepancy in incidence rates might be explained by undercertification of pancreatic cancer deaths in Japan or by certification of some such cases as gastric cancer, a more common tumour in that country

(Jablon et al, 1966; Haenszel and Kurihara, 1968). Similar confusing migrant studies have been carried out on the negro populations of America; in one study, northwards migrating negroes born in the south of the USA, assumed a higher incidence rate than negroes born in the northern state of Ohio (Mancusco and Sterling, 1974).

Some religious groups experience a higher than average susceptibility to the development of pancreatic cancer. In studies based in New York City, pancreatic cancer occurred more frequently among Jews than Catholics or Protestants (MacMahon, 1960; Newill, 1961). Similar findings were reported by Seidman (1970); differences were particularly striking between Jewish and non-Jewish women. Enstrom (1978) reported that Utah Mormons had a lower risk of developing pancreatic cancer than non-smoking white American males although a previous Utah-based survey demonstrated low rates of pancreatic cancer in both Mormons and non-Mormons (Lyon et al, 1976).

1.3 Aetiology/Epidemiology

a) Smoking

Many epidemiological studies have indicated that the most consistent risk factor for the development of pancreatic cancer is cigarette smoking. In a study of US veterans, Kahn (1966) reported a relative risk in cigarette smokers compared to non-smokers of 1.84:1. In the same year, Best (1966) demonstrated a dose-response relationship between the number of cigarettes smoked and the mortality rate from pancreatic cancer. This finding has been confirmed by others (Hammond, 1966; Hirayama, 1977; Mack et al, 1986). In 1976, cigarette smoking male doctors were shown to have a relative risk of dying from pancreatic cancer of 1.6:1 when compared to non-smoking controls (Doll and Peto, 1976). A Swedish group reported a mortality ratio of 3.1:1 for cigarette smoking males and 2.5:1 for females (Cederlof et al, 1975). It is therefore generally accepted that cigarette smoking doubles the risk of developing pancreatic cancer present in a non-smoking population (Fraumeni, 1975). The increased risk of developing pancreatic cancer does not seem to apply to pipe or cigar smokers (Kahn, 1966; MacMahon et al, 1981) although not all authors have agreed with this assertion (Best, 1966; Wynder et al, 1973). Krain provided circumstantial evidence in support of the role of cigarette smoking by indicating that the

increase in cigarette smoking from 1940 to 1970 in the USA paralleled the increase in pancreatic cancer (Krain, 1970). This conclusion was supported in a study of smoking trends and pancreatic cancer mortality rates in the USA between 1920 and 1978 (Weiss and Bernarde, 1983). It demonstrated that the mortality rate for pancreatic cancer closely shadowed rises and falls in the incidence of cigarette smoking. This relationship was more striking in males than in females. Unfortunately, both of these papers can be criticised on methodological grounds in that the figures used to calculate mortality trends were not entirely comparable.

Despite a substantial body of published data supporting the relationship between smoking and pancreatic cancer, there have been a number of studies which have failed to demonstrate this relationship. In a study of cancer incidence and mortality published in 1975, Armstrong and Doll failed to demonstrate any relationship between smoking and pancreatic cancer. Surprisingly, they also failed to demonstrate a strong relationship between lung cancer and cigarette smoking and admitted that there were several problems with the analytical methods used in their study. They also emphasised that failure to demonstrate a correlation between two variables in a population did not necessarily exclude a relationship in individuals. Binstock and colleagues (1983) reported a weak but

statistically non-significant relationship between pancreatic cancer and smoking in a study concerned primarily with the putative link with coffee consumption. In a retrospective case-control study, Durbec et al (1983) reported an association between pancreatic cancer and alcohol consumption and smoking taken together, although alcohol seemed to be more important than tobacco.

While it seems likely that cigarette smoking does increase the risk of developing pancreatic cancer, the mechanism whereby this occurs remains obscure. Wynder (1975) hypothesised that tobacco smoke might influence the development of pancreatic cancer in three ways; a) carcinogens in tobacco excreted in bile might reflux into the pancreatic duct, b) tobacco carcinogens might reach the pancreas via the blood stream and c) cigarette smoking might increase blood lipids which have been shown to influence the development of pancreatic cancer. While certain nitrosamine chemicals have been shown to be carcinogenic in experimental animals, none of the nitrosamines in cigarette smoke have yet been shown to cause pancreatic cancer in this setting. The suggestion that reflux of carcinogen-containing bile into the pancreatic duct might be responsible for the development of pancreatic cancer is plausible and pancreatic cancer in man occurs most frequently in the head of the gland (Cubilla and Fitzgerald, 1979). In

addition, it has been demonstrated in man that certain variations in the anatomy of the bile and pancreatic ducts at the sphincter of Oddi can allow bile to reflux from the bile duct into the pancreatic duct (Armstrong and Taylor, 1985). DiMagno and colleagues (1982) demonstrated an increase in pancreatic ductal epithelial abnormalities in patients who lacked a prominent pancreatic and bile duct common channel, although Armstrong and Taylor suggested that bile reflux into the pancreatic duct was more likely in patients with a long functioning common channel. It remains unclear whether this mechanism is relevant in the development of pancreatic cancer in man.

b) Diet

Demographic differences in the incidence of pancreatic cancer throughout the world has prompted an extensive search for dietary factors which might be implicated in the disease. In particular, the consumption of a "Western diet" seems to increase the relative risk; Doll and Peto (1981) suggested that in the USA, 35% of all cancer deaths could be attributed to dietary factors.

In 1967, Lea reported a significant correlation between the incidence of pancreatic cancer in various countries and per capita consumption of fats, oils, sugar, animal protein, eggs and milk. There was a significant positive correlation between pancreatic

cancer and high meat, low vegetable intake in men, and low vegetable intake in women in a Japanese study published in the following year (Ishii et al, 1968). Unfortunately, dietary histories in this latter study were obtained from relatives by questionnaire in many instances. Certainly, there is a significant correlation between pancreatic cancer death rates in different countries and the average per capita daily consumption of fat (Segi et al, 1969; F.A.O., 1969).

In a large follow-up study from Japan, Hirayama (1981) reported that daily, or more frequent consumption of meat was associated with an increased risk (1.5:1) of developing pancreatic cancer. This association, although weak, was statistically significant. It has been suggested that the great increase in the incidence of pancreatic cancer in Japan might be related to Westernisation of the Japanese diet with documented increases in protein and fat consumption (Wynder et al, 1973; Hirayama, 1975).

In a case-controlled study, Gold and colleagues (1985) reported an association between pancreatic cancer and the consumption of white bread and decreased risk with the consumption of raw fruits, vegetables and "diet soda".

It is interesting to note that in animals, dietary fat and protein have been shown to increase the incidence of nitrosamine-induced pancreatic cancer (Birt et al, 1981; Birt et al, 1983b; Longnecker et

al, 1985b) while retinoids inhibit carcinogenesis (Longnecker et al, 1982; Longnecker et al, 1986).

Exactly why dietary factors should influence the development of pancreatic cancer remains unclear. Dietary fat and protein are potent stimulants of the release of many gastrointestinal hormones. Many workers have shown that in animals, exogenous administration of some of these hormones can cause pancreatic hypertrophy (Mainz, Black and Webster, 1973; Barrowman and Mayston, 1974; Folsch and Wormsley, 1976) and potentiate the development of pancreatic cancer (Howatson and Carter, 1985). Diets rich in raw soya flour, a potent trypsin inhibitor, can promote the effect of the pancreatic carcinogen azaserine in rats (Morgan et al, 1977). It has been suggested that diets rich in fats and protein stimulate the release of various gastrointestinal hormones which act to increase pancreatic cell turnover, and thus increase the susceptibility of the gland to the effects of carcinogens (Morgan and Wormsley, 1977).

Nitrosamines, which induce pancreatic cancer in hamsters, have been shown to be formed during cooking and in the stomach from nitrites and nitrates used in the preservation of meats (Weisberger and Williams, 1975; Miller and Miller, 1986). The average daily intake of volatile nitrosamines has been shown to vary from between 0.6 to 2ug per person per day, although

actual values vary depending on local eating habits and food preparation (Bartsch et al, 1982; Preussman and Eisenbrand, 1984).

The role of vitamins in the development of cancer of various organs has received considerable attention in recent years. Vitamins C and E have been shown to inhibit or reverse nitrosation in experimental conditions (Mirvish, 1986) and this might explain the inverse correlation between pancreatic cancer risk and fruit and vegetable consumption noted earlier (Gold et al, 1985). Low serum levels of vitamin A (retinol) have been reported as being associated with an increased risk of cancer (Wald et al, 1980) although a subsequent report from the same group suggested that low serum retinol levels might be a consequence of cancer metabolism rather than a risk factor for cancer development (Wald, Boreham and Bailey, 1986).

In the last 20 years, the amount of nitrates and nitrites in food have diminished and vitamin C has been added to all nitrate and nitrite preserved foods in the USA, to prevent the nitrosation reaction taking place (Preussman and Eisenbrand, 1984; Mirvish, 1986).

The American Cancer Society have issued seven guidelines on nutrition to reduce cancer risk; avoid obesity, reduce fat intake, eat more high fibre foods, eat more foods rich in vitamins A and C, eat more

cruciferous vegetables, e.g. cabbage, consume alcohol in moderation and consume salt-cured, smoked and nitrite cured foods in moderation (Weinhouse, 1986). It seems likely that adoption of these guidelines could reduce the risk of pancreatic cancer.

c) Coffee Consumption

The role of coffee in the aetiology of pancreatic cancer has been the subject of much controversy in recent years. In a comparison of age adjusted death rates from various cancers and annual consumption of cigarettes, tea and coffee in a number of countries, Stocks (1970) reported a positive correlation between coffee consumption and pancreatic cancer in males but not in females. In a large case-controlled study, a highly significant association was also found between coffee consumption and the risk of pancreatic cancer even when the data were adjusted to take cigarette smoking, age and sex into account (MacMahon et al, 1981). There seemed to be a dose response relationship; risk increased in proportion to the number of cups of coffee consumed. MacMahon subsequently urged caution in the interpretation of his initial data, in that the control group was not ideal (MacMahon, 1982). In a subsequent case-controlled study, MacMahon's group reported a slightly increased risk of pancreatic cancer only in those whose coffee consumption exceeded five cups per day

(Hsieh et al, 1986). They concluded that any association between pancreatic cancer and coffee consumption was not as strong as their initial data had suggested.

Mack and colleagues failed to show a consistent link between coffee consumption and pancreatic cancer in a subsequent case-controlled study in Los Angeles (Mack et al, 1986). In addition, they found no link between pancreatic cancer and previous consumption of tea, carbonated drinks, beer or spirits. Similar results were reported by Gold and associates from Baltimore, although there seemed to be a dose response relationship with coffee drinking in women, albeit not of statistical significance (Gold et al, 1985).

In 1981, Lin and Kessler reported a relationship between decaffeinated coffee and pancreatic cancer. More recent studies have failed to confirm this relationship (Jick and Dinan, 1981; Hsieh et al, 1986; Wynder et al, 1986). Wynder and his group showed no relationship between decaffeinated coffee consumption and pancreatic cancer in males; in females an association existed for consumption of 1-2 cups per day but was lost at higher levels of consumption (Wynder et al, 1986).

d) Chronic Pancreatitis

A familial form of chronic pancreatitis was first reported in 1952 (Comfort and Steinberg, 1952). This disease seems to be inherited as an autosomal dominant trait with incomplete penetrance and other affected families have since been reported (Gross, Gambill and Ulrich, 1962; Whitten, Feingold and Eisenklam, 1968; Appel, 1974). Approximately one third of affected patients develop pancreatic cancer although family members without pancreatitis also develop cancer (Castleman, Scully and McNeeley, 1972). The relationship between chronic non-familial pancreatitis and pancreatic cancer is unclear. In 1950, Mikal and Campbell reported the presence of chronic pancreatitis in 49 of 100 cases of pancreatic cancer at autopsy. Similarly, Gambill (1971) reported histological chronic pancreatitis in 10% of 255 consecutive patients with pancreatic and ampullary cancer, although in an earlier study he failed to demonstrate pancreatic cancer in 56 patients with chronic pancreatitis after 16 to 20 years of follow-up (Gambill, Baggenstoss and Priestley, 1960). In 1970, Robinson and colleagues were only able to find 15 authenticated cases of chronic pancreatitis and pancreatic cancer occurring together. There have been other reports of pancreatic cancer developing in patients with longstanding chronic pancreatitis (Bartholomew, Gross and Comfort, 1958; Mohr et al,

1975) but most authorities on pancreatic disease are of the opinion that the relationship is probably fortuitous (Wynder et al, 1973; Morgan and Wormsley, 1977). It is possible that both diseases share common aetiologies; they certainly share the same symptom pattern and diagnostic differentiation is problematic in many cases. In addition, it is well recognised that obstructing pancreatic cancers may cause pancreatitis as a consequence of pancreatic duct obstruction (Gordis and Gold, 1984).

e) Diabetes

The relationship between pancreatic cancer and diabetes mellitus has been difficult to determine with any certainty because of problems in establishing cause and effect. Medical students are taught that pancreatic cancer can worsen carbohydrate homeostasis in established diabetics and can cause diabetes to develop de novo. Whether diabetes gives rise to an increased risk of developing pancreatic cancer is less clear.

The earliest reported association of pancreatic cancer and diabetes was made before the metabolic role of the pancreas was completely understood (Mirallie, 1893). Since then there have been many reports of the association of the two diseases. In 1941, from collected statistics, Berk reported glycosuria in 9.4% and hyperglycaemia in 19.4% of patients with

pancreatic carcinoma. Bell (1957) reported 38 cases of diabetes mellitus in 587 cases of pancreatic cancer at autopsy. The incidence of diabetes in the pancreatic cancer group was twice that of the general autopsy population for males and 50% greater for females. He pointed out, however, that the average duration of the diabetes in the 38 pancreatic cancer cases was only 3.4 years and was less than 1 year in ten of the cases. When the 13 cases where the symptoms of pancreatic cancer antedated or coincided with the development of diabetes were excluded from the analysis, the incidence of diabetes in the pancreatic cancer group fell to that of the general autopsy population, both in males and females.

In a further autopsy study, Green and colleagues (1958) reported that 4.3% of 209 pancreatic cancer patients had diabetes prior to the onset of the symptoms of pancreatic cancer; 15.3% were found to have diabetes after the onset of such symptoms and a further 29% were found to have glycosuria. No conclusion was reached as to whether diabetes predisposed to pancreatic cancer. Clark and Mitchell (1961) reported on 65 patients with pancreatic cancer, ten of whom were diabetic; in all but one of these cases the interval between diagnosis of pancreatic cancer and diabetes was less than one year. Karmody and Kyle (1969) also found that the interval between diagnosis of diabetes and pancreatic cancer was less

than one year in 80% of their 51 cases. In six cases, diabetes was diagnosed more than two years prior to the development of pancreatic cancer symptoms; these authors concluded that the incidence of pancreatic cancer in such diabetics was greater than could be accounted for by chance.

The most exhaustive study of the relationship between diabetes and pancreatic cancer was published by Kessler in 1970. Cancer mortality among 21,447 diabetic patients during a 26 year period ending in 1956 was reported. Male diabetics were found to have a lower than expected mortality from all forms of cancer while in females, there was no difference from the expected rate. A statistically significant excess of deaths, in both males and females, was observed for pancreatic cancer; the standardised mortality rate (SMR) for males was 1.47 and for females it was 2.13. When the deaths of patients in whom it could not be established that the diabetes had preceded the cancer, were excluded from the analysis, the SMR's fell to 1.27 for males and 1.82 for females. This study has been criticised on the grounds that the SMR's were calculated using data from the general Massachusetts population, which may not have been appropriate to the Boston group of patients under study. Kessler made the interesting proposition that exogenous animal insulins should be examined closely for carcinogenic effects in humans given their antigenic and

teratogenic potential in animals. In a subsequent review, Kessler urged caution in the interpretation of the many reports and studies of diabetes and pancreatic cancer given the wide variation in study design and the bias that is often present in autopsy studies (Kessler, 1971).

Green and Jensen (1985) followed the entire insulin-dependent diabetic community in Fyn County, Denmark for eight and a half years and reported a significant excess of pancreatic cancer cases (6 vs 2.4 expected) during this time. When they excluded those patients where diabetes was an early sign of the development of the tumour, this excess was reduced (4 vs 2.4). They concluded that there was no association between pancreatic cancer and prior diabetes. In a case-control study based on three Swedish hospitals, patients with pancreatic cancer were more likely to have diabetes than either hospital based (relative risk 19.7) or population based controls (relative risk 3.3) (Norell et al, 1986). When cases where the diabetes was diagnosed less than five years prior to development of pancreatic cancer were excluded, the increased relative risk remained (6.9 compared to hospital controls, 2.4 compared to population controls) although the number of cases in each group was small.

Part of the problem in studying the link between pancreatic cancer and diabetes is a failure in most

reports to distinguish between insulin-dependent (IDDM) and non-insulin-dependent diabetes (NIDDM). It might be postulated therefore that any increased risk of pancreatic cancer in the two subgroups should be different. Animal work has only confused the situation. Bell and Strayer (1983) demonstrated that Syrian hamsters, made diabetic by destruction of insulin-secreting islet cells by streptozotocin, do not develop pancreatic cancer when injected with carcinogen. They subsequently examined the incidence of chemically induced pancreatic cancer in genetically diabetic and non-diabetic Chinese hamster strains and reported that tumours only develop in the non-diabetic animals (Bell and Pour, 1987).

There has been considerable interest in recent years in the relationship between pancreatic cancer and carbohydrate intolerance. In a prospective study of 99 patients suspected of having pancreatic cancer, Schwartz and colleagues (1978) measured blood glucose, serum insulin, serum c-peptide and plasma glucagon during an oral glucose tolerance test. They demonstrated an increased incidence of carbohydrate intolerance in the 32 patients found subsequently to have pancreatic cancer (81%) compared to the 67 control patients (36%) suggesting that abnormalities in carbohydrate metabolism were more widespread in patients with pancreatic cancer than had previously been realised. Insulin secretion was lower during the

glucose tolerance test in the pancreatic cancer patients than in the controls. Unfortunately, the picture here may again be clouded in that patients with obstructive jaundice may exhibit abnormalities in carbohydrate metabolism during oral glucose tolerance tests (Ozawa et al, 1975).

The nature of the abnormality of carbohydrate metabolism in these patients remains unclear. While Schwartz and colleagues (1978) exhibited low serum insulin levels in humans in response to an oral glucose tolerance test, in a recent study of pancreatic cancer induced in Syrian golden hamsters, production of insulin in response to infusions of glucose and arginine in an isolated preparation of the malignant pancreas was unaffected (Bell et al, 1986).

f) Gallstones and Cholecystectomy

In an autopsy study of 609 cases of pancreatic cancer, Bell (1957) noted that 14% of male and 38% of female cases had gallstones co-existent with the tumour. Despite the fact that the overall incidence of cholelithiasis in these autopsy cases (21%) exceeded that of a general autopsy population, Bell concluded that there was no obvious association between pancreatic cancer and gallstones. Wynder and colleagues (1973), in a retrospective case-controlled study of 142 patients with pancreatic cancer, noted a higher incidence of prior cholecystectomy in female

cancer patients (15%) than in controls (8%), although this difference did not reach statistical significance. Wynder (1975) subsequently reviewed the results of his group's initial 1973 study, and cautioned that the association between prior cholecystectomy and pancreatic cancer in women was based on a relatively small number of patients. Haines and colleagues (1982) failed to demonstrate a relationship between cholecystectomy and pancreatic cancer in patients who underwent cholecystectomy more than five years prior to the development of pancreatic cancer. In a review of 586 randomly selected patients with abdominal malignancy, a Finnish group suggested that there was a significantly increased frequency of prior cholecystectomy in patients developing pancreatic cancer, with the peak incidence of pancreatic cancer occurring five years after surgery (Hyvarinen and Partanen, 1987).

In a small study from Athens, cholelithiasis was associated with an increased risk of pancreatic cancer (relative risk = 3.5) (Manousos et al, 1981). In a more recent case-controlled study from Stockholm, it was concluded that the relative risk of pancreatic cancer for subjects who reported suffering from gallstone disease was 1.7 compared with hospital controls and 2.7 compared with population based controls (Norell et al, 1986). The relative risk values were 1.2 and 2.9 following exclusion of

patients who reported gallstones only in the five years prior to the diagnosis of pancreatic cancer, and this group concluded that the risk of pancreatic cancer was increased in patients with gallstone disease.

It has been shown, in the hamster, that cholecystectomy results in an increase in circulating plasma cholecystokinin levels, pancreatic weight, DNA content and DNA synthesis rate and in hyperplasia and hypertrophy of pancreatic cell populations (Rosenberg et al, 1983; Rosenberg et al, 1984). In a Japanese study, the incidence of pancreatic cancer induced by the nitrosamine N-nitrosobis(2-hydroxypropyl)amine (BHP) was increased in animals which had undergone cholecystectomy one week prior to the first injection of carcinogen (Ura et al, 1986). This difference did not reach statistical significance. When cholecystectomy was combined with the administration of the secondary bile salt lithocholic acid, the incidence of pancreatic carcinoma was significantly greater than the control group which received carcinogen only.

While cholecystectomy may increase the risk of development of experimental pancreatic cancer when undertaken immediately prior to exposure to chemical carcinogens (the mechanism perhaps involving transient increase in cell turnover mediated by increased circulating levels of CCK), it remains to be seen

whether this mechanism would apply to humans who had undergone cholecystectomy many years prior to their presentation with pancreatic cancer. Why cholelithiasis should predispose to pancreatic cancer is unclear.

g) Gastric Surgery

In 1982, McLean-Ross and colleagues reported on the results of a follow-up study of the mortality rate and causes of death in 779 patients who had undergone peptic ulcer surgery. Eighty-six percent of the patients had undergone some form of gastrectomy and the remainder vagotomy and drainage. The authors found that the life expectancy of patients following peptic ulcer surgery was reduced in all age groups studied. One of the surprise findings of this study was a statistically significant increase in the number of patients dying of pancreatic cancer (11 actual deaths compared with 3.9 expected, $p<0.01$). They concluded that part of this increase in mortality following gastric surgery was related to continued cigarette smoking, and this could explain the increase in pancreatic cancer cases.

Further evidence to suggest a relationship between previous gastric surgery and pancreatic cancer was provided by Mack and colleagues (1986). In a large case-controlled study, they demonstrated a relative risk of developing pancreatic cancer

following gastric surgery (mainly partial gastrectomy) of 7.4 in non-smokers and 8.1 for current or recent smokers (the relative risk for smokers without gastrectomy was 2.3). A three-fold risk of pancreatic cancer following gastric surgery has been reported by other groups (Caygill et al, 1985; Offerhaus et al, 1987). In the Offerhaus study corrections were made for cigarette consumption in case and control groups, and the relationship persisted. This group postulated that the cause for the increase in pancreatic cancer might be increased production of carcinogens such as nitrates and nitroso-related compounds in the gastric remnant. Nitrosamines have been used for many years to induce pancreatic cancer in experimental animals (Pour and Wilson, 1980).

In a follow-up study of 336 patients who underwent gastric surgery for benign peptic ulcer disease, a group from the Mayo Clinic reported no relationship between pancreatic cancer and previous gastric surgery (Marighini et al, 1986). Only one patient developed pancreatic cancer 33 years after a Billroth II subtotal gastrectomy compared with 1.6 expected (relative risk = 0.64).

h) Familial Cases

Cases of pancreatic cancer occurring in families have appeared sporadically in the literature. MacDermott and Kramer (1973) reported three brothers

and one sister who all developed pancreatic cancer between the ages of 59 and 72 years. Another group of four affected brothers, who all smoked cigarettes, was reported three years later (Friedman and Fialkow, 1976). In both of these reports, the affected individuals all developed pancreatic cancer in the high incidence age groups. Another case report published in 1973 described a father and son who presented with pancreatic cancer who had both previously been exposed to toxic chemicals (Reimer et al, 1977). There have been four other case reports of familial pancreatic cancer in the 12 years to 1987 (Danes and Lynch, 1982; Dat and Sontag, 1982; Grajower, 1983; Ehrenthal et al, 1987). One of these reports documented the familial occurrence of pancreatic cancer in three women of consecutive generations (Ehrenthal et al, 1987). The women were European and Jewish and two died at the young ages of 29 and 42 years with pathologically proven disease (the daughter and grandaughter in the series). Two of the women were smokers.

Despite these case reports, it is generally accepted that such cases are rare and that the association could still be due to chance (MacMahon, 1982).

i) Industrial Exposure

Since Potts' original observation of an increase in the incidence of scrotal cancer amongst chimney sweeps, many investigators have looked for an association between occupational chemical exposure and human pancreatic cancer.

In a study of 639 men employed in 1938 or 1939 in a company manufacturing B-naphthylamine and benzidine and followed up until 1965, Mancusco and El-Attar (1967) reported six cases of pancreatic cancer. These six cases accounted for almost one third of malignant gastrointestinal tumours recorded in the study and represented a mortality rate for pancreatic cancer in the cohort of 39/100,000, compared with 7.9 for men of similar ages in the general Ohio population.

Li and colleagues (1969) examined 3,637 of 4,644 death certificates of members of the American Chemical Society who died between 1948 and 1967, and compared the mortality rate from pancreatic cancer with the mortality rate of a general population of age-matched professional men. They reported 56 deaths from pancreatic cancer compared to 35 expected but were not able to implicate any one chemical substance as responsible for this increase. The authors advised caution in the interpretation of their results, given that 22% of the death certificates were missing, and secondly, the relative frequency method used to make

the comparisons was thought to have the potential for erroneous results. Two subsequent studies have failed to reveal such an excess of pancreatic cancer cases in chemists (Hoar and Pell, 1981; Searle et al, 1981).

There has been one report suggesting an excess of pancreatic cancer in British radiologists but the excess was small and of marginal statistical significance (Smith and Doll, 1981).

In a study of 94 cases of pancreatic adenocarcinoma, lifetime occupational histories were obtained by interviewing the subjects and age and sex-matched controls (Lin and Kessler, 1981). The proportion of male patients with pancreatic cancer who had been employed in the dry cleaning business or in jobs involving close exposure to petrol was significantly higher than for male controls and the risk of pancreatic cancer was increased by a factor of five in men in these occupations for more than ten years.

Other high-risk occupations have been reported; metal industry and aluminium milling workers, particularly those exposed to coal tar pitch derivatives (Turner and Grace, 1938; Dorken, 1964) and coke plant workers (Redmond et al, 1976).

j) Alcohol Consumption

Alcohol was initially suggested as a risk factor for pancreatic cancer by Burch and Ansari in 1968. In their study, 65% of 83 pancreatic cancer patients admitted to moderate to heavy alcohol consumption for at least 15 years, compared to less than 15% of the control group. Ishii and co-workers (1968) also reported that the risk of pancreatic cancer was twice as high in males who drank alcohol every day compared to non-drinkers. In a case control study, Durbec and colleagues (1983) showed an increased risk of pancreatic cancer with high fat diet and alcohol intake. In a prospective study of 16,713 Norwegians, Heuch and co-workers (1983) found a relative risk of developing pancreatic cancer of 5.4 for frequent alcohol consumption.

Studies of alcoholics in many countries have failed to demonstrate a link between alcohol consumption and pancreatic cancer (Hakulinen et al, 1974; Monson and Lyon, 1975; Robinette, Hrubec and Fraumeni, 1979; Schmidt and Popham, 1981). Many case-controlled studies have similarly failed to demonstrate any such link between alcohol consumption and pancreatic cancer (Wynder et al, 1973; MacMahon et al, 1981; Haines et al, 1982; Mack et al, 1986) while others have suggested a weak link with wine consumption in males (Lin and Kessler, 1981).

Some authors have attempted to overcome the positive correlation between cigarette smoking and alcohol intake by attempting to standardise for tobacco use (Wynder et al, 1973). No association between pancreatic cancer and alcohol consumption was demonstrated in this study. In a case-control study in Baltimore, consumption of wine in both males and females seemed to be significantly protective against pancreatic cancer (Gold et al, 1985).

Despite the established relationships of alcohol and cancers of the mouth, pharynx, oesophagus, larynx and liver, the link between pancreatic cancer and alcohol consumption is weak and inconsistent and suggests that any increased risk is small (Gordis and Gold, 1984).

k) Oncogenes and Pancreatic Cancer

As molecular biologists and geneticists become more familiar with the human genome and techniques with which to explore it, chromosomal abnormalities associated with specific disease states are being reported with increasing frequency. This is the case in pancreatic cancer.

Hirai and colleagues demonstrated that a human pancreatic cancer cell line, T3M-4, contained activated c-Kirsten(Ki)-ras oncogene resulting in amplification and overexpression (Hirai et al, 1985). Yamada and co-workers (1986) demonstrated a point

mutation at codon 12 in the same oncogene in a primary pancreatic adenocarcinoma and a lymph node metastasis and reported a three to six-fold amplification of the oncogene in both samples, suggesting that the point mutation had occurred early in the tumour process.

1.4 Human Pathology

The pathological classification of pancreatic cancer proposed by Cubilla and Fitzgerald is generally accepted as definitive for descriptive purposes (Cubilla and Fitzgerald, 1975; Cubilla and Fitzgerald, 1979). In their initial paper (Cubilla and Fitzgerald, 1975) they reviewed the histology of 406 cases of non-endocrine pancreatic carcinoma. Duct cell adenocarcinoma accounted for 76% of cases; other histological types were much rarer, namely giant cell carcinoma (5%), microadenocarcinoma (4%), adenosquamous carcinoma (4%), mucinous adenocarcinoma (2%), anaplastic carcinoma (2%), cystadenocarcinoma (1%), acinar cell carcinoma (1%), pancreaticoblastoma (<1%) and unclassified (7%). The proportion of each type of tumour has undergone minor alteration in subsequent publications but the general distribution remains the same.

Ductal Adenocarcinoma

This histological type of pancreatic carcinoma predominates in the elderly (sixth and seventh decades of life). Cubilla and Fitzgerald (1979) made a number of observations regarding this tumour type which remain relevant. It was found most commonly in the head of the pancreas (61%) followed by the body (13%) and tail (5%); in the remainder the pancreas was diffusely involved with tumour. In approximately two-

thirds of patients, overt metastases were present at the time of diagnosis, most commonly in the liver, regional lymph nodes, peritoneum, lungs and pleura. Less than 10% had tumours apparently confined to the gland at diagnosis. In 60% of the patients with head of pancreas disease, the tumour diameter exceeded 5 cm. Lesions in the body and tail were generally larger (averaging 10 cm in diameter).

Histologically ductal pancreatic adenocarcinoma resembles adenocarcinomata seen in other sites in the body such as the gastrointestinal tract, uterus and lung. Glands of different sizes and degrees of differentiation are found together with a variable amount of fibrous tissue. In most tumours, appropriate histological stains reveal the presence of mucin, either in the cell apex or in extracellular pools. Foci of haemorrhage, necrosis and fat necrosis are also seen. In cases where the main pancreatic duct is occluded, acinar and ductular atrophy occur associated with ductular ectasia.

Tumours involving the body and tail of the pancreas have been reported to result in a different clinical spectrum from head of pancreas cancer. Jaundice is less common while thromboembolism and metastases (particularly subcutaneous metastases) are more frequent (Die Goyanes, Pack and Bowden, 1971; Cubilla and Fitzgerald, 1978). In a prospective study of 11 patients with pancreatic body and/or tail cancer

abdominal pain was present in 10 patients and back pain in nine (Fitzgerald et al, 1978).

In their initial publication, Cubilla and Fitzgerald (1975) also reported on the incidence of other associated histological abnormalities found in the pancreatic ducts of 195 patients with duct cell adenocarcinoma. Mucous cell hypertrophy, pyloric gland metaplasia and papillary hyperplasia were all more commonly found in patients with pancreatic cancer than controls and atypical duct hyperplasia and carcinoma-in-situ were only found in the cancer group. In an autopsy study, hyperplastic, preneoplastic and frankly neoplastic lesions were found in the pancreas of 83 military veterans, only two of whom were known to have pancreatic cancer, while three were known to have metastatic pancreatic carcinoma (Pour, Sayed and Sayed, 1982). Ten of these patients were found to have neoplastic exocrine lesions including unsuspected early pancreatic carcinoma (n = 2), ductal carcinoma-in-situ (n = 1) and ductular carcinoma-in-situ (n = 7). No less than 47 patients (57%) had pancreatic ductal hyperplasia and 32 (39%) had ductular hyperplasia; 48% exhibited squamous metaplasia in the ductules.

The importance of the diagnosis of in-situ carcinoma of the pancreas has recently been emphasised in a paper from Australia, which reported two such cases both of which were successfully treated by

surgical excision (Smith, Kneale and Goulston, 1986). In the first patient, laparotomy was undertaken to relieve obstructive jaundice; per-operative biopsy of an area of hard pancreas demonstrated cellular atypia in a papillary tumour but no invasion and total pancreatectomy was performed. In the second patient, pre-operative investigations had revealed an abnormal area in the head of the pancreas; per-operative biopsy suggested the presence of adenocarcinoma and a Whipple procedure was performed. Subsequent examination of the excised pancreas failed to reveal invasive adenocarcinoma. In neither case was the diagnosis made pre-operatively. In-situ carcinoma of the pancreas is not included in Cubilla and Fitzgerald's classification.

In 1985, Kloppel proposed that pancreatic duct cell adenocarcinomata should be subdivided into three major grades of malignancy using glandular differentiation, nuclear size, anaplasia and mitotic activity as histological criteria for the classification. The grades of malignancy reported correlated with the tumour doubling times in explants in nude mice and with the median survival time after resection of tumour in 75 patients assessed retrospectively. In a subsequent ultrastructural study of 51 pancreatic adenocarcinomata, increasing grades of malignancy correlated with a progressive loss in cell polarity and a loss of association of

tumour cells with the basement membrane (Kern et al, 1987).

Giant Cell Carcinoma:

This tumour type occurs in the same age group as ductal adenocarcinoma and has a similar sex distribution. Body and tail cancers are relatively more common. Tumours tend to be relatively large with considerable haemorrhage and necrosis. Histologically, the tumours contain large, polypoid mono-nucleated tumour giant cells and malignant spindle cells. A small, round, uniform-sized cell with little cytoplasm is usually present in sheets or clusters. Epithelial glands are often present. The prognosis for patients with this tumour is similar to that of ductal adenocarcinoma (Cubilla and Fitzgerald, 1979).

Microadenocarcinoma:

This is an uncommon and rapidly fatal type of pancreatic cancer. It is usually large and necrotic and histologically it resembles the carcinoid tumour. Metastatic disease is common. Glands are smaller than in ductal adenocarcinoma and are often found in sheets. Fibrosis is less prominent than in ductal adenocarcinoma (Cubilla and Fitzgerald, 1979).

Adenosquamous Carcinoma:

This tumour type, found most frequently in males, comprises two elements; an adenocarcinoma and a squamous carcinoma. Glandular areas differ widely in degrees of differentiation. Squamous areas have abundant cytoplasmic keratin and intercellular bridges. Metastases to liver, lymph nodes and peritoneum are common (Cubilla and Fitzgerald, 1975; Cubilla and Fitzgerald, 1979).

Mucinous Adenocarcinoma:

This tumour was initially reported only in males and found most frequently in the head of the pancreas. It is characterised histologically by large cystic spaces filled with mucin and frequently lined by tall, columnar, nonpapillary, glandular epithelium. The prognosis for patients with this type of pancreatic cancer is poor (Cubilla and Fitzgerald, 1975; Cubilla and Fitzgerald, 1979).

Anaplastic Carcinoma:

This subgroup includes those tumours which cannot be classified under any of the other headings. The pancreas is frequently involved in a diffuse manner. Tumours exhibit many different histological appearances including a large-cell group which resembles anaplastic adenocarcinoma, a group with small uniform cells which resembles malignant lymphoma

and a third type which resembles renal carcinoma (Cubilla and Fitzgerald, 1979).

Cystadenocarcinoma:

This tumour occurs most commonly in young women and most frequently involves the tail of the pancreas. The tumours are frequently large and present as a palpable abdominal mass or following a complication such as haemorrhage, rupture or secondary infection. The tumour is grey in colour with numerous multiloculated cysts which vary in diameter from approximately 1 cm to 20-30 cm. The cyst wall comprises tall columnar cells with abundant clear cytoplasm filled with mucigen granules. Prominent intracystic papillary projections of cells are a feature of all tumours of this type. Following resection, usually of the tail of the pancreas, the prognosis for this tumour is much better than for ductal adenocarcinoma (Compagno and Oertel, 1978; Cubilla and Fitzgerald, 1979; Kaufman et al, 1986).

Acinar Cell Carcinoma:

This tumour occurs in a younger group of patients than duct cell adenocarcinoma. Necrosis is a prominent feature. Tumour cells frequently form acinar structures with a small acinar lumen. The cells are large and polyhedral with a basal nucleus and abundant cytoplasm containing coarse cytoplasmic

granules. Areas in the tumour can show formation of giant cells, anaplasia and groups of small round cells resembling lymphoma. Tumour dissemination is common (Cubilla and Fitzgerald, 1979).

Pancreatico-blastoma:

This rare tumour has been reported by a number of authors (Moynan, Neerhout and Johnson, 1964; Frable, Still and Kay, 1971; Tsukimoto et al, 1973). In one case a three year old Peruvian boy was affected (Taxy, 1976). The tumour was initially diagnosed as a neuroblastoma but following Whipple resection of the involved head of pancreas, the 4 x 3 x 2 cm tumour was reclassified. Histologically the lesion comprised sheets of small cells with clear, scanty cytoplasm. Some areas contained spindle cells, chondroid, osteoid and bone tissue as well as epithelial components (Cubilla and Fitzgerald, 1979).

Unclassified (mixed cell types):

This group, which is distinct from the anaplastic group, comprises those tumours which contain a number of cell types, thus making classification difficult. Some cases show evidence of mucin production while most show some attempt to form gland or duct-like structures (Cubilla and Fitzgerald, 1975).

Metastatic Cancer:

Many tumour types can metastasize to the pancreas. The commonest primary sites reported are lung, melanoma of the skin and lymphoma (Cubilla and Fitzgerald, 1975).

1.5 Animal Models of Pancreatic Cancer

The natural history of pancreatic cancer in humans makes direct scientific observation and manipulation difficult. The tumour usually presents late, is irresectable and carries a dismal prognosis. Largely as a consequence of these factors, workers in the field have turned to animal models in an attempt to increase our knowledge and understanding of the basic biology and biological behaviour of pancreatic cancer.

Spontaneous exocrine pancreatic tumours occur in many species of animal, but are rare, accounting for much less than 1% of tumours reported (Pour and Wilson, 1980). The hamster seems to be most susceptible to spontaneous exocrine pancreatic cancer; 1-4% of all tumours in this species are pancreatic cancers with a ductal or ductular morphology which is similar to that of the human tumour (Fortner, 1957; Kirkman, 1962; Pour et al, 1976a).

a) Rat

Chemically induced pancreatic neoplasia was first reported in animals during an attempt to find safer organic substitutes for inorganic insecticides (Wilson, DeEds and Cox, 1941). The compound 2-acetylamino-fluorene was fed to albino rats whose organs were subsequently examined for carcinogenic effects. Nodular hyperplasia was demonstrated in the

liver and the pancreas; 17 of 39 rats exhibited pancreatic lesions and one developed an acinar cell carcinoma. Neoplastic lesions were also reported in many other organs. Similar findings have been reported in Buffalo rats fed the same substance for six to seven months (Morris et al, 1961). In the same paper, almost all of a group of Buffalo rats fed 2,7-fluorenylenebisacetamide develop similar lesions after four months of treatment.

Many other chemicals have been tested in rats since the initial 1941 report of induced pancreatic neoplasia. A low incidence of neoplastic pancreatic lesions was reported after long term administration of p-dimethylaminoazobenzene in Wistar rats (Hoch-Ligeti, 1949). In 1955, Hendry and colleagues reported acinar cell adenomata in three of 23 albino rats injected subcutaneously with 4'-fluoro-4-aminobiphenyl. Intravenous injections of 4-hydroxyaminoquinolone-1-oxide to Sprague-Dawley rats yielded a much higher incidence of similar adenomatous lesions (Hayashi and Hasegawa, 1971; Shinozuka and Konishi, 1974).

All of these substances suffer from the disadvantages of low tumour yield, production of predominantly benign tumours, long tumour latency and lack of organ specificity. In addition, lesions produced are acinar cell in type, whereas in human pancreatic cancer, ductal morphology predominates, although there is debate about the actual cell of

origin of human pancreatic adenocarcinoma.

Azaserine (o-diazoacetyl-L-serine), an analogue of glutamine with antimetabolite purine-synthesis-inhibiting properties, was reported to cause toxic pancreatic acinar cell injury (Hruban, Swift and Slesers, 1965). Using tritiated labelling, azaserine was found to localise particularly to the kidney and pancreas (Longnecker and Curphey, 1975). Weekly or twice weekly administration of azaserine intraperitoneally to Wistar rats, resulted in the development of a large number of hyperplastic acinar-type nodules and adenomata after 6 months of treatment (Longnecker and Crawford, 1974). These atypical acinar cell nodules (AACN's) appeared after eight weeks of treatment and increased in size and number during subsequent months (Longnecker, 1981). In a longer term study, rats were injected once or twice weekly for six months and sacrificed and autopsied at 6, 9, 12 and 18 months after initiation of treatment or when weight loss or obvious morbidity made survival unlikely (Longnecker and Curphey, 1975). All rats autopsied exhibited AACN's and in 25% the pancreas contained discrete encapsulated adenomata. Of the 60 animals injected twice weekly, 23 survived more than one year; nine of these exhibited pancreatic adenocarcinomata (20%) and three more had lesions suspected of being adenocarcinoma. Of the 60 animals injected once weekly, 18 were autopsied after one year

and five exhibited pancreatic adenocarcinomata (8%). Of the 18 animals with adenocarcinoma or suspected adenocarcinoma, 16 were male. Five animals had developed liver metastases, one pulmonary metastases and one diffuse tumour spread in peritoneal fat. Tumours also occurred in kidney, breast, neck, liver, pituitary gland, subcutaneous tissues and rectum. Variations in the susceptibility to the effects of azaserine of various strains of rats have been reported (Roebuck and Longnecker, 1977). With respect to the induction of AACN's, F344 rats were least susceptible, W/LEW rats exhibited a 100% response and Wistar rats an intermediate response. In addition it was noted that male rats developed almost twice the number of lesions as female rats. Since these initial reports, azaserine has become the standard substance used to study various aspects of pancreatic carcinogenesis in the rat.

b) Syrian golden hamster

The high rate of spontaneous ductal pancreatic tumours in the Syrian golden hamster prompted a number of investigators to study this animal in more detail with a view to developing a model of pancreatic cancer with the same ductal morphology as the human tumour.

In 1974, chemicals of the nitrosamine family were reported to induce pancreatic cancer in the Syrian hamster (Kruger, Pour and Althoff, 1974; Pour

et al, 1974a). A large number of related nitrosamines have now been studied for their carcinogenic effects in the hamster. N-nitroso-dipropylamine (DPN) (Pour et al, 1973), N-nitrosomethyl-n-propylamine (MPN) (Pour et al, 1974b), N-nitroso-(2-hydroxypropyl)-n-propylamine (2-HPPN) (Pour et al, 1974c), N-nitroso-(2-oxopropyl)-n-propylamine (OPPN) (Pour et al, 1974d), and N-nitroso-2,2'-dihydroxy-di-n-propylamine (DHPN or BHP) (Pour et al, 1975a) all proved to be carcinogenic to a greater or lesser extent. Adenomatous lesions predominated although the incidence of pancreatic cancer was much higher in the BHP-treated animals. Tumours were induced in many organs in addition to the pancreas suggesting that the effect of this group of carcinogens was non-specific. In a subsequent study, groups of hamsters were given weekly subcutaneous doses of each of the five substances mentioned above for life and sacrificed when moribund (Pour et al, 1975b). DPN failed to produce any carcinogenic effects in the pancreas. OPPN, 2-HPPN and MPN were all associated with a low incidence of ductal adenomata, BHP induced ductal adenomas in all animals, ductal carcinomas in 80-100% of animals and acinar cell carcinomas in 17%.

Pour's group then investigated other metabolites of the original nitrosamines in an attempt to develop carcinogens which had a more specific effect on the pancreas. These compounds were manufactured by

further substitutions on the B-aliphatic side chains of the original nitrosamine compounds. N-nitrosobis(2-acetoxypropyl)amine (BAP) exhibited a similar incidence of tumour and organ specificity to BHP (Pour et al, 1975c; Pour et al, 1976b). N-nitrosobis(2-oxopropyl)amine (BOP) was found to be more toxic than any of the other substances tested, resulted in tumour development as early as 13 weeks and seemed to have a more specific effect on the pancreas (Pour et al, 1975d). In a subsequent study, BOP was administered to male and female hamsters, either in a single subcutaneous dose (500, 250, 125 and 62.5 mg/kg body weight) or once weekly (10, 5 and 2.5 mg/kg body weight) for life (Pour et al, 1977a). The incidence of pancreatic neoplasms was between 50% and 80% for those animals injected once and between 67% and 100% for those animals injected weekly. Tumours occurring in other organs (particularly lung and liver) were more common in the animals injected only once. The incidence of pancreatic adenocarcinoma was highest (95%) in male hamsters receiving a weekly dose of 5 mg/kg body weight and in female hamsters receiving half of this dose. All pancreatic tumours in the single injection groups were benign but the incidence of liver neoplasm was much higher than in the multiple injection groups. Some BOP-treated hamsters also developed ascites, diarrhoea, weight loss, icterus and vascular thrombosis, similar

symptoms to those reported in man.

Different dose regimes have been studied in attempts to maximise the yield of pancreatic tumours and minimise the development of tumours in other organs. In a study where hamsters were injected for six weeks with 10 mg/kg body weight of BOP, and sacrificed at two-weekly intervals, no tumours were recorded at 8 weeks, 50% had pancreatic tumours at 10 weeks and at 16 weeks all animals examined had pancreatic tumours (Pour et al, 1977b). The incidence of lung tumours was low (only 12% of all animals examined) and other tumours only developed in females after 18 weeks; all males had died by this stage in the experiment. Single low doses of BOP (40, 20, 10, 5 and 2.5 mg/kg body weight) administered subcutaneously resulted in the development of pancreatic and other neoplasms (Pour, Salmasi and Runge, 1978). Pancreatic tumours were more frequent at higher doses but the incidence of tumours in other organs was also higher. Pancreatic tumours were induced even at low doses (2.5 mg/kg body weight) indicating the high carcinogenic potency of BOP in the hamster pancreas.

A large number of substances have now been tested for their carcinogenic potential in the Syrian hamster although none has exhibited the specificity and high tumour induction rates observed with BOP (Pour and Wilson, 1980; Pour and Raha, 1981).

Many studies have been carried out on the metabolism of the nitrosamine group of chemicals in an attempt to understand why they should have such an affinity for the pancreas in the Syrian hamster. Gingell and colleagues (1976b) administered BHP, BOP and HPOP (N-nitroso(2-hydroxypropyl)(2-oxopropyl) amine) intraperitoneally to male hamsters and studied blood levels and urinary excretion of their metabolites. HPOP and BHP were both detected in the urine after administration of BHP and BOP, but the amount of HPOP obtained was significantly greater after BOP administration. BOP was only obtained in the urine in very small quantities after BOP administration. HPOP appeared in the blood stream more rapidly after BOP administration than after BHP. The authors suggested that the carcinogenic effects seen after BOP or BHP administration, might be mediated through the proximate carcinogen HPOP, given that the amount of HPOP appearing in both blood and urine following BOP and BHP administration correlated with the tumour yield of these compounds in the hamster pancreas. They also pointed out that the cyclic form of HPOP resembled the pyranose form of hexose sugars and suggested that this similarity might provide a route for HPOP to enter pancreatic cells. HPOP had previously been shown to be a metabolite of another hamster pancreatic carcinogen, N-nitroso-2,6-dimethylmorpholine (Gingell et al, 1976a) and has

since been shown to possess a carcinogenic potency similar to that of BOP (Pour et al, 1979b).

Different routes of administration also influence the effect of BOP on the hamster pancreas. Oral administration results in more bile duct tumours, few pancreatic tumours and no lung or renal tumours (Pour et al, 1977c). Oral and intraperitoneal (IP) administration of BOP were compared with regard to excretion of metabolites in the bile and pancreatic juice in addition to blood levels and urinary excretion (Gingell and Pour, 1978). Urinary excretion of HPOP and BHP was greater after IP administration than after oral administration as were blood concentrations. All three substances were detected in gallbladder bile although IP administration again resulted in higher concentrations. In contrast, only HPOP and BHP were detected in pancreatic juice with higher levels again following IP administration; BOP was not detected in the pancreatic juice after oral or IP administration. These results were interpreted as further proof that HPOP may be the proximate carcinogen in BOP carcinogenesis. The authors were unable to explain the different tumour spectrum after oral BOP.

Further work by Gingell and colleagues (1979) to investigate carcinogen metabolism involved IP administration of BOP, BHP and HPOP labelled with radioactive ^{14}C in hamsters and rats. For BHP in the

hamster, 74% of radioactivity was excreted in the urine and 19% as CO_2; in the rat 73% was excreted in urine and 4% as CO_2; for BOP in the hamster, 12% in urine and 74% as CO_2; in the rat 23% in the urine and 55% as CO_2: for HPOP in the hamster, 57% in the urine and 24% as CO_2. In this study trace amounts of BHP and HPOP were detected in pancreatic juice in hamsters after BOP administration, which taken with their earlier experimental work, led the authors to suggest that the effects on the pancreas were mediated through blood-borne carcinogens and not through exposure of the pancreatic duct to carcinogens in pancreatic juice or in refluxed bile. They also suggested that metabolic activation of carcinogens seemed to be by an oxidative pathway.

It is interesting to note that although nitrosamines seem to be metabolised in rats in a similar way to hamsters, they do not cause pancreatic tumours in rats. BHP causes tumours to develop in the nasal cavity, lungs, oesophagus, liver and kidneys of rats (Reznik and Mohr, 1976; Mohr, Reznik and Pour, 1977) and BOP causes tumours to develop in the intestines, liver, urogenital tract and respiratory tract (Pour, 1978a). A later report from Longnecker and his group (1985a), however, reported successful induction of pancreatic neoplasms including carcinomas (of acinar cell type) in 77% of rats after IP administration of a single dose of HPOP, although the

incidence of tumours in the liver and lungs was as high as that in the pancreas.

In 1980, Scarpelli and colleagues postulated three mechanisms by which the carcinogens could be activated namely (a) activation by liver microsomal mixed function oxidase (MFO) and release of active carcinogen into the blood stream, (b) activation in the liver followed by release of carcinogen into the bile and (c) activation of the carcinogens by MFO's in the pancreas and release of carcinogen into the pancreatic ductules and ducts. In a complex study to determine whether hamster pancreas contained the necessary MFO enzymes to activate carcinogen they investigated the mutagenic effects of BOP and another potent hamster pancreatic carcinogen, 2,6-dimethylnitrosomorpholine (DMNM), following activation by the post-mitrochondrial fraction of hamster pancreas (S-9), on Salmonella typhimurium TA 1535. They demonstrated that after activation, both carcinogens became more mutagenic to the bacteria. Interestingly, DMNM was more mutagenic than BOP despite the fact that BOP is the more potent carcinogen in the hamster. Mutagenicity was enhanced by pre-treatment of the hamsters with B-naphthoflavone and 2,3,7,8-tetrachlorodibenzo-p-dioxin, but not by 3-methylcholanthrene and this mirrored changes in aryl hydrocarbon hydroxylase activity. Mutagenicity was inhibited by pre-treatment with alpha-naphthoflavone

and 2,5-diphenyloxazole and there seemed to be an absolute requirement for nicotinamide adenine dinucleotide phosphate (NADPH). These findings taken together suggest that the enzymes responsible for carcinogen activation were indeed pancreatic MFO's.

The relative species-specificity of pancreatic carcinogens has intrigued many authors in recent years. Azaserine, BOP and streptozotocin have been examined for toxic effects on rat and hamster acinar cell preparations (Zucker, Chan and Archer, 1986). Using inhibition of protein synthesis as a measure of cellular toxicity, it was demonstrated that while azaserine inhibited synthesis in all tissues examined, BOP and its metabolite HPOP had no effect on any. Streptozotocin inhibited synthesis in islets and in hamster acinar cells. The authors concluded that the specificities of the cellular toxicity of the carcinogens paralleled their tumorigenic effects. Scarpelli's group investigated the capacity of rat liver and pancreas to convert the putative proximate carcinogen HPOP to a mutagenic agent (Mangino, Hollenberg and Scarpelli, 1986). They reported that hamster hepatocytes rather than rat hepatocytes preferentially converted HPOP to a mutagenic metabolite. Pancreatic acinar cells in both animals were poor activators of HPOP and they concluded that hamster liver activated the carcinogen into a mutagenic form.

In 1987, Curphey and colleagues reported on the ability of 11 pancreatic carcinogens to damage the DNA of pancreatic acinar cells of rats and hamsters, both in vivo and in vitro. Azaserine in vivo and in vitro damaged DNA in both rat and hamster pancreatic acinar cells in a dose dependent fashion. BOP only damaged DNA in hamster pancreatic acinar cells in vivo while HPOP damaged DNA in rats and hamsters in vivo but only in hamsters in vitro. The authors suggested that azaserine may well be carcinogenic to hamster pancreas under appropriate conditions and that the acinar cell in both species may well be the target for pancreatic carcinogens despite the apparent ductal morphology seen in the hamster.

c) Other mammals

Mice have not proved to be a useful model for human pancreatic cancer although they have proven invaluable in studying transplanted human and chemically induced tumours.

The rabbit has similarly not been widely investigated with regard to pancreatic carcinogenesis. In one study, small catheters made from 40% dimethylhydrazine were surgically implanted into the pancreatic ducts of New Zealand white rabbits (Elkort, Handler and Mozden, 1975). The catheters did not seem to interfere with pancreatic exocrine or endocrine function, but after 18 weeks, metaplasia, hyperplasia

and dysplasia developed within the pancreatic duct epithelium. After a further 48 weeks, these lesions had developed into adenomatous periductal lesions and adenomas.

Guinea pigs have also been studied for the effects of various chemicals on the pancreas. N-nitroso-N-methylurea was administered to guinea pigs in drinking water five times weekly for life; two of 26 animals exhibited pancreatic adenocarcinomata after 548 and 560 days of treatment (Druckery et al, 1968). In other studies, the same substance was administered intraperitoneally once weekly for life to inbred guinea pigs (NIH strain 13). Approximately one third of animals exhibited pancreatic adenocarcinomata after 28 to 44 weeks of treatment (Reddy and Rao, 1975; Reddy, Scarpelli and Rao, 1979). Despite these results it is generally believed that the guinea pig does not provide a useful model for the investigation of pancreatic carcinogenesis. Long latency periods, low tumour yields and a high animal loss rate during the first six months of treatment make it unsuitable.

d) Guinea Fowl

A recent paper from Bulgaria reported on the successful induction of pancreatic adenocarcinoma in 350 guinea fowls infected with the avian osteopetrosis virus strain Pts-56 (Kirev, Toshkov and Mladenov, 1986). Pancreatic carcinomata were detected four

months post-infection. By six months almost all birds studied exhibited neoplastic changes in the pancreas and over 60% exhibited pancreatic carcinomata. Further work is being undertaken by this group to determine the relevance of these findings in relation to human pancreatic cancer.

1.6 Syrian Golden Hamster Model

a) Anatomy (Takahashi et al, 1977a)

The pancreas of the adult Syrian golden hamster is well defined (unlike other rodent pancreata), lobulated, pink in colour and has three distinct lobes; gastric, duodenal and splenic (Figure 1.1).

The duodenal lobe, the smallest of the three, is closely applied to the posterior aspect of the duodenum; it accounts for approximately 12% of the pancreatic wet weight. It joins the other two lobes at an irregularly shaped head situated medial and dorsal to the duodenum. The gastric lobe extends ventrally from the head region, lies in close apposition to the greater curvature of the stomach and the pylorus, and is easily seen on opening the abdomen. It accounts for 25% of the weight of the organ. The splenic lobe, the largest of the three (40% of organ wet weight), extends dorsally from the head region and lies behind the stomach. It passes the spleen in a caudal position and attaches through a thin membrane to the descending colon, in front of the left kidney. The tail of the gastric and splenic lobes of the pancreas are connected by a long piece of omental fat ("fatty string") which lies lateral to the forestomach.

Each lobe has a single major pancreatic duct (although the gastric lobe sometimes possesses two

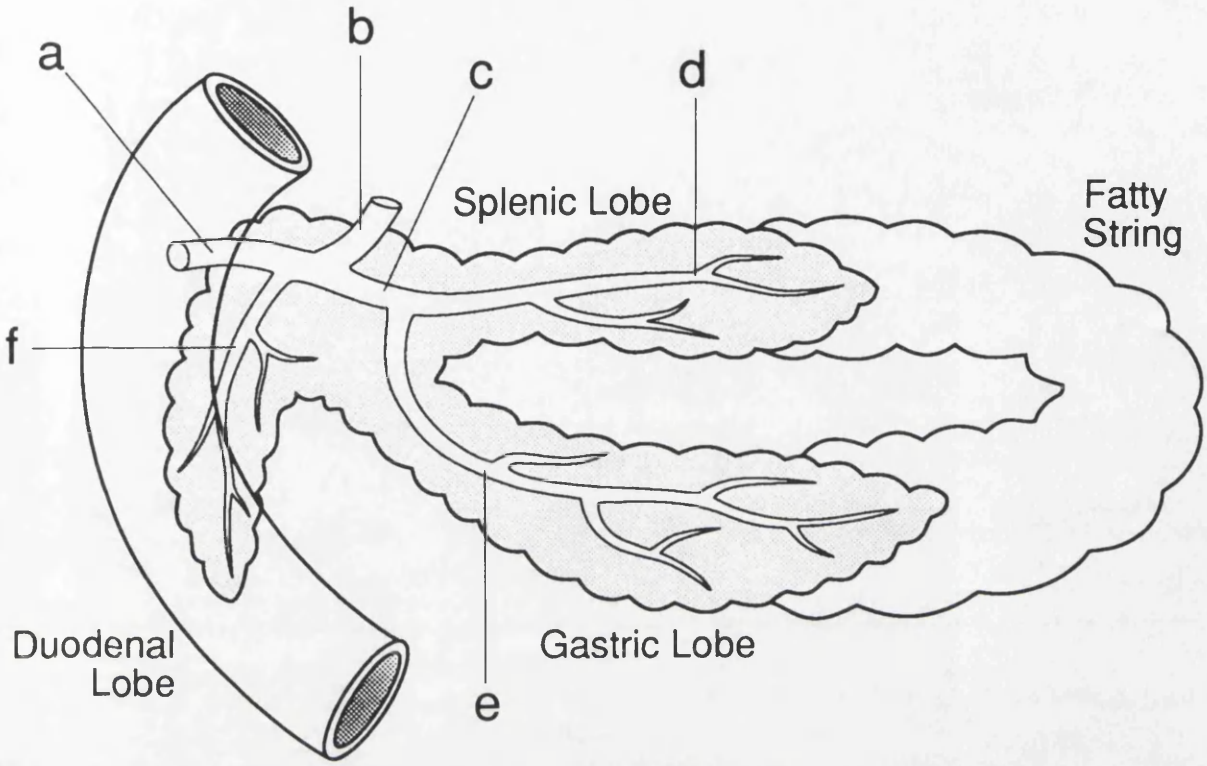

Figure 1.1: Anatomy of the pancreas in the Syrian golden hamster. The pancreas comprises three lobes; gastric, splenic and duodenal. The gastric and splenic lobes, situated anterior and posterior to the stomach, respectively, are attached by a fatty string of tissue. The duodenal lobe lies behind the duodenum. Each lobe contains a major duct (splenic lobe duct (d), gastric lobe duct (e), duodenal lobe duct (f). The gastric and splenic lobe ducts join to form the common pancreatic duct (c). The common bile duct (b) joins the common pancreatic duct which, together with the duodenal lobe duct, forms the common duct (a) which enters the posterior aspect of the duodenum.

such ducts). The ducts of the gastric and splenic lobes join proximal to the head region to form a large main pancreatic duct; this duct joins the common bile duct prior to entry into the duodenum. The duct of the duodenal lobe joins the common bile duct distal to the entry of the main pancreatic duct.

The blood supply of the gland is derived from the splenic, right gastroepiploic, pancreatoduodenal and superior mesenteric arteries. This is not dissimilar to the human situation although there are some variations in vascular pattern specific to the hamster.

Histologically, the hamster pancreas resembles that of other mammals, including the human (Figure 1.2). The gland is composed of both exocrine and endocrine elements. The exocrine tissue is arranged into acini draining into ductules and ducts of increasing diameter. The cells of the acini are pyramidal in shape with basal nuclei. The acinar cells contain eosinophilic zymogen granules in their apical portion (Pour and Wilson, 1980).

The endocrine elements of the pancreas are found in Islets of Langerhans (as in man. The islets are distributed evenly throughout the gland but can vary considerably in size. Islets contain alpha and beta cells in similar proportions and numbers to the human. Delta cells are also present but are much less frequently identified (Pour and Wilson, 1980).

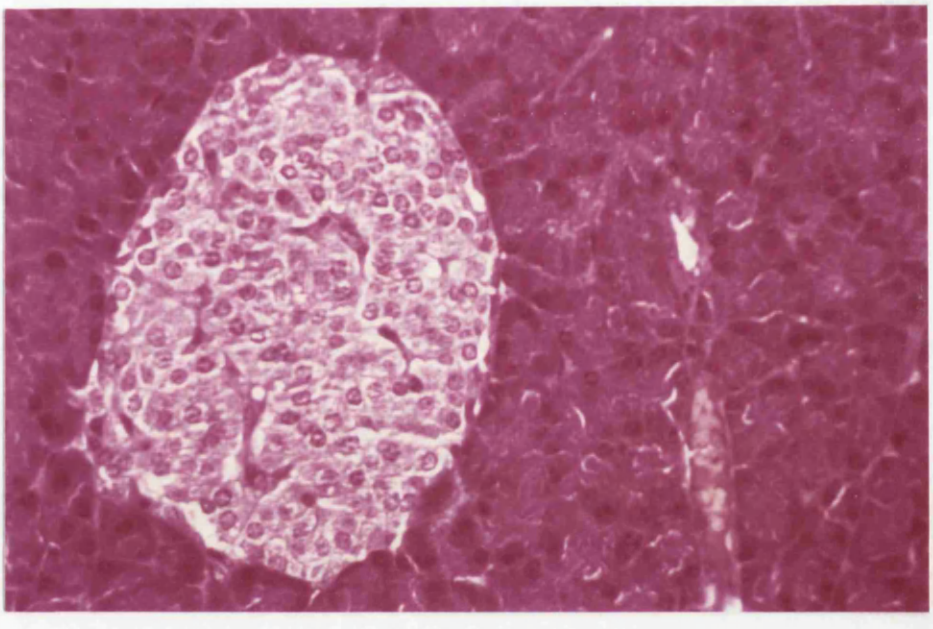

Figure 1.2: Histological appearance of normal hamster pancreas. Acini drain into ductules and ducts (top right of section). The endocrine elements are found in the islets of Langerhans (H&E; x360 magnification).

b) Physiology (Helgeson et al, 1980a; Helgeson et al, 1980b)

In their initial paper, Helgeson and colleagues (1980a) studied various aspects of pancreatic function in starved and fed eight week old male and female Syrian golden hamsters. After the animals were anaesthetised with IP sodium pentobarbital, the common bile duct was ligated and the distal common pancreatic duct cannulated. After a period of recovery from the anaesthetic, pancreatic juice was collected in pre-weighed vials for 20 hours; animals were allowed free access to water but no food. In a second experiment, DNA and protein synthesis rates were measured following IP injection of [^{3}H]phenylalanine or [^{3}H]thymidine. Animals were sacrificed 15 minutes after the injection and radioactivity in DNA or protein was measured.

With regard to pancreatic secretion there was little fluctuation in the pH of pancreatic juice during the 20 hour study period although fed animals exhibited a consistently higher pH than starved animals (range of mean pH's: fed animals 9.11-9.46, starved animals 8.87-9.17). The flow rate of pancreatic juice increased steadily from a basal level in fed animals of approximately 80 ul/hr/kg body weight (BW) to a peak level of approximately 120 ul/hr/kgBW after 10 hours. This was followed by a slight fall in secretion during the night time

collection period. Flow rates in starved animals were generally lower than in the fed animals although the pattern of secretion was similar. The protein content of the pancreatic juice was five times greater in the starved animals than in the fed animals in all periods; protein output gradually fell over the study period in the fed animals but slowly increased in the starved animals before falling during the night. The authors suggested that the higher protein content of pancreatic juice in the starved animals could be explained by cellular debris in the pancreatic ducts caused by cell death as a consequence of starvation and resultant pancreatic atrophy.

Electrolyte concentrations were measured in the pancreatic juice during the study period. Calcium and magnesium content gradually decreased with time. Bicarbonate and chloride concentration exhibited a reciprocal relationship but the combined total increased with time. Phosphate and sulphate exhibited a similar reciprocal relationship but their combined total decreased with time.

DNA synthesis rates were similar in fed and starved male hamsters; females had a consistently higher synthesis rate than the corresponding male group and synthesis rates were higher in the fed group. The protein synthesis rate for fed animals was twice that of the corresponding starved animals.

In a subsequent study, Helgeson's group (1980b) investigated the effects of repeated injection of secretin and pancreozymin in the cannulated hamster pancreatic duct model. The hormones were injected every 30 minutes for a total of six doses (secretin doses 25 iu/kgBW, pancreozymin doses 20 iu/kgBW). The pancreatic juice of animals given secretin was of a consistently and significantly lower pH than that of animals administered pancreozymin. The flow rate of pancreatic juice was two to threefold greater in the secretin-stimulated than in the pancreozymin-stimulated animals during the first three hours in both males and females and in the second three hours in males. Thereafter the flow rates in the two groups were similar. Overall, the flow rate in pancreozymin treated animals paralleled that of unstimulated animals in the previous experiment, whereas in the secretin treated animals the flow rate was initially much greater, gradually falling until the last period when it rose again. The hourly total protein output following administration of secretin or pancreozymin was five to ten times greater than that of unstimulated animals throughout the experiment. The pancreatic juice protein concentration was greater in the pancreozymin treated animals, particularly so between hours six and ten.

DNA and protein synthesis were depressed by secretin administration whereas pancreozymin did not seem to exert any effect.

c) BOP Carcinogenesis

In the original paper describing the carcinogenic effects of BOP in the Syrian golden hamster, Pour and colleagues (1977a) reported on a number of features of the induced pancreatic tumours. Only half were visible to the naked eye; tumour nodules varied in size from two to 15 mm and were frequently multiple. Tumours appeared as early as 13 weeks after initiation of carcinogenesis in male animals and 15 weeks in females.

Histologically the tumours were adenomata, intraductal carcinomata and adenocarcinomata of a similar histological appearance to those induced by other nitrosamines (Pour et al, 1975a; Pour et al, 1975b; Pour et al, 1975e). In these earlier papers, adenomata appeared microscopically to be of ductal origin with a wide variation in appearance; the majority comprised tubular structures lined by a single layer of flattened epithelial cells while others consisted of cystic or cystic-papillary structures lined by a layer of single flattened cuboidal or columnar cells. Hyperplasia and infolding of the larger duct epithelium was seen in almost all animals. Intraductal carcinomata were characterised

by proliferation of relatively uniform cells with areas of secondary glandular formation and necrosis. The frank adenocarcinomata exhibited a wide variation in appearance. Some were cystic-papillary in appearance, some contained large amounts of mucous, and areas comprising transitional or squamous cell epithelium and signet ring cells were noted. Some poorly differentiated tumours resembled sarcomata and some areas of acinar cell carcinoma were reported. Some adenocarcinomata invaded adjacent structures and metastases to lymph nodes and other organs were occasionally seen. Carcinoma-bearing animals exhibited marked loss of weight and some developed ascites, thromboses and fat necrosis.

In the BOP paper (Pour et al, 1977a) no acinar cell tumours were reported but mixed ductal cell and islet cell neoplasms were noted in two male hamsters. Local invasion of adjacent structures was common and metastases were reported in a few animals. Three female hamsters developed obstructive jaundice, haemorrhagic ascites developed in three females, diarrhoea in two males and portal vein thrombosis in two females and two males. Fat necrosis was not seen.

In a detailed study, Pour and colleagues (1977d) examined the sequential effect of weekly subcutaneous injection of BOP (10 mg/kgBW) on the hamster pancreas. Male and female eight-week old Syrian golden hamsters were divided into three groups; group one received

carcinogen weekly for six weeks, group two received carcinogen weekly for life and group three were injected with normal saline as control. Group one animals were sacrificed in batches every second week from week two, and group two animals every second week from week eight. The pancreas from each animal was excised with extrahepatic ducts attached, fixed, cut into step sections (seven sections for each pancreatic lobe) and stained with haemotoxylin and eosin. Morphological changes were reported in the common duct, in the main and lesser pancreatic ducts, in the ductules and, to a lesser extent, in the islets.

From two weeks following initial exposure to carcinogen, focal hypertrophy and hyperplasia of common duct epithelium was reported. Goblet cell metaplasia was also seen at this stage. This was followed by stratification of the epithelium during the next eight weeks with cellular atypia and an increasing number of mitotic figures. Papillary proliferation was seen from 12 weeks until the termination of the experiment. Benign and malignant tumours within the duct were uncommon; two such periampullary carcinomata accounted for only 1.7% of all malignant neoplasms in the experiment.

Similar changes occurred in the main and lesser pancreatic ducts from week four. Epithelial cells became enlarged and encroached on the duct lumen, often resulting in a pseudobridging effect. Focal

areas of periductal inflammation and fibrosis were seen from six weeks. Progressive hyperplasia and heaping up of cells with increasing cellular atypia and mitotic figures resulted in areas of carcinoma-in-situ and intraductal carcinoma from 10 weeks. Frank invasion was seen from 12 weeks.

Ductular hypertrophy and hyperplasia were recorded from week two. Thereafter the carcinogen seemed to affect the ductules in a different manner from the larger ducts; ductular proliferation was seen from week six. These proliferating ductules formed adenomatous patterns from week eight and adenomata from week 10. Occasionally such adenomatous ductular proliferation could affect a whole pancreatic lobule. As the adenomata became more atypical, a pseudocapsule developed, often surrounded by a rim of lymphocytes. These lesions eventually breached the pseudocapsule and became invasive adenocarcinomata.

Changes in the pancreatic islets were also observed during exposure to BOP. Islet cells seemed to undergo hyperplasia resulting in an increase in islet size to four or five times normal. Similar changes in the islets were observed in the control animals, although to a much lesser extent. Intrainsular ductular formation was consistently observed during the carcinogenic process from eight weeks. These new ductules proliferated in a similar way to the normal ductules resulting in adenoma

formation from week 10. Islets seemed to atrophy as ductular proliferation progressed. Subsequent changes were similar to those seen with respect to the ductules.

Throughout the carcinogenic process, acinar cells underwent degeneration and death, often being replaced by proliferating ductules or other atypical cells. All pancreatic lesions reported were thought to have originated in the pancreatic ductal system. It proved difficult to determine the origin of many of the adenocarcinomata because of the simultaneous development of ductal and ductular lesions in close proximity.

In a subsequent study, Pour's group attempted to identify which part of the pancreatic ductal system was most susceptible to the effects of BOP (Takahashi et al, 1977b). Male and female hamsters were injected weekly with BOP (10 mg/kgBW) for five, seven and nine weeks and sacrificed in groups for examination two weeks after the last injection and every two weeks thereafter. In an attempt to determine whether cystic ductular changes occurred as a consequence of ductal or ductular obstruction, India ink was injected down the pancreatic duct of each animal immediately after death and the pancreas was photographed and prepared histologically as before. At week seven, areas of focal necrosis of acinar cells were reported. One hamster exhibited focal ductal hypertrophy and

hyperplasia with moderate periductal inflammation but all other ducts and ductules showed no changes and contained India ink. At nine weeks, changes of epithelial hyperplasia, hypertrophy and stratification were noted in the common duct in three animals and focal hyperplasia in the pancreatic duct in four of six animals. India ink was noted in all ducts and ductules including proliferated ductules and a secondary ductule which led to a small peripheral adenocarcinoma, although there was no ink within the adenocarcinoma itself. At 11 weeks there was still no ductal obstruction and all proliferated ductular structures contained ink. Two small adenocarcinomata showed no communication with ductal or ductular structures.

Despite the progression of all lesions during this experiment, ductal or ductular obstruction was an infrequent finding and usually occurred only when a large adenocarcinoma occluded one of the main ducts within the pancreas. The authors concluded that ductular alteration particularly affecting the peri- and intrainsular ductules was the most common response of the hamster pancreas to the carcinogen BOP. The majority of adenocarcinomata induced seemed to be ductular in origin. Ductal and ductular occlusion did not seem to play an important part in the genesis of cystic ductular proliferative lesions. They suggested that the cells of the ductular epithelium were the

primary progenitor cells of induced pancreatic cancer in the Syrian hamster model.

In 1978, Pour published the results of further observations relating to the role of pancreatic islets in the genesis of ductal adenocarcinomata in the hamster model (Pour, 1978b). Using biological material from previous studies in addition to pancreatic tissue from six further groups of hamsters injected weekly with BOP (10 mg/kgBW) for up to nine weeks and two groups receiving single injections of BOP (20 and 40 mg/kgBW), Pour carefully examined the morphology of islets and intra- and peri-insular ductules during the carcinogenic process. Carcinogen-treated animals exhibited a marked increase in numbers of islets associated with hyperplasia and hypertrophy of intralobular and interlobular ductal cells; new islets were usually hypovascular and composed almost exclusively of beta cells.

Pour concluded that ductular cells, particularly those of peri-insular and intrainsular origin were most susceptible to the effects of carcinogen. The carcinogenic process seemed to be initiated with hyperplasia of intralobular ductular and interlobular ductal cells associated with newly formed islets, followed by excess formation of mature and immature islet cells and islet cell percursors in the periphery of the islets. This was associated with the appearance, distension and multiplication of

periinsular and intrainsular ductules. The relationship between islet cell precursor cells and proliferating peri- and intrainsular ductular cells was emphasised and Pour suggested that they arose from a common undifferentiated precursor cell in response to the effect of the carcinogen. Subsequent changes in these cells result in malignant transformation and the establishment of the carcinogenic process.

Further support for this assertion came in another paper from Pour's group (Pour, Salmazi and Runge, 1978). Single subcutaneous injections of BOP (40, 20, 10, 5 and 2.5 mg/kgBW) were administered to male and female Syrian hamsters. Animals were observed for life and sacrificed when moribund. Most tumours of the pancreas occurred in the highest dose group and seemed to occur most frequently along the line of the main pancreatic ducts, where islets were most frequently found, although neoplastic change was seen in all groups, emphasising the high carcinogenic potency of BOP for the hamster pancreas. Periinsular and intrainsular ductular proliferation was seen in all treated hamsters whereas ductal hyperplasia was rarely seen. Similarly, intraductal carcinomata were rare indicating that the hyperplastic response of the ductal epithelium requires a higher threshold dose of carcinogen than proliferating ductular cells.

In a histological study of three human pancreatic cancers, Pour and colleagues (1979a)

demonstrated similar morphological changes in the affected pancreata to those seen in the Syrian hamster BOP model. All three human cases exhibited ductular and ductal hyperplasia and preneoplastic and neoplastic changes similar to those found in the animal model. Nesidioblastosis was also a common feature in association with hyperplastic and atypical intrainsular ductules. This paper concluded that cells of small ducts and ductules represented a source of human as well as experimental pancreatic tumours.

Flaks and colleagues (1980a) have questioned Pour's conclusions with regard to the cell of origin of experimental pancreatic cancer in hamsters. They pointed out that having initially claimed a ductal histogenesis for experimental pancreatic cancer in hamsters, Pour and colleagues subsequently indicated that ductular cells were the cells of origin followed by work suggesting that precursor cells arose from intrainsular ductules associated with newly formed islets. This confusion has been heightened by work from others which suggested that BHP induces exclusively ductal neoplasms (Levitt et al, 1977; Levitt et al, 1978). Flaks' group injected hamsters subcutaneously with BHP (250 mg/kgBW) weekly and sacrificed the animals in groups after two, four and six weeks of treatment and thereafter at three weekly intervals. Grossly visible pancreatic tumours were apparent from 12 weeks and all animals had tumours by

the end of the experimental period. No pancreatic lesions were seen until nine weeks of treatment. After 12 weeks, increasing numbers of abnormal acini were noted and as treatment progressed these acini became distended with a decrease in the size of the acinar cells. In the later stages of the experiment the acini were replaced by small ductule-like structures. From 15 weeks cystic foci became increasingly evident progressing into cystadenomata. Ductal changes were limited to luminal dilatation and ductal cell hyperplasia and there was no evidence that neoplasms arose from the ductal epithelium. Flaks group therefore disagreed with Levitt's results regarding a ductal cell of origin for the tumours. They also failed to demonstrate new islet formation or that intrainsular ductules had a role in pancreatic carcinogenesis. They suggested that the cell of origin of the cystic focus was the precursor of hamster pancreatic tumours and that further work was required to clarify the now confused situation.

Further work from Flaks' group has attempted to identify the cell of origin of experimental pancreatic cancer in the hamster model by looking in detail at the various morphological changes which occur during the carcinogenic process. In a study of the cystic lesions seen in the pancreas during carcinogenesis induced by BHP, using high resolution light microscopy and electron microscopy, it was suggested that the

cystic lesions, consisting of cells similar to those found in pancreatic adenomata and adenocarcinomata, were indeed true precursors of these tumours (Flaks, Moore and Flaks, 1980b). They noted, however, that cells possessing acinar cell characteristics could also be found in the cystic lesions and that they too might have an important role in the development of pancreatic tumours.

In a subsequent study, Flaks' group looked at lesions seen at an earlier stage during the carcinogenic process than the cystic lesions reported in their earlier paper (Flaks, Moore and Flaks, 1981). These pseudoductules, which seemed to arise from replacement of acini, appeared to develop from acinar cells which dedifferentiated in response to the carcinogen. The authors suggested that the primary target for BHP might indeed be the acinar cell of the pancreas.

Acinar cell changes were studied in two subsequent papers from Flaks' group (Flaks, Moore and Flaks, 1982a and 1982b). Acinar cells appeared to undergo a process of dedifferentiation leading to the development of a population of cells closely resembling centroacinar and ductular cells. Interestingly, they reported that ductular cells and centroacinar cells were not themselves affected by BHP. These findings supported the assertion that the acinar cell was the target for BHP carcinogenesis.

In 1983, Moore and co-workers suggested that cystic lesions were not true precursors of malignant pancreatic tumours induced by BOP and di-hydroxy-di-n-propyl nitrosamine (DHPN) (Moore et al, 1983a; Moore et al, 1983b). They proposed that the important carcinogenic sequence was ductal or ductular proliferation, epithelial atypia and dysplasia and carcinoma. This sequence appeared to be different from the ductular proliferation, benign multilocular cyst, cystadenoma sequence studied in depth by Flaks' group and shed doubt on the significance of Flaks' findings. Increase in the activity of glucose-6-phosphate dehydrogenase was established by Moore's group as a useful histochemical marker for early atypical proliferation.

In 1983, in a further attempt to determine whether ductal adenocarcinoma arose from acinar cells in the hamster model, Scarpelli's group reported on the effects of the administration of BOP during ethionine-induced pancreatic regeneration (Scarpelli, Roa and Subbarao, 1983). BOP was adminstered when the maximal number of acinar cells were in the S-phase of the cell cycle. Hamsters were fed with a methionine deficient diet and injected intraperitoneally once each day with DL-ethionine for two weeks to induce pancreatic degeneration, followed by a single IP injection of L-methionine to induce pancreatic regeneration. Pancreatic tumour induction was

augmented significantly in those animals injected with carcinogen during pancreatic regeneration. Despite the fact that the cells in the pancreas most affected during regeneration were acinar cells, neoplastic lesions induced were exclusively ductular and ductal in morphology. The authors hypothesised that either the acinar cells were unable to metabolise BOP to an active carcinogenic form or that exposure to BOP led to a phenotypic alteration of acinar cells to duct-like cells which subsequently underwent malignant change. They concluded that acinar cells were intimately involved in the pathogenesis of pancreatic ductal adenocarcinoma in the hamster model.

In a further study to determine the cell of origin of BOP induced hamster pancreatic cancer, Bell and Ray (1987) stained normal and neoplastic hamster pancreata with antiserum against the epithelial cell marker cytokeratin. In normal pancreas the monoclonal antiserum against cytokeratin stains centroacinar, ductular and ductal epithelium but did not stain acinar cells. In pancreata exposed to BOP the antiserum strongly stained cells of all BOP associated lesions including cysts, pseudoductules, hyperplasia, dysplasia and adenocarcinomata. Acinar cell staining was not seen in any pancreas exposed to BOP. The authors concluded that BOP-induced lesions arose from ductal epithelium and not from acinar cells.

d) Modifying the Carcinogenic Process

Since the establishment of the Syrian golden hamster model, investigators have attempted to elucidate the aetiology of pancreatic cancer by identifying factors which alter the development of tumour in response to carcinogens.

In 1978, Pour's group published the results of a study which investigated the effects of operative biliary diversion on BOP carcinogenesis in hamsters (Pour and Donnelly, 1978). The rationale for these experiments lay in the widely supported hypothesis that pancreatic cancer, most frequently reported as occurring in the head of the organ in humans, was caused by reflux of bile into the pancreatic duct. Hamsters underwent laparotomy and ligation and division of the common bile duct above the junction with the pancreatic duct. Thereafter, a small hole was made in the gallbladder fundus and the whole gallbladder was inserted through the duodenal wall into the gut lumen, five to ten millimetres distal to the opening of the common duct. Bile was thus diverted from the lower end of the common bile duct and away from its junction with the pancreatic duct. In two hamsters the integrity of the cholecystoduodenal anastomosis was confirmed by operative cholangiography. Hamsters then received BOP (5 mg/kgBW/week) for 20 weeks. Only animals which were deemed to be in a normal condition one week after

surgery were included in the study.

The mean survival of treated hamsters was 32 ± 4 weeks and at death all treated hamsters had developed pancreatic ductal adenocarcinomata. The distribution of neoplasms in the pancreas was similar to that previously reported and, in addition, over 90% of tumours exhibited ductular morphology. The authors concluded that bile diversion did not alter the pattern of experimental pancreatic carcinogenesis in the hamster. The distribution of tumour in the pancreas was proportional to the size and weight of the pancreatic segments in which the tumours developed; bigger segments generated more tumours. They suggested that the high frequency of head of pancreas cancer in humans could have a similar simple explanation.

In a subsequent study using the carcinogen BHP, biliary diversion using the same surgical technique as Pour did not alter the extent or distribution of pancreatic tumours in treated hamsters (Andren-Sandberg, Dawiskiba and Ihse, 1982).

In a further attempt to determine whether reflux of intestinal contents into the pancreatic duct increased the development of carcinogen-induced pancreatic cancer, Pour's group conducted an experiment where the neck of one of the pancreatic lobes was ligated, the distal part of the same lobe was divided, and both cut ends inserted into the

colonic lumen through a small hole, thus fashioning a partial pancreatico-colostomy (Pour et al, 1983b). The integrity and function of this anastomosis was established in ten pilot animals examined two weeks after surgery. BOP was administered as a single subcutaneous dose (20 mg/kgBW) shortly after surgery and animals were sacrificed 46 weeks after injection.

While the overall tumour yield was much less in this study than that reported in animals receiving carcinogen in weekly doses, partial pancreatico-colostomy did not alter the distribution, frequency or morphology of BOP induced lesions in the anastomosed pancreatic lobe. Interestingly, the frequency of lesions in a group of control animals not undergoing surgery was significantly greater than operated or sham-operated animals, confirming a previous finding that surgery seems to exert a protective effect when carcinogen is administered shortly after the operation (Pour et al, 1983a). The authors concluded that reflux of intestinal contents, including bile, did not have an aetiological role in the development of pancreatic cancer in the hamster.

Partial pancreatectomy (70% of organ excised) in hamsters given a single subcutaneous dose of BOP does have an effect on tumour development depending on when the carcinogen is administered in relation to the time of surgery (Pour et al, 1983a). The highest tumour yield occurred in animals injected with BOP one week

after surgery and it was lowest in those injected with BOP 30 minutes after surgery ($p<0.01$). Pancreatectomised animals given BOP one week after surgery developed significantly more cancer in the residual duodenal lobe and head of the gland than non-operated control animals in the same regions of the pancreas. The authors concluded that the higher incidence of tumour in the animals injected one week later occurred because the carcinogen was adminstered at a time when the pancreas was undergoing regeneration; they cited previously published work in rats which showed that post-partial pancreatectomy, DNA synthesis was maximal after 36 hours (Lehv and Fitzgerald, 1968; Konishi et al, 1978) and that by two months, pancreatic segments reach a size 21-78% greater than the segments of non-pancreatectomised rats (Pearson, Scott and Torrance, 1977). Pour's group assumed for their experiment that the same regenerative process would occur in hamster pancreata. They also observed that most tumours developed at the excision line (which was oversewn) and postulated that local inflammatory or degenerative processes could have an influence on response to carcinogen.

In an attempt to look in more detail at the effects of pancreatic degeneration and regeneration on BOP carcinogenesis, Pour's group investigated the effects of experimentally induced pancreatitis, following temporary pancreatic duct ligation, at

various times during this disease (Pour et al, 1983c). Pilot studies identified that the optimal duration of duct ligation for inducing pancreatitis, with minimal mortality, was 48 hours. BOP was administered subcutaneously in one dose (20 mg/kgBW) 30 minutes before duct ligation in one group of animals and at three days (peak time for cellular degeneration), one week (peak time for cellular regeneration) and eight weeks (healing phase) after duct ligation in three other groups. A fifth group underwent duct ligation four weeks after injection of BOP and again at four weekly intervals thereafter (recurrent pancreatitis group). BOP produced significantly fewer tumours than control groups when administered during cellular degeneration (day 3 group) and during healing (week 8 group). When administered before duct ligation or during cellular regeneration (week 1 group) BOP did not seem to influence the tumour pattern compared to controls. The incidence and size of pancreatic carcinomata was significantly higher in the recurrent pancreatitis group than controls.

The authors were surprised that BOP administration during the regenerative phase of acute pancreatitis did not increase tumour yield, but suggested that difficulties in identifying one week as the time of maximal ductal cell regeneration in the pilot studies, which often occurred with the phase of degeneration, might have resulted in carcinogen being

administered before or after the real regenerative phase. In addition, the mortality in the one week animals was extremely high (only 16% survived to the end of the experiment compared to 65% of one of the comparable control groups). On-going inflammation and regeneration in the animals with recurrent pancreatitis was suggested as a possible cause for the increase in pancreatic cancer in this group.

Cholecystectomy has been suggested as a risk factor for the development of pancreatic cancer in humans (Wynder et al, 1973). Cholecystectomy in hamsters has been shown to increase circulating levels of cholecystokinin and increase pancreatic weight, DNA content and synthesis rate and protein content (Rosenberg et al, 1983). In a subsequent study the same group of authors demonstrated, using morphometric and autoradiographic techniques, that cholecystectomy resulted in hyperplasia and hypertrophy of the pancreas (Rosenberg et al, 1984). In the light of evidence quoted above that regenerating pancreatic tissue is more at risk from the effects of carcinogen, these observations might explain the increased risk of pancreatic tumours in cholecystectomised humans, although there is no published evidence in man to support this thesis, particularly given the time delay between cholecystectomy and the development of pancreatic cancer many years later.

Cholecystectomy alters bile acid composition by reducing the circulating pool of primary bile salts and increasing the proportion of secondary bile salts (Hepner et al, 1974; Pomare and Heaton, 1979). Exogenous dietary administration of the secondary bile salts lithocholic acid (LCA) and deoxycholic acid (DCA) during BHP carcinogenesis, both significantly enhance the development of pancreatic cancer in the hamster (Makino et al, 1986). In the same study administration of phenobarbital did not alter the incidence of pancreatic carcinomata.

In 1986, Ura et al studied the effects of combining cholecystectomy with dietary administration of LCA in the BHP hamster model. The incidence of pancreatic cancer was significantly increased only in those animals subjected to cholecystectomy and LCA supplemented diet. Cholecystectomy increased the yield of pancreatic cancer from 28% to 56% but this did not reach statistical significance. LCA dietary supplementation alone did not increase the incidence of pancreatic cancer compared to those animals receiving standard feed. The dose of BHP used in Ura's study was only 250 mg/kgBW/dose for five doses compared to 500 mg/kgBW/dose for five weeks in Makino's study; this might explain the failure of Ura's study to demonstrate any effect of LCA.

Throughout 1983, Pour's group published the results of a series of experiments looking at the

effects of dietary factors on experimental pancreatic carcinogenesis in the hamster model. Hamsters fed a protein free diet for 28 days developed significantly fewer pancreatic tumours compared to controls regardless of when the carcinogen was administered during the dietary period (Pour et al, 1983d). In addition to inhibition of tumour development, hamsters injected with BOP on day 18 of the protein free diet did not develop any benign or malignant tumours in the pancreas, suggesting that dietary protein may also be important in the initiation of BOP-induced pancreatic cancers.

Diets high in unsaturated fat have been shown to enhance both the initiation and the promotion of BOP-induced pancreatic cancer in hamsters (Birt, Salmasi and Pour, 1981). In a later study, Birt and Pour (1983a) reported an increase in the yield of renal adenocarcinomata, pulmonary adenomata and benign biliary cystic adenomata in animals exposed to BOP and a high-fat diet. In a further study hamsters were fed diets low or high in fat and protein in combination for eight weeks before or after a single subcutaneous BOP injection (10 mg/kgBW) (Birt et al, 1983b). When BOP was administered after the special diets, pancreatic cancer yield was highest in those animals fed a high fat/high protein diet, less in those fed a high fat/low protein diet, and least in those on the low fat/high protein and low fat/low protein diets.

In those animals where BOP was administered before the special diets the development of pancreatic cancer was inhibited in the low fat/low protein group and greatest in the high fat/high protein group (although not as great as in the high fat/high protein group given BOP after the special diet). Animals fed high fat diets either before or after BOP injection had on average a 25% greater calorie intake and a 25% greater intake of other dietary substances including protein compared to animals in the low fat groups. Whether this observation is important in explaining the differences reported is not clear. The authors concluded that both dietary fat and protein influenced experimental pancreatic carcinogenesis.

In 1987, Woutersen and colleagues in an attempt to develop a short four month protocol of BOP treatment which would allow more careful study of the pre-neoplastic lesions seen during carcinogenesis, reported that diets high in saturated fat resulted in a greater yield of large ductal complexes, ductal hyperplasia and ductal atypia. Dietary fat did not seem to influence main pancreatic ductal changes. They concluded that saturated fat in the diet enhanced pancreatic carcinogenesis in the hamster.

A subsequent study into the effects of different levels of dietary protein alone produced confusing results (Pour and Birt, 1983e). Hamsters were fed diets with low or high protein content (given as

casein) before or after BOP treatment. The low protein diet inhibited carcinogenesis only in female hamsters whereas the high protein diets did not affect the development of pancreatic cancer in any way. The authors were unable to offer a convincing explanation for these unexpected results.

Alcohol has been proposed as a possible aetiological factor in human pancreatic cancer and its effects have been studied in the BOP-hamster model. When administered in the animals' drinking water in a concentration of 25% weight/volume, ethanol inhibited pancreatic cancer induced by a single subcutaneous injection of BOP administered two weeks after commencing alcohol ingestion (Tweedie et al, 1981). In a subsequent study, 5% ethanol administered in drinking water either before or before and after a single subcutaneous injection of BOP, failed to have any effect on the development of pancreatic cancer (Pour et al, 1983f). The authors of the second of these papers suggested that the effect of ethanol could be dose-related and that the higher concentration could be exerting some sort of toxic effect on the pancreas which somehow interfered with the carcinogenic process.

Other dietary components which might exert a protective influence with regard to the development of pancreatic cancer in humans, have been examined for their effect on BOP-induced pancreatic carcinogenesis

in the hamster model. Natural and synthetic retinoids have been shown to inhibit the later stages of carcinogenesis in animal models of skin, breast and bladder cancer (Bollag, 1972; Moon et al, 1977; Becci et al, 1978). In a large study of the effects of four synthetic retinoids, Longnecker and co-workers (1986) demonstrated a significant reduction in the yield of pancreatic cancer in male hamsters fed N-4-propionyloxyphenylretinamide or retinylidene dimedone. A lower yield of pancreatic cancer was seen in 12 of the 14 retinoid-fed groups although this did not reach significance in the remainder. The authors urged caution in the interpretation of these results particularly since the overall yield of pancreatic neoplasms in the animal groups was relatively low.

Dietary cabbage supplementation of a high-fat diet produced more BOP-induced pancreatic cancer in hamsters than a low-fat, cabbage-supplemented diet or a high-fat diet without supplementary cabbage, in a study published in 1987 (Birt et al, 1987). The authors of this paper suggested that further work was required before the general public should be urged to increase their consumption of green vegetables in an attempt to reduce cancer risk.

Interest in the effect of dietary changes on experimental pancreatic carcinogenesis and the implication of diet in the aetiology of human pancreatic cancer has stimulated further

investigations into possible mechanisms for these observations. Cholecystokinin, a peptide hormone released from the duodenal mucosa in response to a diet rich in fat and protein, has been studied for its effect in the hamster model. Howatson and Carter (1985) reported that a six week course of CCK, administered subcutaneously in a gelatin carrier twice daily on three days each week around the time of weekly BOP administration, significantly increased the yield of pancreatic cancer in a group of hamsters after 15 weeks of carcinogen, when compared to animals injected with BOP alone. In the same paper they demonstrated that 15 consecutive days of twice daily CCK administration and the same schedule of CCK injections as used in the carcinogen part of the study significantly increased pancreatic DNA content and pancreatic wet weight compared to control animals. The authors postulated that CCK acted as a co-carcinogen or promotor of pancreatic carcinogenesis by influencing pancreatic cell turnover and in so-doing, increased the susceptibility of the dividing cells to the effects of BOP.

Not all investigators have agreed with these findings. Johnson and co-workers (1983) reported that CCK administered before or simultaneously with BOP exerted an inhibitory effect on pancreatic tumour development. Caerulein, an analogue of CCK has also been studied for its effects in the hamster model.

Andren-Sandberg and colleagues (1984) failed to demonstrate an effect of caerulein administration on experimental pancreatic carcinogenesis while a later study reported a statistically significant increase in pancreatic cancer in hamsters injected with weekly caerulein and the nitrosamine carcinogen BHP (Satake et al, 1986). Differences in experimental design, particularly with regard to the timing of CCK/caerulein administration in relation to carcinogen administration, make interpretation of these conflicting results difficult.

Secretin, another gastrointestinal peptide hormone, released in response to acid in the duodenum, stimulates pancreatic secretion primarily by stimulating ductal rather than acinar cells. In a study of similar design to their CCK study, Howatson and Carter (1987) demonstrated a significant increase in pancreatic cancer yield in hamsters injected with secretin in addition to BOP providing further evidence for a link between the effects of diet and pancreatic cancer.

Exogenous administration of epidermal growth factor (EGF) during the carcinogenic process in the BOP hamster model promotes the development of pancreatic cancer (Chester et al, 1986). After 19 weeks of carcinogen and four weeks (weeks five to eight) of EGF, 75% of EGF treated hamsters exhibited histological pancreatic cancer compared to only 44% of

these receiving BOP ($p = 0.016$). Interestingly the incidence of bronchial carcinoma increased in the EGF treated animals.

1.7 Cholecystokinin, Secretin and the Pancreas

a) Cholecystokinin

The main actions of cholecystokinin were initially described earlier this century by two independent groups of workers; instillation of fat into the proximal intestine of the hog stimulated gallbladder contraction (Ivy and Oldberg, 1928) and a substance released from the duodenal mucosa stimulated pancreatic secretion (Harper and Raper, 1943). Subsequent purification of these two substances demonstrated that they were an identical peptide comprising 33 amino acids (Jorpes and Mutt, 1962; Jorpes, Mutt and Toczko, 1964; Jorpes, 1968), the C-terminal tetrapeptide of which is identical to gastrin. Ivy and Oldberg confirmed the hormonal nature of CCK in a series of carotid-to-carotid cross-perfusion studies (Ivy and Oldberg, 1928).

Since these original observations, CCK has been shown to exhibit considerable molecular heterogeneity, occurring in several different forms (CCK-58, CCK-39, CCK-33, CCK-8, CCK-5, CCK-4 and probably CCK-21 and CCK-12). The sulphated form of CCK (sulphated on the tyrosine residue of position 7 in CCK-33) is more potent with respect to gallbladder contraction than the desulphated form (Amer, 1969). PRO-CCK, a 95 amino acid precursor of the CCK family and PRE-PRO-CCK of 115 amino acid residues have both been described

and characterised (Gubler et al, 1984; Beinfeld, 1985; Deschenes et al, 1985).

Cholecystokinin is present throughout the gastrointestinal tract (Ogden et al, 1982) and in the central and peripheral nervous systems (Brownstein and Rehfeld, 1985). Interestingly, different CCK receptors have been described in the brain and pancreas; the pancreas possessed 300 times more binding sites than the brain (Innis and Snyder, 1980). Sulphated CCK had a higher affinity for both types of receptor in this study.

In 1928, ingestion of fat was shown to cause gallbladder contraction (Boyden, 1928). Since then it has been shown by many investigators that CCK is released in response to the presence of fat and protein digestion products in the gut lumen. L-isomers of the amino acids, particularly tryptophan and phenylalanine, and straight-chain fatty acids are particularly potent in this respect (Lin, 1975; Marx et al, 1987). Postprandial release of CCK in response to food or intraduodenal fat occurs after 5 minutes in humans, 20 minutes in pigs and after 2 hours in dogs (Lilja et al, 1982). In a study using ultrasonography to assess gallbladder contraction, Wiener and colleagues (1981) demonstrated that intraduodenal instillation of a medium-chain triglyceride increased plasma CCK to maximal levels within 16 minutes and reduced gallbladder volume to minimum in 18 minutes.

Infusion of exogenous CCK in humans to physiological plasma levels has been shown to stimulate pancreatic secretion and gallbladder contraction (Kerstens et al, 1985). Interestingly, in rats, only intact proteins but not amino acids, fats or carbohydrate seem to stimulate CCK release (Liddle et al, 1986).

In addition to the stimulation of gallbladder contraction and pancreatic enzyme secretion, CCK has been shown to exert a number of other effects. It stimulates bicarbonate release and insulin secretion from the pancreas, intestinal motility, the contraction of the resting stomach and pylorus and bile production in the liver, amongst others; it inhibits lower oesophageal sphincter contraction and contraction of the sphincter of Oddi (all cited in Marx et al, 1987).

In 1967, Rothman and Wells found that synthesis of pancreatic enzymes in the rat was stimulated by CCK. Although their study was not designed to demonstrate trophic influences on the pancreas, they did note that pancreatic weight was significantly increased in the CCK group. Other investigators have confirmed the trophic effect of CCK (Barrowman and Mayston, 1973; Brants and Morisset, 1976; Howatson and Carter, 1985). Chronic administration of CCK was associated with increases in pancreatic protein, RNA and DNA content and in stimulation of [^{14}C]thymidine incorporation into DNA in a study by Mainz and co-

workers (1973). Caerulein (an amphibian skin decapeptide structurally and functionally related to CCK) was shown to be trophic to rat pancreas, and when administered with exogenous secretin, the trophic effect was greater than expected by simple summation of the effects of each individually (Solomon et al, 1978). The potentiated trophic effect on the pancreas of combined administration of caerulein and secretin was confirmed by Dembinski and Johnson (1980). Subsequent workers have confirmed these early observations although there have been some interesting species differences and variations in response depending on dose schedules and CCK preparations used.

b) Secretin

The study of endocrinology started in 1902 when Bayliss and Starling described the secretion of pancreatic juice in response to the infusion of hydrochloric acid into a devervated loop of small intestine. The 27 amino acid sequence of secretin was not described or synthesised for another 60 years (Bodanszky et al, 1966; Mutt et al, 1965).

Secretin is found predominantly in the duodenum and proximal small intestine (Curtis, Rayford and Thompson, 1976; Straus and Yalow, 1978). More recently, immunoreactive secretin has been discovered in the central nervous system of rats and pigs (O'Donohue et al, 1981).

Despite a search for dietary and other stimulants of secretin release, duodenal acidification with hydrochloric acid remains the only consistent such agent (Rayford et al, 1976); secretin release is proportionate to the amount of acid in the duodenum. In the dog, perfusion of an isolated segment of jejunum with hydrochloric acid or sodium oleate stimulated secretin release (Fujimura et al, 1984a; Fujimura et al, 1984b). In the rat, acid perfusion of the distal ileum increased plasma secretin and pancreatic bicarbonate secretion (Shinomura et al, 1983).

In addition to the stimulation of pancreatic bicarbonate secretion, secretin stimulates the production of colonic mucin, gastric pepsin secretion and increases serum parathormone; it inhibits colonic contraction, gastric acid secretion, gastric emptying, lower oesophageal sphincter tone and gastrointestinal motility (cited in Doyle, Luis and Rayford, 1987).

Secretin has also been reported as having a trophic effect on the pancreas. Eight-hourly subcutaneous injections of secretin (25 ug/kgBW) for 5, 10 and 15 days resulted in a significant increase in pancreatic weight only in the 15 day treatment group in a study by Solomon and co-workers (1978). Pancreatic DNA content in the secretin-treated animals was not increased in this study although there was a significant increase in RNA and lipase content at 15

days. Protein content was also increased in the secretin treated rats, but this did not reach statistical significance. The authors concluded that secretin caused pancreatic hypertrophy alone, given the lack of increase in DNA content. In 1980, Dembinski and Johnson reported that chronic IP injection of 50 U/kgBW of secretin eight-hourly for seven days in rats, resulted in a significant increase in pancreatic DNA and RNA concentration compared to control animals. Pancreatic DNA synthesis was also significantly increased in the secretin-treated group. The authors concluded in this study that secretin stimulated both pancreatic hypertrophy and hyperplasia.

In 1981, in a study of the effects of endogenous secretin on the pancreas, Johnson reported that five days of continuous duodenal perfusion with hydrochloric acid (known to stimulate the release of secretin) resulted in an increase in pancreatic weight and in pancreatic DNA content in Sprague-Dawley rats. He concluded that endogenous secretin had a similar trophic effect on the pancreas as exogenous hormone. Unfortunately, Johnson was unable to assay secretin to ensure that duodenal perfusion with acid had resulted in stimulation of secretin secretion.

1.8 Somatostatin and Analogues

a) Physiology

Somatostatin, a tetradecapeptide with a molecular weight of 1637 was first described by Brazeau and colleagues (1973) during a search for an ovine hypothalamic growth hormone (somatotropin) release inhibiting hormone (SRIF or GHRIH). Brazeau's group were able to purify, sequence and synthesize SRIF and renamed it somatostatin. Schally and colleagues (1976) isolated and characterised somatostatin from porcine hypothalami and confirmed that its amino acid sequence was identical to somatostatin of ovine origin. Identical substances have since been isolated and purified from pigeon pancreas (Speiss et al, 1979), from the pancreatic islets of anglerfish (Noe et al, 1979) and from the pancreas of Sprague-Dawley rats (Benoit et al, 1980). Somatostatin-28 has been identified in porcine intestine (Pradayrol et al, 1980) and in ovine hypothalamus (Esch et al, 1980). Even larger forms of somatostatin have been identified and sequenced (Bethge, Diel and Usadel, 1982).

In 1975, Polak and colleagues described the presence of GHRIH-like immunoreactivity in the D-cells of the pancreatic islets and in cells in all parts of the upper gastrointestinal mucosa. GHRIH staining cells were localised mainly to the midzone of mucosal

glands, but also to cells in the basal region of the glands and the tips of the villi. Since then, somatostatin has been found in the nervous system, in various body fluids, in most of the tissues of the body and in some tumours (Efendic, Hokfelt and Luft, 1978; Larsson, 1981; Bethge et al, 1982). In the gastrointestinal tract, which contains approximately 70% of the body's total somatostatin (Patel et al, 1981), somatostatin has been demonstrated in the antrum, body and fundus of the stomach, the duodenum, jejunum, ileum, colon and pancreas, with particularly high activity in the pancreas and the stomach (McIntosh et al, 1978). In man, its half-life in plasma is short; between 1.69 minutes (Bethge et al, 1981) and 3 minutes (Bauer et al, 1982).

In the gastointestinal tract somatostatin release is influenced by vagal tone, prostaglandins, circadian rhythm, cholinergic and adrenergic receptors and fluctuations in the migrating motor complex; post-prandial plasma levels are increased by meals, intraluminal acid and bile, and circulating nutrients, and are modulated by vagal tone, prostaglandins, and cholinergic, adrenergic, histaminergic and opiate receptors. Somatostatin release is stimulated by CCK, gastrin, gastric inhibitory polypeptide (GIP), secretin, bombesin, substance P and vasoactive intestinal polypeptide (VIP) (Newman, Lluis and Townsend, 1987).

The actions of somatostatin in the gastrointestinal tract are numerous. It inhibits salivary gland, gastric acid, pepsin and intrinsic factor secretion (Arnold and Lankisch, 1980), and increases gastric mucous production (Johansson and Aly, 1982). It inhibits the release of all known gastrointestinal hormones including gastrin, secretin, CCK, motilin, enteroglucagon, GIP, VIP and pancreatic polypeptide (Arnold and Lankisch, 1980). It inhibits the release of insulin and glucagon from the pancreas (Koerker et al, 1974) and when administered exogenously, decreases the release of the thyroid hormones T_3 and T_4 (Lins, Efendic and Hall, 1979). Amino acid residues 7 to 10 are essential for biological activity (Bauer et al, 1982).

The effects of somatostatin on the exocrine pancreas are less clear. Pharmacological doses of somatostatin in dogs resulted in competitive inhibition of the effects of secretin but not CCK (Konturek et al, 1976), and when administered as a bolus in rats seemed to stimulate pancreatic secretion (Arnold and Lankisch, 1980); it was suggested that identical amino acids comprising the 5-8 positions in the secretin molecule and the 10-13 positions in the somatostatin molecule might explain this phenomenon. The majority of studies, however, report an inhibition of the secretion of protein, bicarbonate and volume of pancreatic juice in response to somatostatin

administration (Arnold and Lankisch, 1980).

b) Somatostatin Analogues

The half-life of somatostatin in plasma in man is only three minutes (Bauer et al, 1982). Therapeutic application is therefore difficult; continuous infusions have been required to arrest upper gastrointestinal haemorrhage from peptic ulcers (Kayasseh et al, 1980; Magnusson et al, 1985) and from oesophageal varices (Jenkins et al, 1985). This short half-life and multiple inhibitory effects have prompted the search for analogues of somatostatin with longer durations of action and more specific inhibitory effects.

Des[Ala^{1},Gly^{2},Asn^{5}]-somatostatin was shown to inhibit the release of insulin, and to a lesser extent growth hormone, without inhibiting glucagon release (Sarantakis et al, 1976). D-Cys^{14}-somatostatin and [D-Trp^{8},D-Cys^{14}]-somatostatin, selectively inhibit growth hormones and glucagon more than insulin release (Brown, Rivier and Vale, 1977; Gordin et al, 1977). Since these initial reports, other somatostatin analogues have been developed with more selective inhibitory actions (Schally, Coy and Meyers, 1978). SMS 201-995 (Sandoz) is an octapeptide analogue of somatostatin [H-(D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr-ol, acetate]. It has a plasma half-life of 45 minutes following the termination of an intravenous infusion

and is a more potent inhibitor of growth hormone, glucagon, insulin and gastric acid secretion than somatostatin. In rats, it has been shown to prevent the development of acute pancreatitis (Baxter et al, 1985a), stimulate the reticuloendothelial system (Baxter et al, 1985b) and reduce the effect of endotoxaemia (Baxter et al, 1985c).

c) Somatostatin Analogues and Pancreatic Cancer

The inhibitory effects of somatostatin and its analogues on various aspects of pancreatic endocrine and exocrine function prompted some investigators to examine its effects on the growth and development of pancreatic tumours. Redding and Schally (1984), using hamster-ductal and rat-acinar pancreatic cancers transplanted into other hamsters and rats respectively, demonstrated that chronic administration of a number of somatostatin analogues could inhibit tumour growth. [L-5-Br-Trp^{8}]-somatostatin-14, which is equipotent with somatostatin with respect to gastric acid secretion suppression, significantly decreased tumour weight and volume in rat and hamster tumour-bearing animals. A cyclical hexapeptide analogue of somatostatin (cyclo-Pro-Phe-D-Trp-Lys-Thr-Phe) inhibited hamster ductal pancreatic cancer growth but had no effect in rats. Somatostatin-28 had no effect on tumour growth in the rat model. This work prompted the authors to suggest that pancreatic cancer

in humans might respond to similar hormonal manipulation.

In a study of a human pancreatic cancer cell line (MIA PaCa-2), Liebow and co-workers (1986) demonstrated that somatostatin could eliminate growth stimulation of the tumour cells by EGF in cell culture. They had previously demonstrated that membrane-bound and cytosolic receptors for somatostatin existed in this cell-line and that binding of somatostatin dephosphorylated a phosphotyrosyl-membrane protein whose phosphorylation had been promoted by EGF (Hierowski et al, 1985). They suggested that this mechanism might explain somatostatin's inhibitory effect on pancreatic cancer and prompt further investigation into possible therapeutic applications of this information.

In 1987, Paz-Bouza, Redding and Schally reported the results of an investigation into the effects of somatostatin analogues on BOP-induced ductal pancreatic adenocarcinoma in the Syrian hamster. The cyclical analogue RC-160 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2 with a disulphide bond between the two Cys amino acids) was administered in slow release microcapsules (liberating 5 ug/day of RC-160) to female hamsters after 18 weeks exposure to BOP. Treated hamsters exhibited a striking decrease in tumour weight and volume and an increased percentage survival after 45 days of treatment compared to

controls.

As yet, there has been no published investigation into the effect of somatostatin or its analogues on pancreatic carcinogenesis in animal models.

1.9 Summary and Aims of Thesis

Cigarette smoking is the most consistent factor so far implicated in the aetiology of human pancreatic cancer. Nitrosamines, which we know to be present in cigarette smoke, can induce exocrine pancreatic cancer in animal models, although, to date, none of the specific nitrosamine carcinogens so far detected in cigarette smoke have been shown to induce such malignant change. Given the morphological similarity between nitrosamine-induced hamster and human pancreatic cancer, and the likely implication of nitrosamine-containing cigarette smoke in the aetiology of the human tumour, the nitrosamine-hamster model of pancreatic cancer would appear to be reasonably valid for investigating aspects of pancreatic carcinogenesis.

There is increasing evidence to suggest that dietary factors may explain some of the differences in the incidence of pancreatic cancer throughout the world. Diets with a high fat and protein content have been implicated most frequently in the increasing incidence of pancreatic cancer. The influence of some dietary factors on experimental pancreatic carcinogenesis has been investigated in animal models, and generally has confirmed that diets with a high fat and protein content increase the susceptibility of the pancreas to the effects of carcinogens.

Given that dietary fat and protein are amongst the most powerful stimulants of the release of various gastrointestinal hormones, which have been shown by many investigators to influence pancreatic physiology and morphology, the question arises as to whether the effects of diet on pancreatic cancer susceptibility might be mediated through some of these hormones. Cholecystokinin and secretin have previously been shown to increase the susceptibility of animal pancreata to the effects of carcinogens. Whether they can be implicated in the human situation is less clear.

Somatostatin, an inhibitory gastrointestinal hormone, has been shown to inhibit both the release and the effects of secretin and CCK. Unfortunately somatostatin has a short half-life and this has prompted the development of many somatostatin analogues. Some investigators have demonstrated that some somatostatin analogues, usually in pharmacological doses, can inhibit the progression of induced animal pancreatic cancer. They have speculated that some of the longer acting analogues may have a role in treating established human pancreatic cancer.

There is little published information on the effect of somatostatin analogues administered during the carcinogenic process in animal models. In addition, there is little information on the

interaction between somatostatin analogues and some of the other gastrointestinal hormones in this respect. As a consequence, the aims of this thesis were:

1) to establish the nitrosamine (BOP) - hamster model of pancreatic carcinogenesis in our laboratory;

2) to investigate the effects of intravenously infused physiological doses of secretin, CCK-8 and the long-acting somatostatin analogue SMS 201-995, alone and in combination, on exocrine pancreatic juice output in the Syrian golden hamster;

3) to determine the effects of subcutaneous injections of secretin, CCK-8 and SMS 201-995, alone and in combination, on pancreatic wet weight and pancreatic DNA content, in the Syrian golden hamster;

4) to determine the effects of subcutaneous injections of secretin, CCK-8 and SMS 201-995, alone and in combination, on BOP carcinogenesis in the Syrian golden hamster.

CHAPTER 2

Induction of pancreatic adenocarcinoma in the Syrian golden hamster: intraperitoneal versus subcutaneous administration of N-nitrosobis(2-oxopropyl)amine (BOP)

2.1 Introduction

The nitrosamine-hamster model of pancreatic cancer has been used by many authors to study various aspects of carcinogenesis since its development by Pour in the 1970's. In order to determine that the model could be reproduced in our own laboratories using the dosing schedules previously described, a pilot study was undertaken.

When the model had been used previously in the University Department of Surgery at Glasgow Royal Infirmary, considerable morbidity had been experienced by the animals as a consequence of repeated subcutaneous injections of the carcinogen N-nitrosobis (2-oxopropyl)amine (BOP) (D. Bell, personal communication). This morbidity took the form of extensive skin ulceration and infection. In addition, there was no available published data on the effect of intraperitoneal injection of carcinogen on the pancreas. It was decided therefore to embark on a small comparative study of intraperitoneal versus subcutaneous administration of carcinogen, to compare the degree of morbidity experienced by the animals in the two groups and to determine whether the morphological changes developed in the pancreata were comparable.

2.2 Materials and Methods

Sixty male WO(GD) strain Syrian golden hamsters (Wrights of Essex) were divided into two groups of 30 animals each. All animals were kept in standardized animal facility conditions with a 12 hour light dark cycle, 21% humidity and caged in groups of four. All animals were fed standard small rodent diet (Rat and Mouse No. 1 Modified Maintenance Diet, Special Diet Services, Witham, Essex) and water ad libitum.

N-nitrosobis(2-oxopropyl)amine (BOP: Ash Stevens Inc., Detroit, USA) was administered once weekly to all animals in a dose of 5mg/kg body weight (kgBW) per injection. Group A received carcinogen intraperitoneally and Group B subcutaneously (SC). The site for SC administration was rotated through right thigh, left thigh, right flank and left flank in an attempt to minimise morbidity from skin ulceration due to repeated injection of carcinogen at one site.

Carcinogen was made up into solution with normal saline (0.9%) to a concentration of 1mg/ml of solution, within one hour of administration, as BOP is known to degrade if left in solution for longer periods. A filter mask was worn during weighing of carcinogen and making up of the solution, and any spillage was immediately cleaned with liberal amounts of water. All staff handling the animals receiving carcinogen wore gloves and animal waste was incinerated.

Animals in both groups were weighed weekly on the day prior to injection. The carcinogen dose administered was calculated on the basis of the animal weight on the previous day, and rounded up or down to the nearest 0.05ml.

Animals were sacrificed in groups after five, 10, 15 and 20 weeks of carcinogen administration. A full post-mortem examination was performed at time of sacrifice and gross lesions in the pancreas and other organs sought. The pancreas was then excised with the duodenum attached to the duodenal lobe and a segment of spleen attached to the splenic lobe to allow identification of the lobes. The entire excised pancreas was fixed in 4% formal saline solution.

After fixation, the pancreas was cut and blocked in paraffin wax. Three blocks were cut from the gastric and splenic lobes and one from the duodenal lobe. Three serial sections 4um thick were cut from each block, mounted on chrome alum microscopic slides and stained with haematoxylin and eosin (H&E). Gross lesions in the pancreas were excised, fixed and stained separately.

All sections were examined for the presence of abnormalities when compared to normal hamster pancreas. In particular, evidence of acinar cell death, acinar-ductular (A-D) transformation, cystic ductular complexes, ductular carcinoma-in-situ (CIS), ductular microcarcinoma, ductal dysplasia, ductal CIS

and frankly invasive adenocarcinoma was sought.

Acinar cell death was recorded when a cell appeared to lose its staining characteristics, when the nucleus appeared pyknotic or when an acinus appeared distorted and small, indicating the death of a number of cells in the acinus. Acinar cell death was often accompanied by an increase in the number of ductular or centroacinar cells. The loss of solid acinar structure and its replacement by or degeneration into a cystic structure lined by a thin rim of cellular material was recorded as AD transformation. When three or more areas of AD transformation appeared together the lesion was described as a cystic ductular complex. The term ductular CIS was used to describe a lesion composed entirely of atypical, dysplastic ductules. These ductules varied greatly in size and shape and were composed of cells with pleomorphic nuclei with frequent mitotic figures. These lesions were usually encapsulated by a zone of inflammatory cells and involved only one pancreatic lobule. A ductular microcarcinoma was essentially a large ductular CIS which had breached the boundaries of a lobule.

The first change seen in larger ducts in response to BOP was the development of ductal hyperplasia. The neatly arranged unilayer of cells lining the duct became heaped up and the cells seemed to lose their polarity. A more disordered appearance

associated with nuclear pleomorphism and the appearance of the occasional mitotic figure was recorded as ductal dysplasia. Ductal CIS was characterised by intraluminal proliferation of atypical epithelium, often of papillary type, with crossbridging across the duct lumen and large numbers of mitotic figures and pleomorphic nuclei. An increase in surrounding inflammatory cells was also a feature.

Invasive adenocarcinoma exhibited all the characteristics of malignancy in any organ, namely a high mitotic rate, nuclear pleomorphism, disordered architecture and invasion.

The presence of a lesion in any section of any one pancreas was recorded as positive for that pancreas. In addition, in an attempt to gauge the extent of any particular histological lesion in the pancreata, results have been expressed as present or absent in each block in any group.

The nature of the histological lesions reported by the author was confirmed in a series of 100 histological blocks examined blindly by Dr. David J. Harrison, Senior Lecturer in the Department of Pathology, University of Edinburgh. The number of lesions present in the SC and IP groups at each time point was compared using Fisher's exact test. Animals weights were compared using a two sample analysis of variance.

2.3 Results

Table 2.1 depicts changes in mean body weights in the two groups of animals over the 20 week experiment. Mean body weight in the two groups increased from 89.3g (IP) and 93.1g (SC) at week 0 to a maximum of 126.2g (IP) and 125.9g (SC) at week 15. Mean body weight thereafter fell in both groups, presumably as a result of the development of malignant lesions in the pancreas. The body weights of the two groups were significantly different at week 0 ($p<0.05$). There was no statistical difference between animal weights in the two groups at any other time point during the experimental period.

Three animals in the intraperitoneal group exhibited weight loss in excess of 10% of body weight in one week, which, under the terms of the animal project licence necessitated humane sacrifice at week four (n=1) and week eight (n=2). All three animals were found at post-mortem examination to have a necrotic testis and associated abscess. It is likely that this was caused by accidental administration of BOP into a retracted abdominal testis during intraperitoneal injection. There was no injection morbidity in the SC group and the remaining hamsters in the IP group.

After five weeks of carcinogen injection, six animals in each group were sacrificed. Full post-mortem examination failed to reveal any gross lesions

Table 2.1: Mean body weights of two groups of hamsters injected with the carcinogen BOP either SC or IP, during a twenty week experiment.

Week	Intraperitoneal Group		Subcutaneous Group	
	n	mean body weight (SD)	n	mean body weight (SD)
0	30	89.3g (6.3g)	30	93.1g (8.1g)*
5	29	110.5g (9.6g)	30	115.3g (9.5g)
10	21	122.0g (12.8g)	24	124.8g (13.2g)
15	14	126.2g (19.3g)	18	125.9g (14.7g)
20	7	112.8g (11.3g)	11	119.7g (20.0g)

(n = number of animals, SD = standard deviation of the mean, *$p<0.05$)

in any organ. On histological examination the pancreata of both groups of animals exhibited evidence of patchy acinar cell death and prominence of small pancreatic ductules (Fig. 2.1). There was no discernable difference between the two groups with respect to the severity or extent of these changes. There was also some evidence of mild periductal inflammation in a minority of sections in both groups. One section in the IP group exhibited mild ductal hyperplasia and another mild goblet cell metaplasia in a major pancreatic duct. These changes were not seen in the SC group largely because few of the sections in this group contained large ducts for examination.

Histological changes seen in the hamster pancreata after 10 weeks of carcinogen treatment are shown in Table 2.2. There was no evidence of ductular or ductal CIS, microcarcinoma or invasive adenocarcinoma in any section in either group. Acinar cell death was a prominent feature in almost all sections examined in both groups. Acinar-ductular transformation was seen in almost all animals but was not widespread throughout each pancreas. Cystic complexes were small, comprising usually not more than three or four small cyst-like structures (Fig. 2.2). Periductal inflammation became very prominent at this stage, particularly affecting the larger ducts. A cellular inflammatory infiltrate was seen in many sections, particularly where acinar cell death and

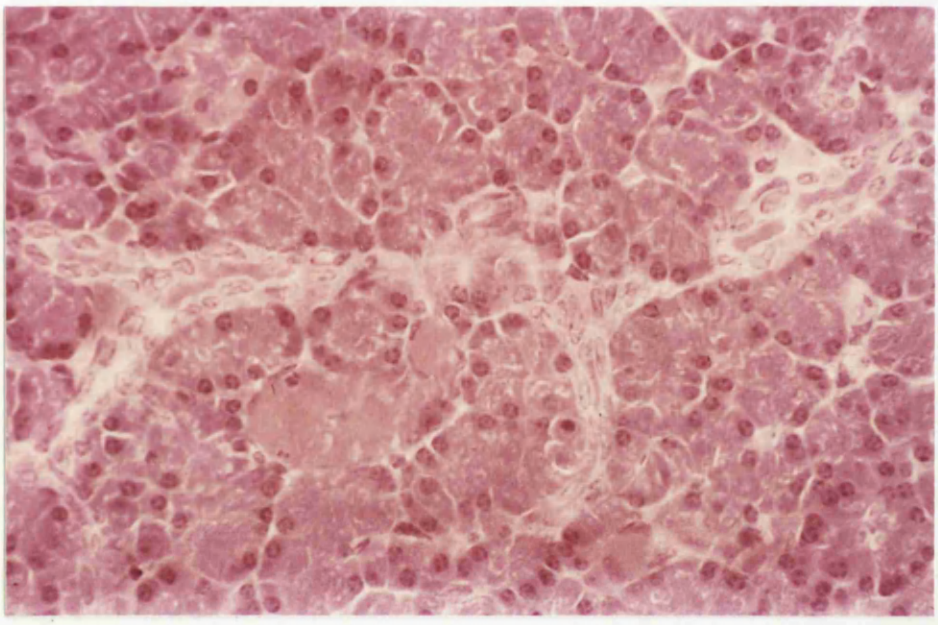

Figure 2.1: Hamster pancreas after 5 weeks IP injection of BOP showing evidence of patchy acinar cell death and prominence of interlobular ductal cells. (H&E; x280 magnification).

Table 2.2: Histological lesions present in hamsters after 10 weeks treatment with BOP.

	Animals		Histological Blocks	
	SC (n=6)	IP (n=6)	SC (n=36)	IP (n=42)
Ductal hyperplasia	1	2	1	3
Ductal dysplasia	0	1	0	1
A-D transformation	5	5	10	18
Cystic complexes	4	3	7	4

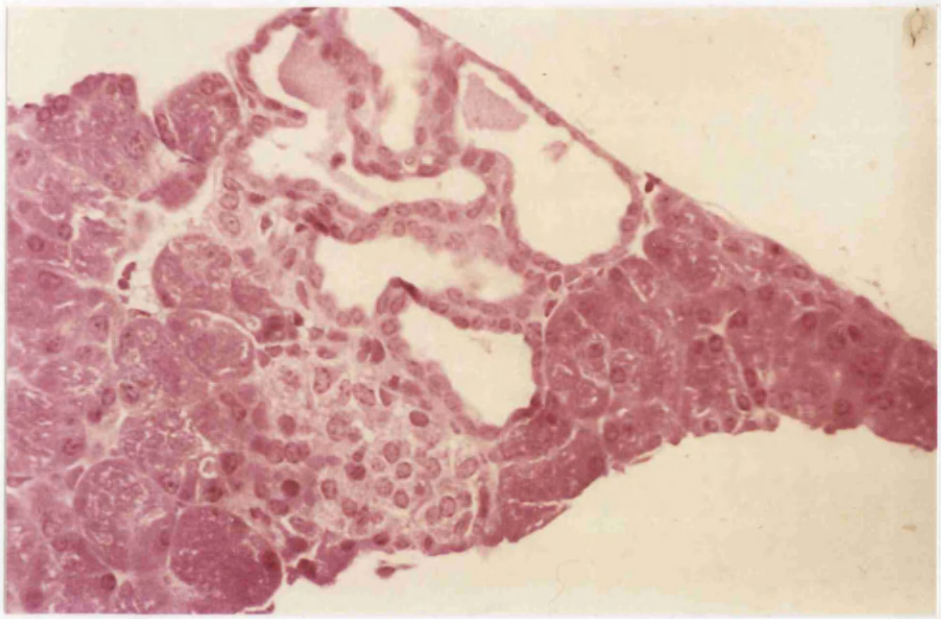

Figure 2.2: Hamster pancreas after 10 weeks SC injection of BOP showing a small cystic ductular complex. The solid appearance of adjacent acini is contrasted by the development of cystic structures lined by a single layer of flattened epithelium. (H&E; x280 magnification).

subsequent proliferation of cells of ductular and centroacinar origin was prominent.

The histological changes seen after 15 weeks of carcinogen treatment in the two groups of animals are shown in Table 2.3. Ductal hyperplasia was present in some sections in both groups, as after ten weeks of carcinogen treatment. Ductal dysplasia (Figs. 2.3 & 2.4) and ductal CIS became a new feature at this stage. Cystic complexes affected all pancreata examined and were present in over half of the blocks examined in both groups (Fig. 2.5). Ductular CIS (Fig. 2.6) was significantly more prominent in the SC group both with regard to affected animals and affected sections. Microcarcinoma (Fig. 2.7) was present in one pancreas in each group but adenocarcinoma was present only in the IP group.

Histological changes seen after 20 weeks of carcinogen treatment in the two groups of animals are shown in Table 2.4. Ductal hyperplasia affected significantly more animals and blocks in the SC group, although this is unlikely to be important given the extent and severity of more advanced pre-malignant changes at this stage. Ductal dysplasia was more widespread in both groups but the amount of ductal CIS did not seem to increase. Cystic complexes, which tended to be even bigger than those seen at 15 weeks, affected nearly all pancreata examined. Ductular CIS affected similar numbers of hamsters in each group but

Table 2.3: Histological lesions present in hamsters after 15 weeks treatment with BOP

	Animals		Histological Blocks	
	SC (n=6)	IP (n=7)	SC (n=34)	IP (n=48)
Ductal hyperplasia	2	3	2	3
Ductal dysplasia	3	2	4	4
Ductal CIS	3	2	5	2
Cystic complexes	6	7	20	25
Ductular CIS	6	3*	13	5+
Microcarcinoma	1	1	2	1
Adenocarcinoma	0	2	0	2

(* = $p<0.05$ versus SC group, Fisher's Exact Test)

(+ = $p<0.005$ versus SC group, Fisher's Exact Test)

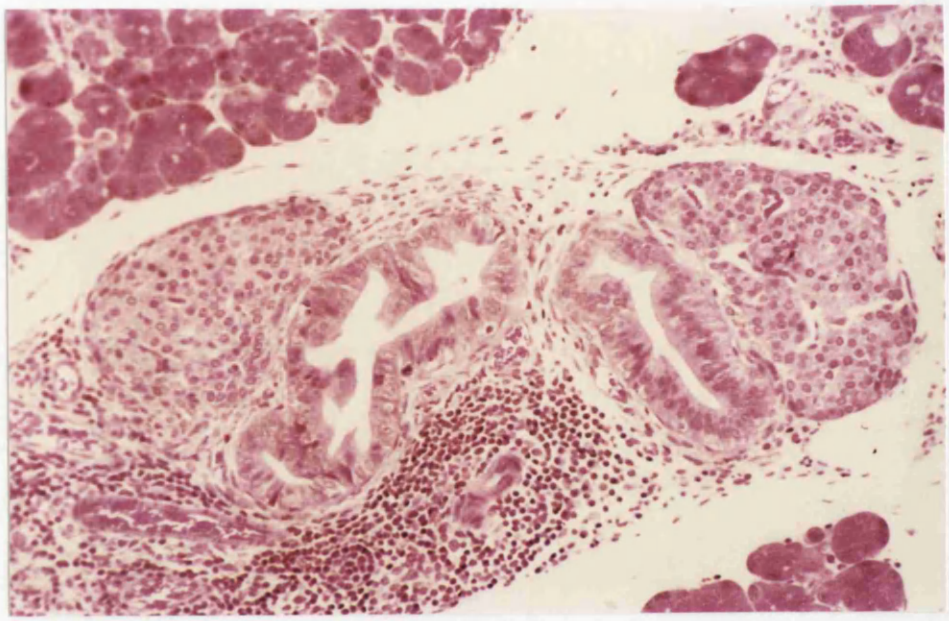

Figure 2.3: Hamster pancreas after 15 weeks SC injection of BOP showing ductal dysplasia in two peri-insular ducts. The cells lining the ducts have lost their order and polarity and there are a number of mitotic figures in evidence. (H&E; x140 magnification).

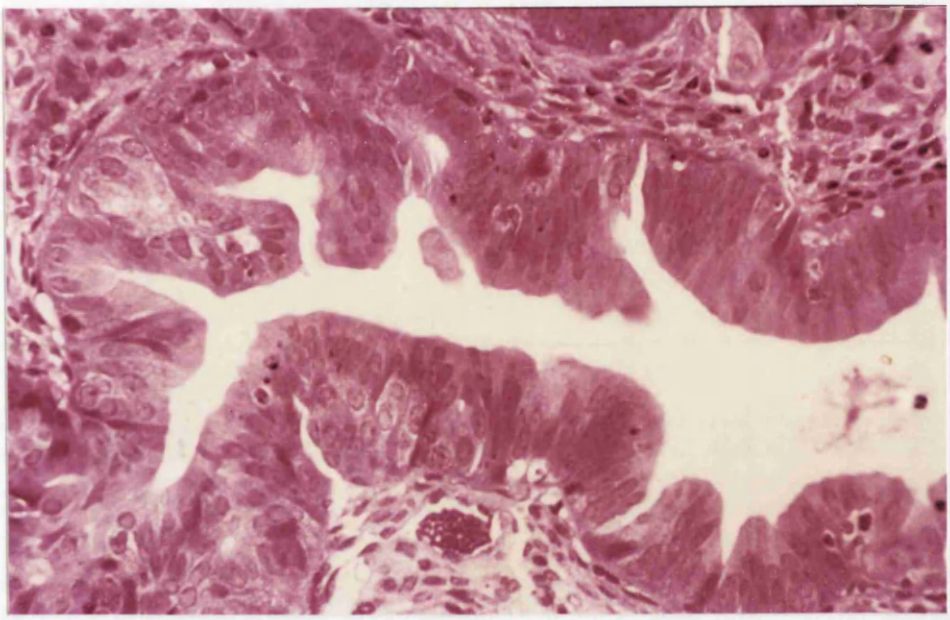

Figure 2.4: Hamster pancreas after 15 weeks SC injection of BOP showing ductal dysplasia in a major pancreatic duct. The cells lining this duct have become disordered with a loss, in some areas, of cellular polarity. Nuclear pleomorphism is evident and there are a number of mitotic figures. (H&E; x280 magnification).

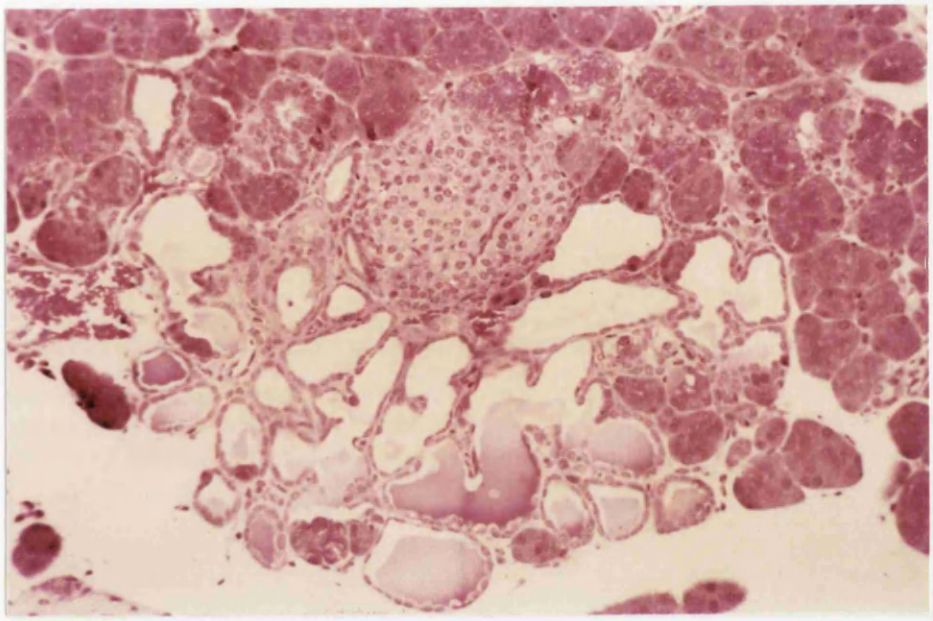

Figure 2.5: Hamster pancreas after 15 weeks SC injection of BOP showing a cystic ductular complex adjacent to an islet of Langerhans (top centre of section). (H&E; x140 magnification).

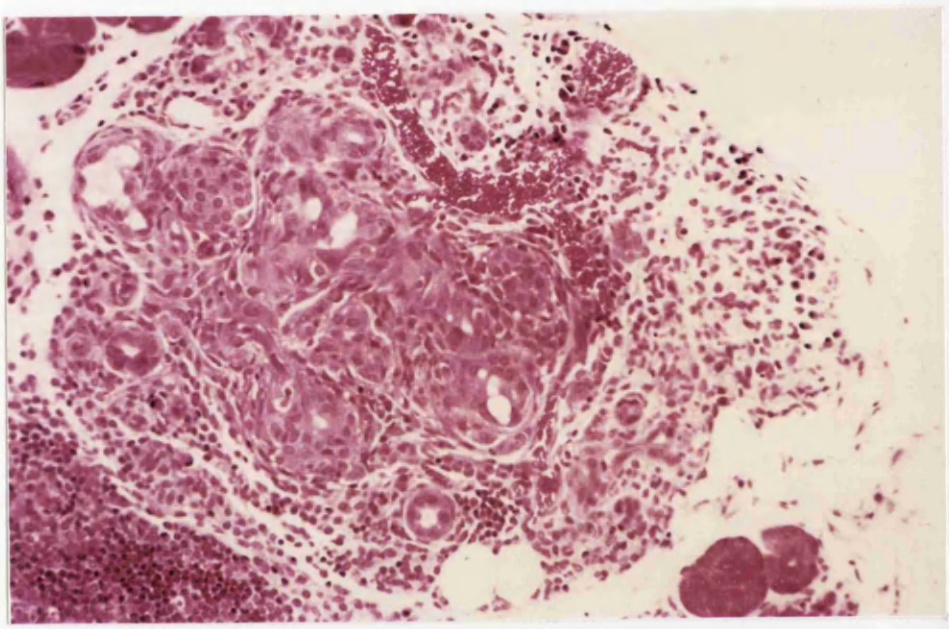

Figure 2.6: Hamster pancreas after 15 weeks SC injection of BOP showing ductular carcinoma-in-situ. This lesion is composed of atypical dysplastic ductules and is surrounded by a halo of inflammatory cells. (H&E; x140 magnification).

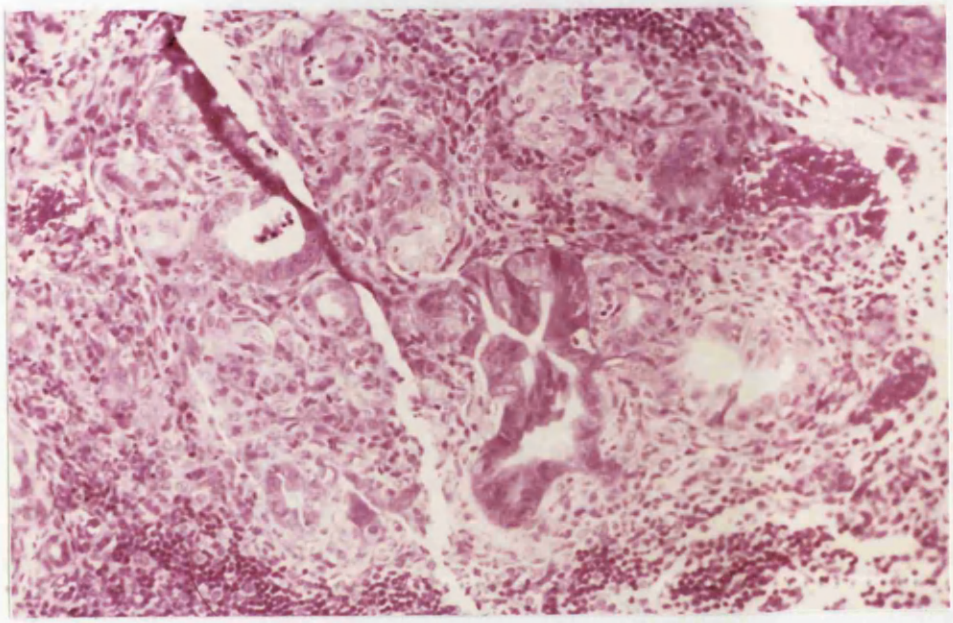

Figure 2.7: Hamster pancreas after 15 weeks SC injection of BOP showing ductular microcarcinoma. The lesion involves adjacent lobules of the pancreas and is surrounded by a halo of inflammatory cells. There is a small dysplastic duct in the centre of the section. (H&E; x140 magnification).

Table 2.4: Histological lesions present in hamsters after 20 weeks treatment with BOP

	Animals		Histological Blocks	
	SC (n=8)	IP (n=7)	SC (n=55)	IP (n=46)
Ductal hyperplasia	5	0#	5	0*
Ductal dysplasia	5	4	5	4
Ductal CIS	3	3	5	5
Cystic complexes	8	6	25	24
Ductular CIS	7	5	19	8*
Microcarcinoma	3	2	4	2
Adenocarcinoma	5	2	15	5*

(# = $p<0.02$ versus SC group, Fisher's Exact Test)

(* = $p<0.05$ versus SC group, Fisher's Exact Test)

was significantly more widespread in the SC group. Microcarcinomata were present in similar numbers in both groups. Invasive adenocarcinoma (Figs. 2.8 & 2.9) affected more hamsters in the SC group than in the IP group.

Although no formal estimation of islet size and number was made at any time point in this experiment, it was not apparent that any obvious changes had occurred in response to BOP administration.

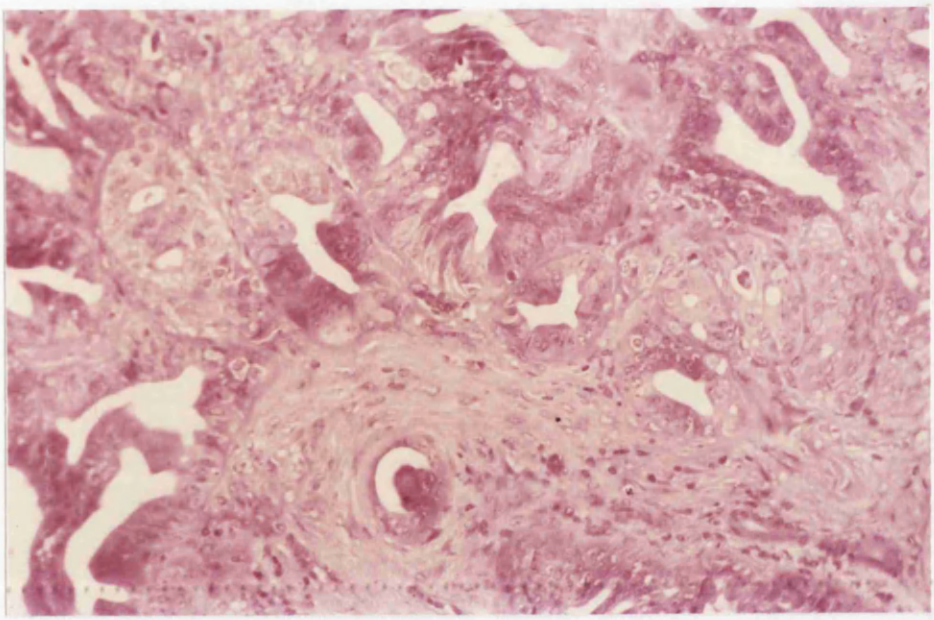

Figure 2.8: Hamster pancreas after 20 weeks SC injection of BOP showing invasive adenocarcinoma I. The tumour in this section exhibits both ductal and ductular morphology. (H&E; x140 magnification).

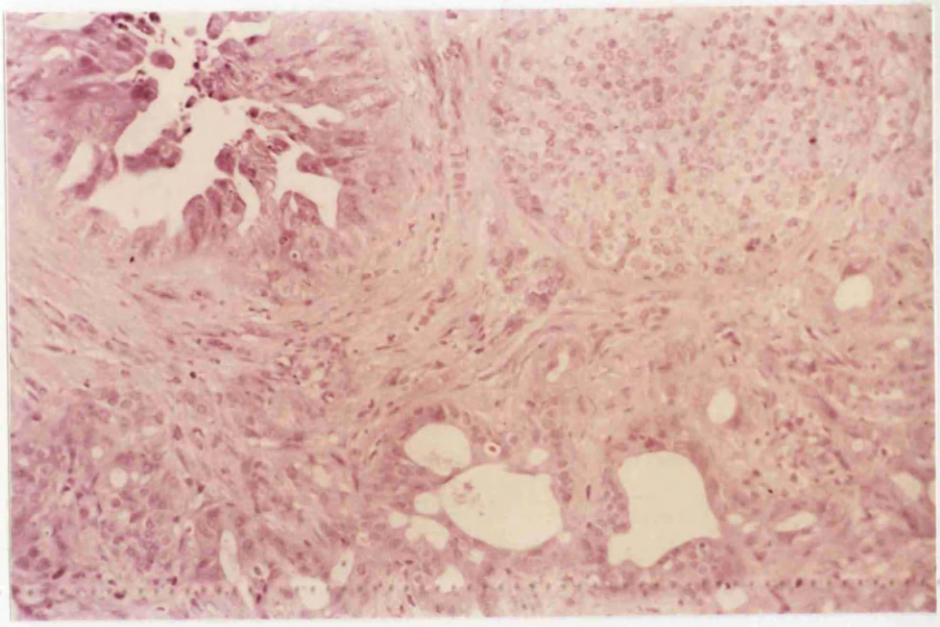

Figure 2.9: Hamster pancreas after 20 weeks SC injection of BOP showing invasive adenocarcinoma II. The tumour in this section exhibits predominantly ductular morphology. The large duct on this histological section (top left) exhibits severe ductal dysplastic change. There is an islet of Langerhans in the top right corner of the section (H&E; x140 magnification).

2.4 Discussion

In this experimental study, the histological changes seen in both groups of animals after exposure to BOP corresponded very closely to those reported in the many early papers by Pour and his colleagues.

The first changes seen were acinar cell death and prominence of small pancreatic ductules after five weeks of treatment. Focal hypertrophy and hyperplasia of large ductal epithelium, reported as occurring within two weeks of carcinogen exposure (BOP 10mg/kgBW) by Pour (1977d) was seen in only one animal in the IP group in this experiment.

The predominant histological abnormalities seen after 10 weeks of carcinogen administration were acinar-ductal transformation and the development of small cystic ductular complexes. The nature of these particular lesions remains the subject of much debate. Ductal and ductular obstruction was initially thought to result in the development of these cystic structures. However, in a study using India ink injection of the main pancreatic ducts, Pour's group demonstrated that ink was able to get through the main ducts and fill the cystic complexes (Takahashi et al, 1977b). Whether the injection pressure used overcame natural obstructing forces is unclear, given that no data relating to injection pressure were presented.

In a more recent electron-microscopic and immunohistochemical study, Pour (1988) suggested that

the earliest changes in the pancreas seen following a single injection of BOP (20mg/kgBW) were hyperplasia and hypertrophy of centroacinar cells. These abnormal centroacinar cells developed long cytoplasmic processes which seemed to separate acinar cells resulting in their degeneration and death. Certainly, centroacinar cell proliferation was only seen in areas where acinar cells had undergone degeneration. After a period, the abnormal centroacinar cells formed into pseudoductular complexes which were lined by a single layer of elongated cells - the cystic complexes reported by many authors and seen in this thesis. The presence of increasing numbers of inflammatory cells during the carcinogenic process could be explained by a need to remove cell debris caused by death of increasing numbers of acinar cells. At no stage did acinar cells seem to transform into ductular cells. Interestingly, some of the cells in these pseudoductular structures became cytologically abnormal with pleomorphic nuclei and plump microvilli, consistent with malignant progression.

After 15 weeks of carcinogen exposure the range of histological abnormalities seen in both groups of animals in the current study increased to include lesions which are generally recognised to be truly pre-malignant or malignant, namely ductular CIS, microcarcinoma and frankly invasive adenocarcinoma. Ductal dysplasia and ductal CIS which both seem to

have the potential to progress to invasive adenocarcinoma became obvious at this stage. These changes agree with previously published work with the BOP model.

Despite the similarities in the nature of histological changes seen in the animals injected SC and those injected IP, the extent and severity of these changes appeared to be worse in the SC group, particularly after 20 weeks of treatment and particularly with respect to the development of ductular CIS, microcarcinoma and invasive adenocarcinoma. Ductal histological changes were similar in both groups.

A possible reason for the differences between the two groups of animals with respect to the extent of the histological lesions developed in response to BOP might lie in the way that the carcinogen is handled after IP or SC administration. The fact that lesions seem to be more frequent in the SC group would suggest that direct contact of the carcinogen with the pancreas (as might occur in the IP group) in an unmetabolised form does not play in important role in the development of the various histological changes reported. To date, there has been no published work comparing the relative effects of IP and SC BOP in the Syrian hamster. From work reported in Chapter 1 of this thesis, it has been generally accepted that BOP acts, regardless of the route of administration, after

absorption into the bloodstream and oxidative metabolism into active compounds, of which HPOP seems to be the most likely candidate.

In a recent study, Mangino and colleagues (1990) attempted to look at the carcinogen metabolising capacity of isolated hamster pancreatic acinar and ductal cells and hepatocytes. They demonstrated that hamster hepatocyte preparations metabolized BOP to HPOP and BHP with a much higher capacity than either hamster pancreatic acinar or ductal cells. In conditions most favourable to BOP metabolism, the ratio of activation products formed by hepatocytes was 14.5 times that formed by acinar cells. Hepatocytes were also shown to activate BOP 106 times more rapidly than ductal cells, as determined from the yield of activation products, or 156 times more rapidly if the ratio for covalently bound metabolites was used for comparison. Pancreatic acinar cells had a higher capacity for BOP activation than ductal cells.

These results led the authors to conclude that alkylation of BOP in other organs, most probably the liver, occurs before any effect on the pancreas was seen. It is unclear whether BOP appearing at the liver via the portal circulation (as is likely in IP injected animals) is handled in a different way to BOP appearing at the liver via the systemic circulation.

In some of Pour's earlier work, great emphasis was placed on the importance of increased numbers of

pancreatic islets and of the importance of peri-insular ductules in the carcinogenic process. In the current study, changes in the number and size of islets were not apparent. Involvement of peri-insular ductules was difficult to determine at the level of magnification used, although some of the larger ductal lesions seen histologically occurred close to or within islets.

Pour and Bell (1989) recently reported on a study of endocrine cell proliferation in hamster pancreas after 6 weeks of BOP treatment. Immunohistochemical staining of pancreatic tissue demonstrated an increase in the number of insulin, glucagon and somatostatin secreting cells after weeks 12 and 33. Almost all of these extra endocrine cells were found outside islets, predominantly in hyperplastic ductal or ductular epithelium. At 12 weeks the number of insulin- and glucagon-secreting cells far outnumbered the somatostatin-secreting cells. At 33 weeks the number of somatostatin-secreting cells increased dramatically. The role of these extra endocrine cells in the carcinogenic process still require explanation and further work on the effect of BOP on the endocrine elements in the pancreas might shed light on the confusing relationship between pancreatic cancer and diabetes.

CHAPTER 3

Part 1

The effect of step-wise increasing doses of cholecystokinin octapeptide (CCK-8), secretin and a somatostatin analogue (SMS 201-995) on pancreatic juice output and composition in the Syrian golden hamster.

Part 2

(a) The effect of simultaneous infusions of cholecystokinin octapeptide (CCK-8) and SMS 201-995 on pancreatic juice output

(b) The effect of simultaneous infusions of secretin and SMS 201-995 on pancreatic juice output

3.1 Introduction

It is well recognised that cholecystokinin and secretin stimulate pancreatic exocrine secretion in both man and animals, under physiological and pharmacological conditions. From our knowledge of the actions of somatostatin, it might be expected that SMS 201-995 would inhibit such exocrine secretion.

In order to determine that cholecystokinin octapeptide, secretin and SMS 201-995 were active in the Syrian golden hamster and to determine the effect of various doses of these peptides on pancreatic exocrine secretion, a series of dose response curves were generated for each peptide based on the effects of intravenous infusions on pancreatic exocrine secretion in the Syrian golden hamster.

Some gastrointestinal hormones can act together to affect gastrointestinal function. Having identified doses of CCK-8, secretin and SMS 201-995 which stimulate or suppress pancreatic secretion when infused intravenously into anaesthetised hamsters, infusion studies of combinations of CCK-8 or secretin and SMS 201-995 were undertaken to determine what effects their interaction have on pancreatic secretion.

Part 1

3.2 Materials and Methods

Male WO(GD) Syrian golden hamsters (Wrights of Essex) 10 - 14 weeks of age were kept in standardised animal facility conditions with a twelve hour light-dark cycle and 21% humidity. Animals were caged in groups of four and fed standard small rodent diet and water ad libitum.

Hamsters were fasted singly in cages but with free access to water for 16 hours prior to the commencement of an infusion. Animals were weighed on the morning of the experiment.

Hamsters were anaesthetised intraperitoneally with pentobarbitone sodium BP (Sagatal, May and Baker Ltd., Dagenham: 60mg per ml) administered in a dose of 0.1ml per 100 grammes body weight. Anaesthesia was maintained throughout the duration of each infusion by further injections of pentobarbitone sodium.

A surgical tracheostomy was fashioned and an intravenous cannula inserted into the right internal jugular vein. Normal saline was infused at a rate of 0.375ml per hour using 5ml glass syringes on an infusion pump (B. Braun Medical Ltd.). A laparotomy was performed through a midline abdominal incision. The pylorus was identified and ligated to exclude gastric contents from the duodenum. The common duct was identified at the point of entry into the posterior aspect of the duodenum (Fig. 1.1). A small

incision was made in the common duct and a cannula (Portex Ltd. 2FG, outer diameter 0.63mm, inner diameter 0.5mm) introduced and passed up into the common bile duct. The cannula was then withdrawn slowly, such that the tip of the cannula came to rest at the lower end of the common duct. The cannula was then firmly ligated in place by passing a suture around the cannula at the lower end of the duct. The common bile duct was then ligated in continuity just below the entry point of the cystic duct, to exclude bile. A large trocar and cannula was then passed through the animal's flank and the trocar removed. The cannula collecting pure bile-free pancreatic juice was then fed through the larger cannula passing through the body wall and left to drain externally. The abdominal incision was closed with a continuous Vicryl suture (Ethicon (UK) Ltd, Edinburgh). Animal temperature was monitored with a rectal thermometer and maintained at 36^{o}C using an overhead heating lamp.

An equilibration period allowed bile-stained pancreatic juice (present in the distal common bile duct prior to ligation of same) to drain out through the pancreatic cannula. Pure pancreatic juice was then collected in pre-weighed tubes placed below the hamster. An initial basal sample was collected for one hour. Subsequent samples were collected over one hour (CCK-8, sulphated CCK fragment 26-33 amide, Sigma Chemical Company Ltd., Dorset, UK.) or 30 minutes

(Secretin, porcine secretin, approximately 98% pure, Sigma Chemical Company Ltd., Dorset, UK.; SMS 201-995, Sandoz Pharma Division, Basle, Switzerland). A 20 minute time period was allowed after the commencement of a new infusion concentration prior to collection of pancreatic juice. This allowed the new infusate to travel down the jugular vein cannula to the animal. Jugular vein cannulae were cut into lengths calculated to clear fluid infused at 0.375ml/hour in 20 minutes, prior to insertion.

Infusates were made up into various concentrations with distilled water, snap frozen in liquid nitrogen and stored at -268^{o}C prior to use. Final dilutions were made on the day of the experiment into a volume of normal saline twice the infusate to be given over the time period and based on the body weight of the animal on that day. All substances were infused at a rate of 0.375ml/hour.

At the end of each time period, the pre-weighed tubes containing collected pancreatic juice were reweighed. The amount of juice produced was calculated by subtracting the weight of the tube prior to collection of pancreatic juice, from the weight of the tube and contents after the collection period. Amounts of pancreatic juice collected over time periods shorter than one hour were multiplied up to give values for hourly collection.

At the end of each experiment, a solution of light green dye was injected into the pancreatic cannula. The abdominal wound was re-opened and the pancreas examined to ensure complete staining of the gland.

Bicarbonate Estimation

The bicarbonate content of the pancreatic juice was estimated using the Natelson Microgasometer (Model 600; Scientific Industries International Inc. (UK) Ltd., Loughborough) (Figure 3.1). Mercury was advanced to the tip of the gasometer pipette. 0.01ml of pancreatic juice was aspirated into the gasometer and sealed in place with 0.02ml of mercury. Lactic acid (0.03ml) was then aspirated into the gasometer followed by 0.01ml of caprylic acid and 0.01ml of mercury. Water (0.1ml) was aspirated into the gasometer followed by mercury to the 0.12ml mark on the reaction chamber. The reaction chamber stopcock was closed and the gasometer piston turned until all the reagents have been drawn into the reaction chamber. The gasometer was agitated for one minute to allow the lactic acid to react with the sample and release CO_2. The gasometer piston was then wound back until the fluid meniscus returned to the 0.12ml mark and the initial pressure (P_1) was read off the manometer; the temperature was also recorded. The piston was then wound back until the mercury column

Figure 3.1: Natelson Microgasometer (Model 600; Scientific Industries International Inc. (UK) Ltd., Loughborough).

reached the top of the manometer. After opening the reaction chamber stopcock, 0.03ml of sodium hydroxide was then drawn into the gasometer pipette followed by mercury to the 0.12ml mark. The stopcock was closed and the piston wound back until all reagents reached the reaction chamber. The stopcock was then closed and the sample agitated again for one minute. Sodium hydroxide reacts with CO_2 thus extracting it from the sample. The piston was then wound back until the meniscus of the reagent mix reached the 0.12ml mark. The second pressure (P_2) was then read on the manometer and CO_2 content calculated by subtracting P_2 from P_1. An adjustment was then made for temperature calculated from standard charts.

Protein Assay

Pancreatic juice protein content was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., Watford, UK.). This assay is based on the colour change induced in a dye in response to various concentrations of protein. Absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 used in the assay shifts from 465nm to 595nm when binding to protein occurs. Spectrophotometric measurement of absorbance at 595nm wavelength allows estimation of protein content to be made.

Samples were diluted with water to required concentrations. Lyophilized bovine serum albumin

standards were diluted to 0.2 - 1.4 mg/ml. 0.1ml of standard and diluted sample solutions were placed in dry test tubes. 0.1ml of sample buffer was placed in a "blank" test tube. 5 ml of diluted dye reagent was added to the samples, the standards and the buffer blank. Test tubes were then agitated on a vortex mixer. After 10 minutes, absorbance at 595nm for the dye-sample complex is measured. Allowance is made for the absorbance of the buffer blanks and the true value obtained for absorbance for each sample and standard. A series of 10 normal calf serum samples were measured during each test run to act as quality control. From the bovine serum albumin standard samples, a standard curve was drawn for each assay run and the protein content of the pancreatic juice samples were read from the curve.

3.3 Results

a) CCK-8 Infusions

Pancreatic juice weight (mg/hour) and pancreatic juice output (mg/kgBW/hour) during the infusion of CCK-8 in six hamsters (mean body weight 116.8g, range 105.9 - 127.7g) are shown in Figures 3.2 and 3.3. The median basal pancreatic juice output over the first hour when saline was infused was 92.1mg/kgBW (Q1:Q3 = 75.5:96.2). This rose steadily to 345mg/kgBW/hour (Q1:Q3 = 321.2:368.6) during infusion of 0.4ug/kgBW/hour of CCK-8 and 460.7mg/kgBW/hour (Q1:Q3 = 316.9:574.8) during infusion of 0.8ug/kgBW/hour of CCK-8. Pancreatic juice output was significantly different from the basal (normal saline) values during infusion with 0.1, 0.2 and 0.4ug/kgBW/hour of CCK-8 (all $p<0.05$, Wilcoxon Rank Test).

Pancreatic juice bicarbonate concentration (mmol/l) is shown in Figure 3.4 and total pancreatic juice bicarbonate output (umol/kgBW/hour) in Figure 3.5. Basal estimations were not possible in any animal due to the small amount of pancreatic juice secreted during the first hour. Median bicarbonate concentration during infusion of 0.1ug/kgBW/hour of CCK-8 was 32.7mmol/l (Q1:Q3 = 30.6:41.2, n = 4). This rose to a maximum of 60.3mmol/l (Q1:Q3 = 52.9:72.0, n = 6) during infusion of 0.2ug/kgBW/hour CCK-8 falling thereafter. Median total bicarbonate output was 8.0mmol/kgBW/hour (Q1:Q3 = 7.5:13.1, n = 4) during

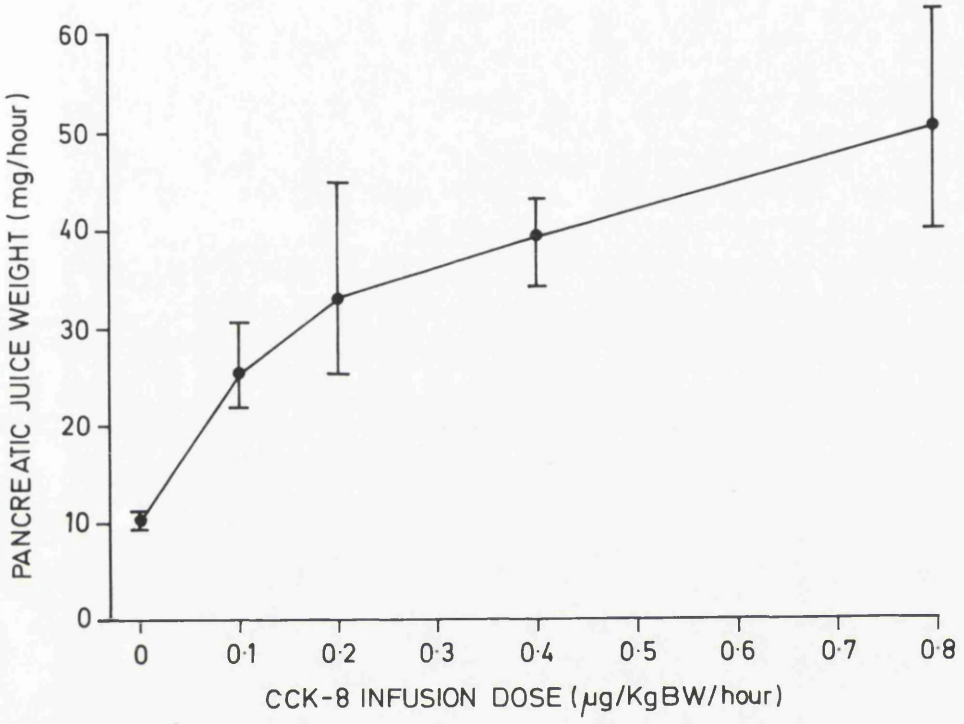

Figure 3.2: Pancreatic juice weight (mg/hour) in six hamsters during infusion of increasing doses of CCK-8 (values expressed as median and interquartile range).

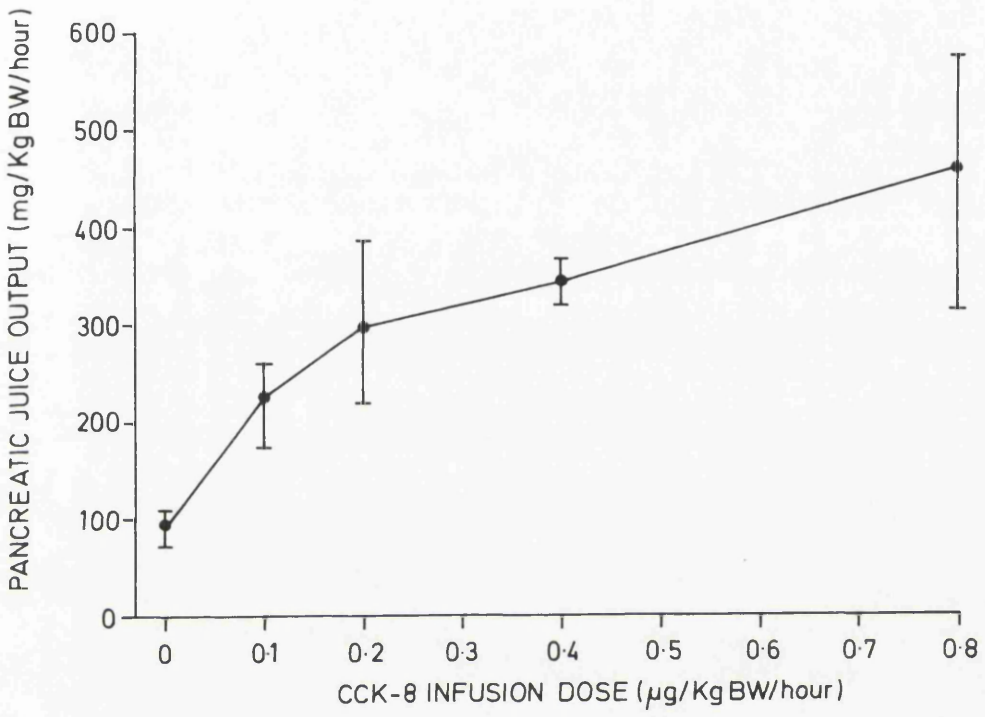

Figure 3.3: Pancreatic juice output (mg/kgBW/hour) in six hamsters during infusion of increasing doses of CCK-8 (values expressed as median and interquartile range).

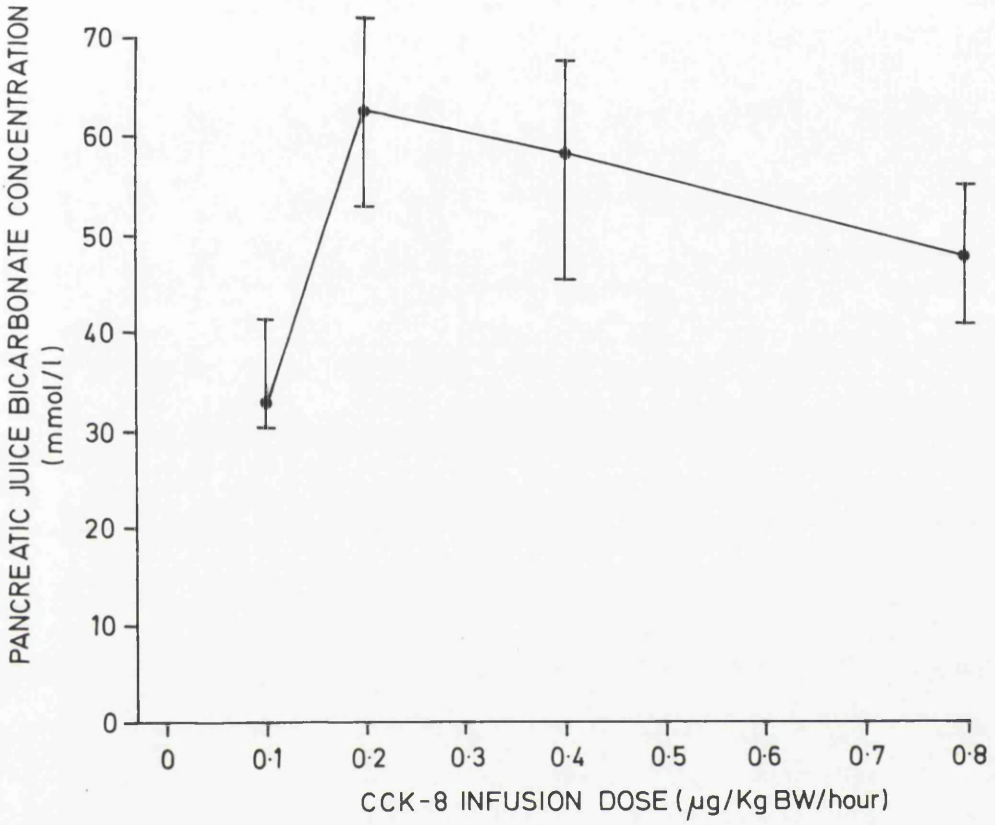

Figure 3.4: Pancreatic juice bicarbonate concentration (mmol/l) in six hamsters during infusion of increasing doses of CCK-8 (values expressed as median and interquartile range).

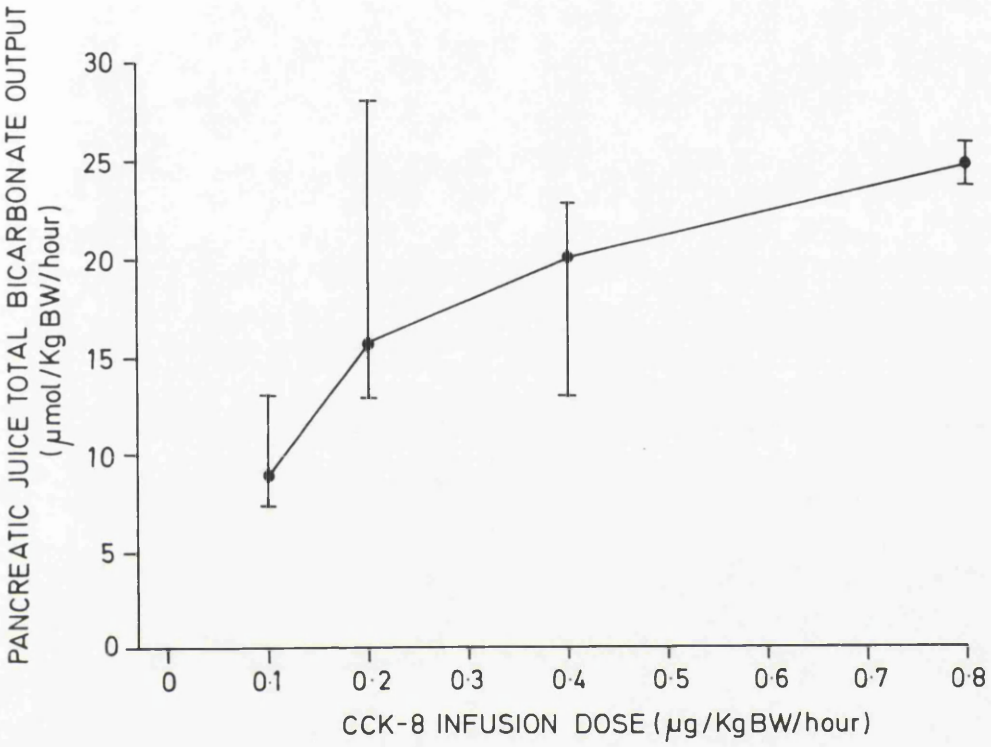

Figure 3.5: Pancreatic juice total bicarbonate output (umol/kgBW/hour) in six hamsters during infusion of increasing doses of CCK-8 (values expressed as median and interquartile range).

infusion of 0.1ug/kgBW/hour of CCK-8 and rose gradually to a maximum of 24.8mmol/kgBW/hour (Q1:Q3 = 23.7:25.9, n = 6) during infusion of 0.8ug/kgBW/hour of CCK-8.

Pancreatic juice protein concentration (mg/ml) is shown in Figure 3.6 and total pancreatic juice protein output (mg/kgBW/hour) in Figure 3.7. Basal estimations were only possible in three hamsters due to small sample sizes. Median basal pancreatic juice protein concentration during infusion of saline was 72.6mg/ml (Q1:Q3 = 15.4:74.4, n = 3). This rose to a maximal value of 90.1mg/ml (Q1:Q3 = 78.1:99.7, n = 6) during infusion of 0.1ug/kgBW/hour of CCK-8, falling thereafter. Median basal total pancreatic juice protein output during saline infusion was 6.9mg/kgBW/hour (Q1:Q3 = 1.8:7.2, n = 3) rising gradually to 29.0mg/kgBW.hour (Q1:Q3 = 22.4:35.2, n = 6) during infusion of 0.4 ug/kgBW/hour CCK-8.

The coefficient of variation for the control calf serum protein samples was 5.6%.

b) Secretin Infusions

Pancreatic juice weight (mg/hour) and pancreatic juice output (mg/kgBW/hour) during the infusion of secretin in six hamsters (mean body weight 103.9g, range 86.0 - 124.3g) are shown in Figures 3.8 and 3.9. The median basal pancreatic juice output over the first hour when saline was infused was 370.2mg/kgBW

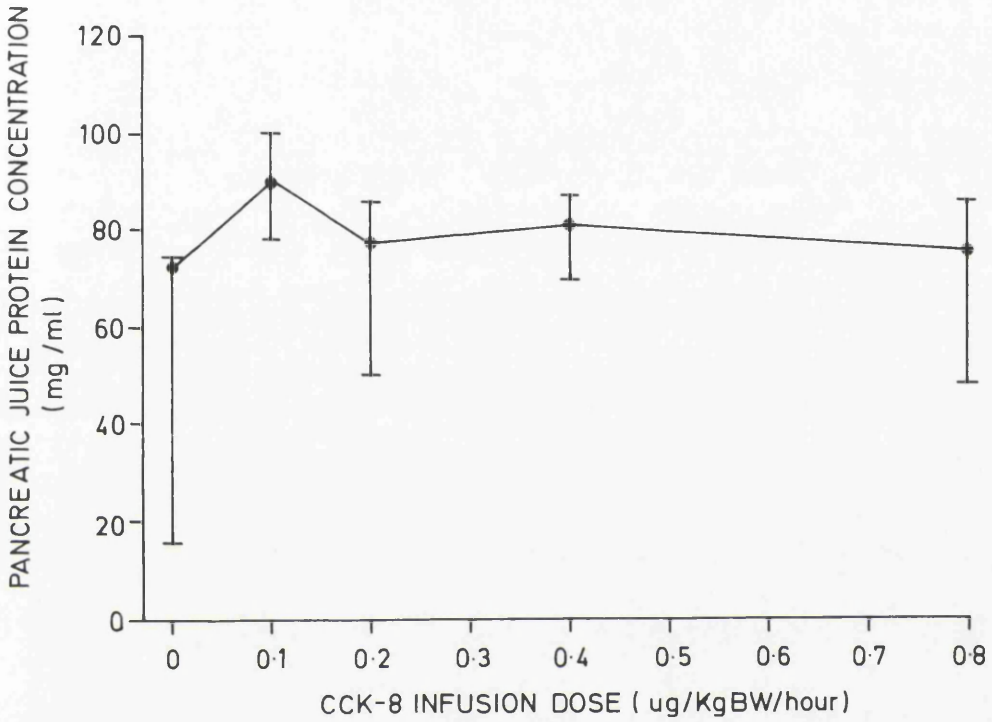

Figure 3.6: Pancreatic juice protein concentration (mg/ml) in six hamsters during infusion of increasing doses of CCK-8 (values expressed as median and interquartile range).

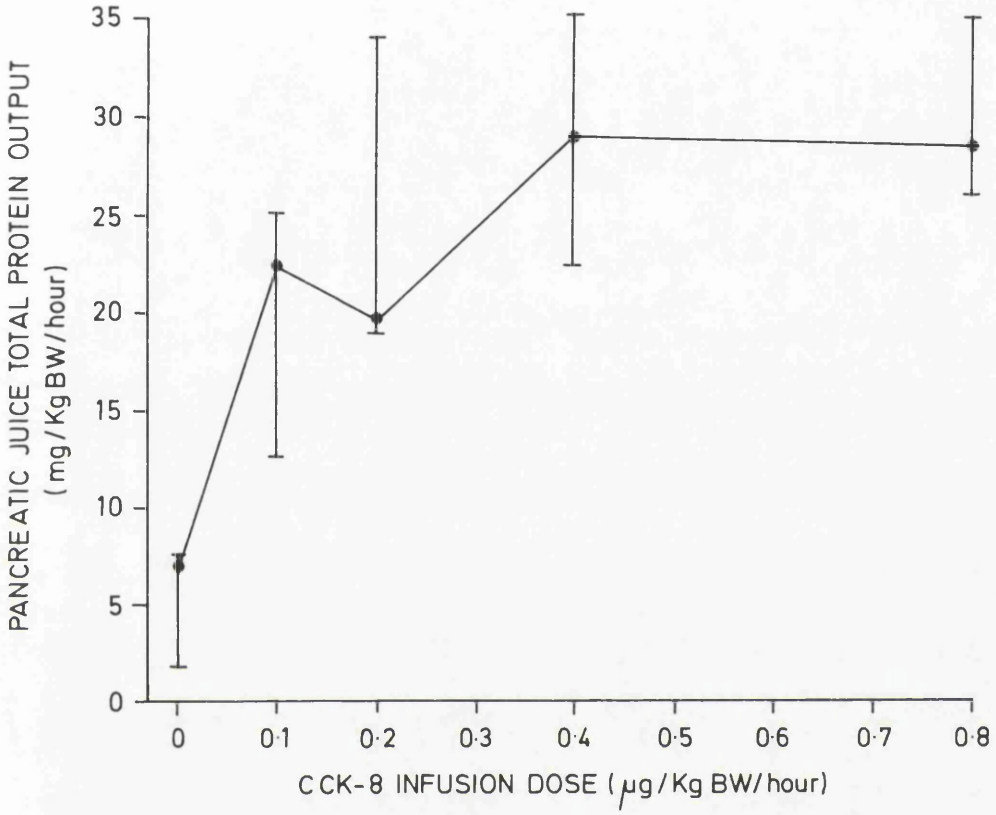

Figure 3.7: Pancreatic juice total protein output (mg/kgBW/hour) in six hamsters during infusion of increasing doses of CCK-8 (values expressed as median and interquartile range).

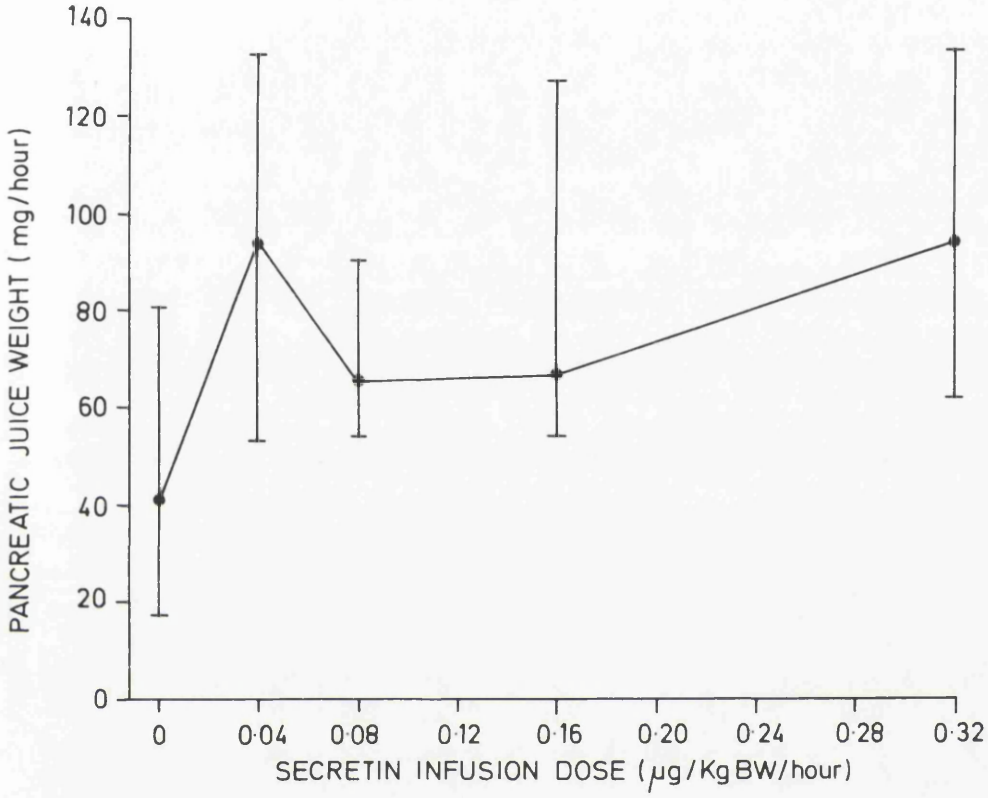

Figure 3.8: Pancreatic juice weight (mg/hour) in six hamsters during infusion of increasing doses of secretin (values expressed as median and interquartile range).

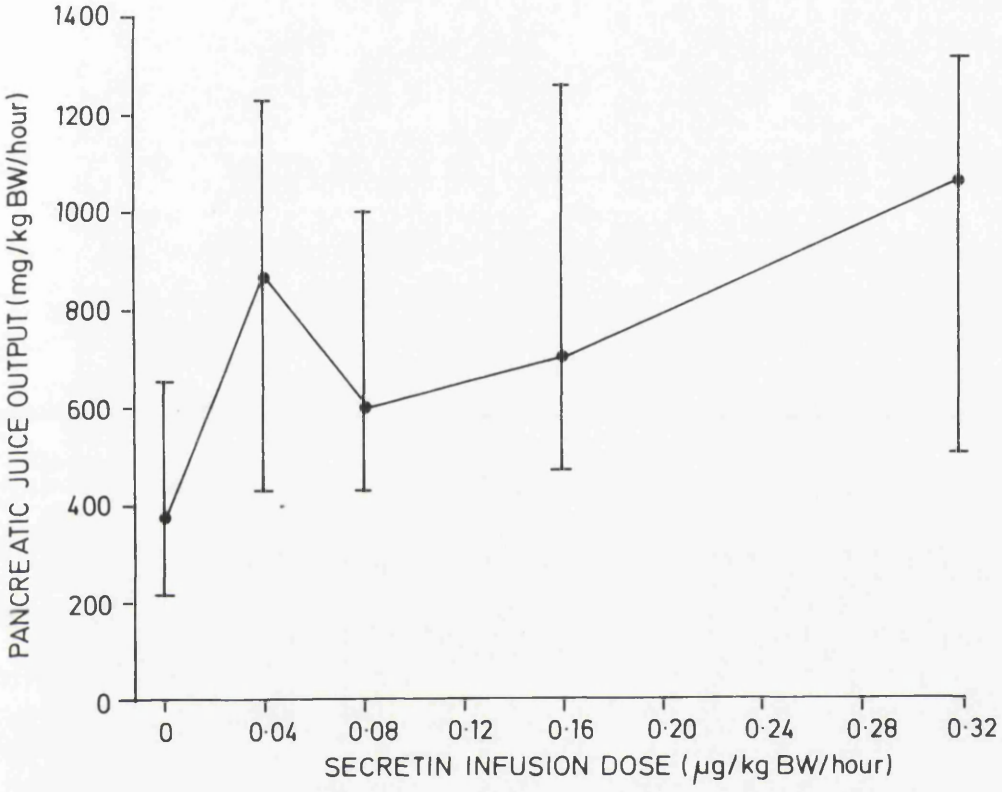

Figure 3.9: Pancreatic juice output (mg/kgBW/hour) in six hamsters during infusion of increasing doses of secretin (values expressed as median and interquartile range).

(Q1:Q3 = 216.0:654.9). This rose to 875.2mg/kgBW/hour (Q1:Q3 = 430.1:1227.8) during infusion of 0.04ug/kgBW/hour of secretin and 1064mg/kgBW/hour (Q1:Q3 = 502.2:1315.0) during infusion of 0.32ug/kgBW/hour of secretin. Pancreatic juice output was significantly different from the basal values during infusion with 0.04ug/kgBW/hour of secretin ($p<0.05$, Wilcoxon Rank Test).

Pancreatic juice bicarbonate concentration is shown in Figure 3.10 and total pancreatic juice bicarbonate output in Figure 3.11. Median basal bicarbonate concentration during infusion of saline was 39.7mmol/l (Q1:Q3 = 36.8:49.2). This rose to a maximum of 55.9mmol/l (Q1:Q3 = 53.7:64.7) during infusion of 0.04ug/kgBW/hour of secretin, falling slowly thereafter. Median basal total bicarbonate output was 19.4mmol/kgBW/hour (Q1:Q3 = 12.8:26.0). This rose to a maximal value of 70.4mmol/kgBW/hour (Q1:Q3 = 37.0:79.0) during infusion of 0.04ug/kgBW/hour of secretin and fell thereafter.

Pancreatic juice protein concentration is shown in Figure 3.12 and total pancreatic juice protein output in Figure 3.13. Median basal pancreatic juice protein concentration during infusion of saline was 98.9mg/ml (Q1:Q3 = 84.8:106.0, n = 4). This fell gradually thereafter during infusion of various doses of secretin. Median basal total pancreatic juice protein output during saline infusion was

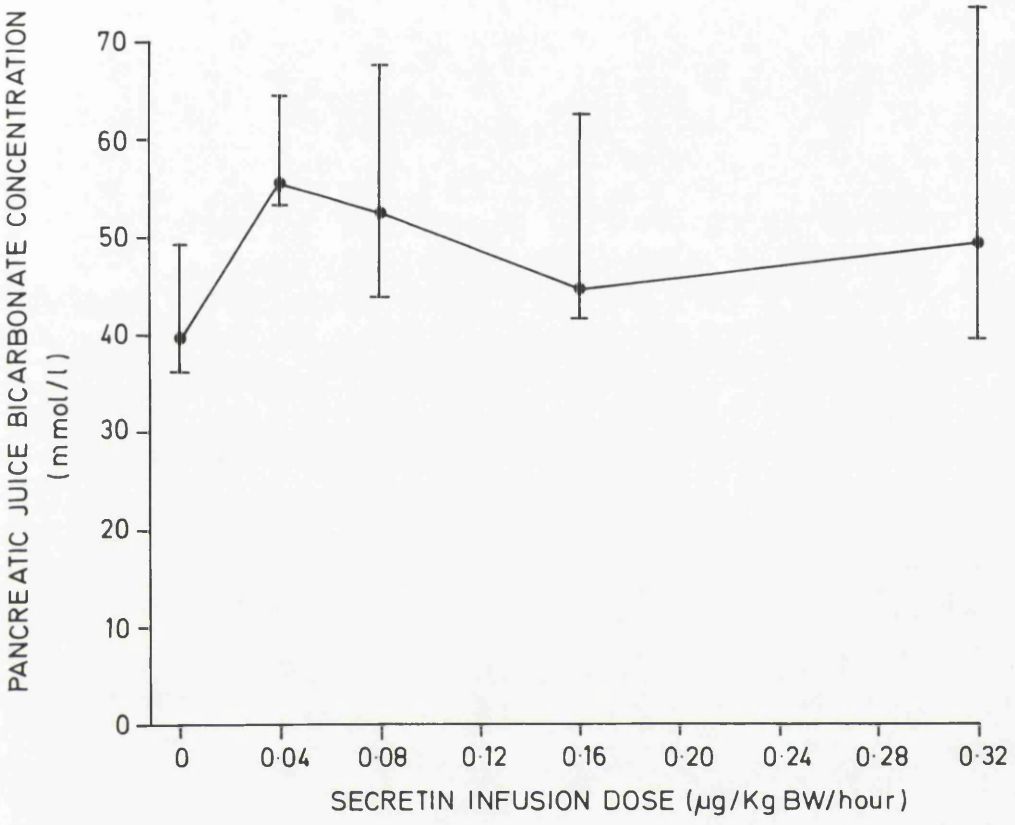

Figure 3.10: Pancreatic juice bicarbonate concentration (mmol/l) in six hamsters during infusion of increasing doses of secretin (values expressed as median and interquartile range).

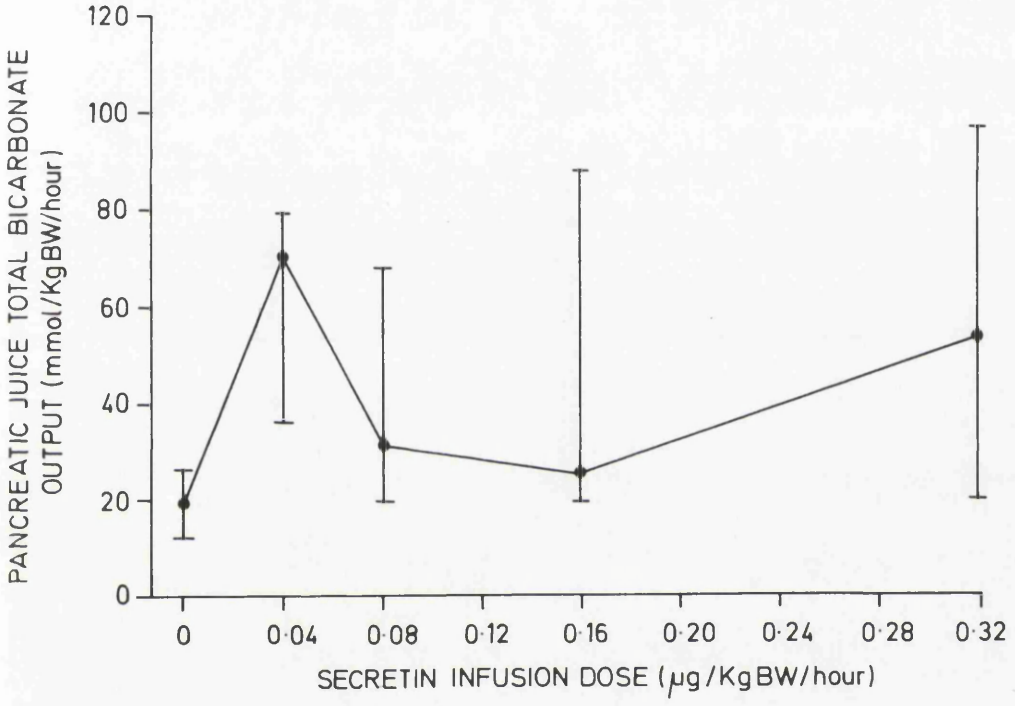

Figure 3.11: Pancreatic juice total bicarbonate output (umol/kgBW/hour) in six hamsters during infusion of increasing doses of secretin (values expressed as median and interquartile range).

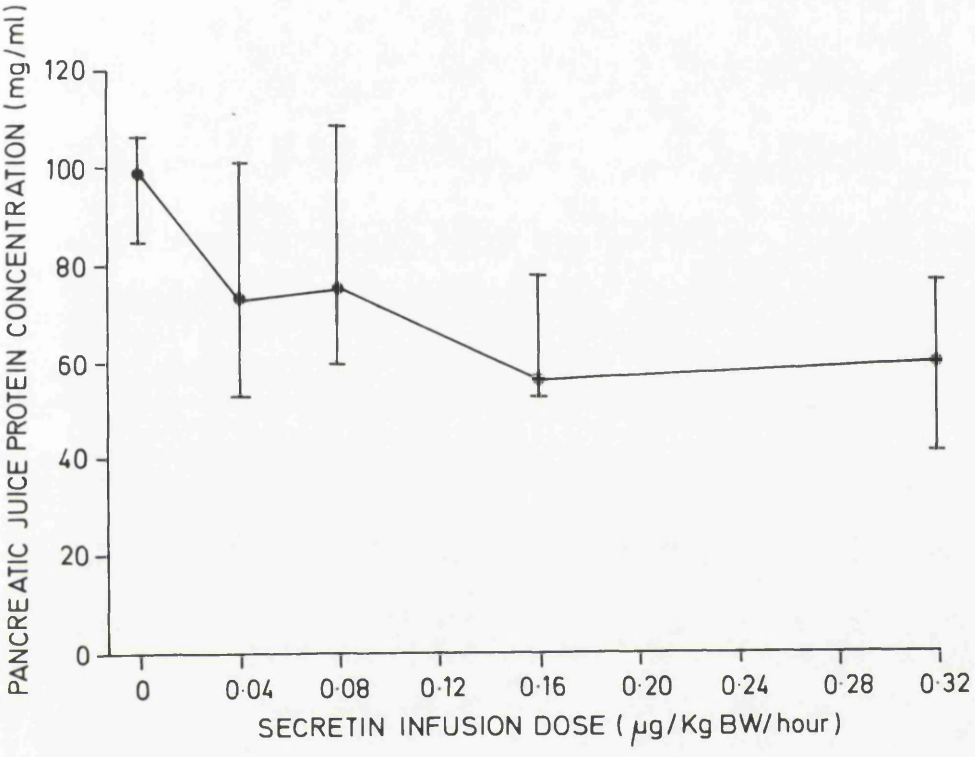

Figure 3.12: Pancreatic juice protein concentration (mg/ml) in six hamsters during infusion of increasing doses of secretin (values expressed as median and interquartile range).

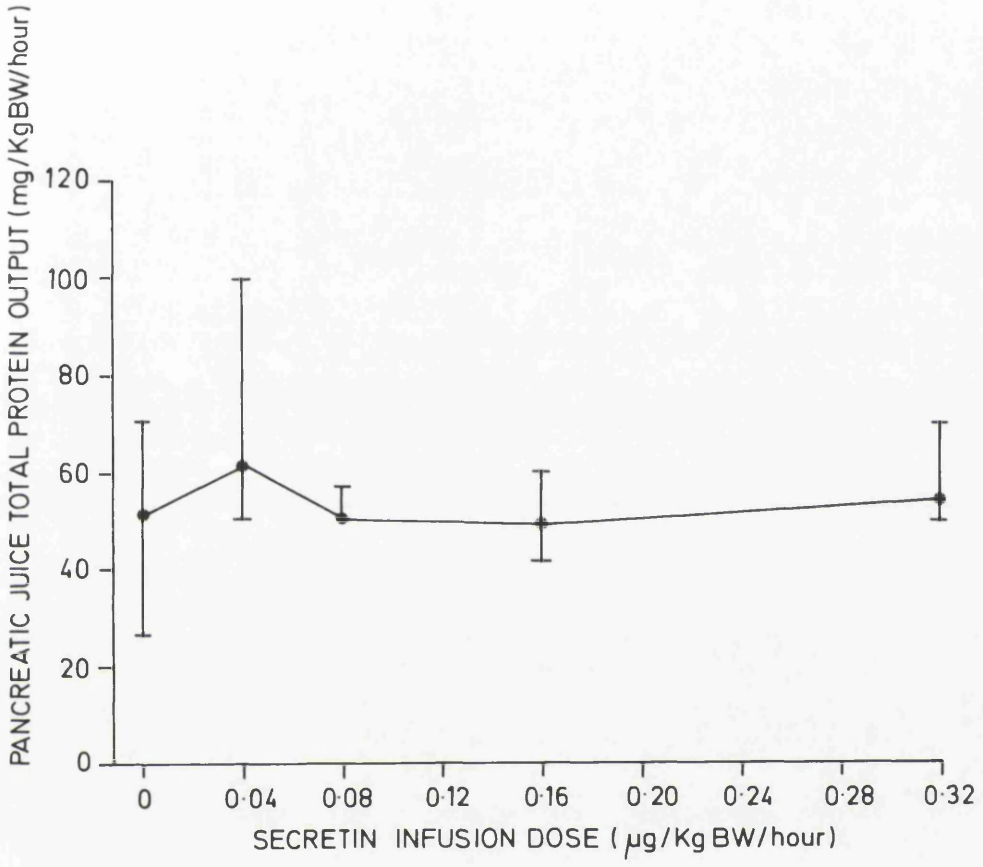

Figure 3.13: Pancreatic juice total protein output (mg/kgBW/hour) in six hamsters during infusion of increasing doses of secretin (values expressed as median and interquartile range).

51.3mg/kgBW/hour (Q1:Q3 = 26.5:70.5, n = 4) rising to a maximum value of 61.7mg/kgBW/hour (Q1:Q3 = 50.6:99.7, n = 4) during infusion of 0.04ug/kgBW/hour of secretin.

The coefficient of variation for the control calf serum protein samples was 8.0%.

c) SMS 201-995 Infusions

Bicarbonate and protein estimations were not possible due to the small volumes of pancreatic juice secreted in this group of six hamsters (mean body weight 110.4g, range 99.7 - 116.6).

Pancreatic juice weight (mg/hour) and pancreatic juice output (mg/kgBW/hour) are shown in Figures 3.14 and 3.15. Median basal pancreatic juice output during saline infusion was 212.0mg/kgBW/hour (Q1:Q3 = 121.1:334.7). This was suppressed during infusion of 5ug/kg/hour of SMS 201-995 to 70.2mg/kgBW/hour (Q1:Q3 = 63.8:102.8, $p<0.05$ Wilcoxon Rank Test) and remained at this level during infusion of higher doses.

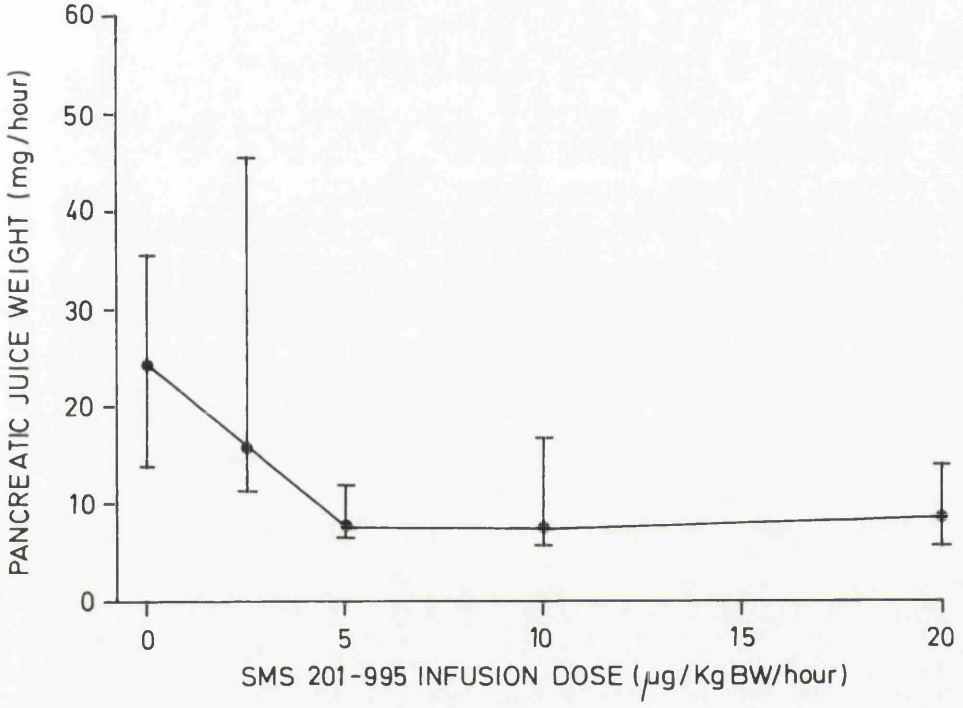

Figure 3.14: Pancreatic juice weight (mg/hour) in six hamsters during infusion of increasing doses of SMS 201-995 (values expressed as median and interquartile range).

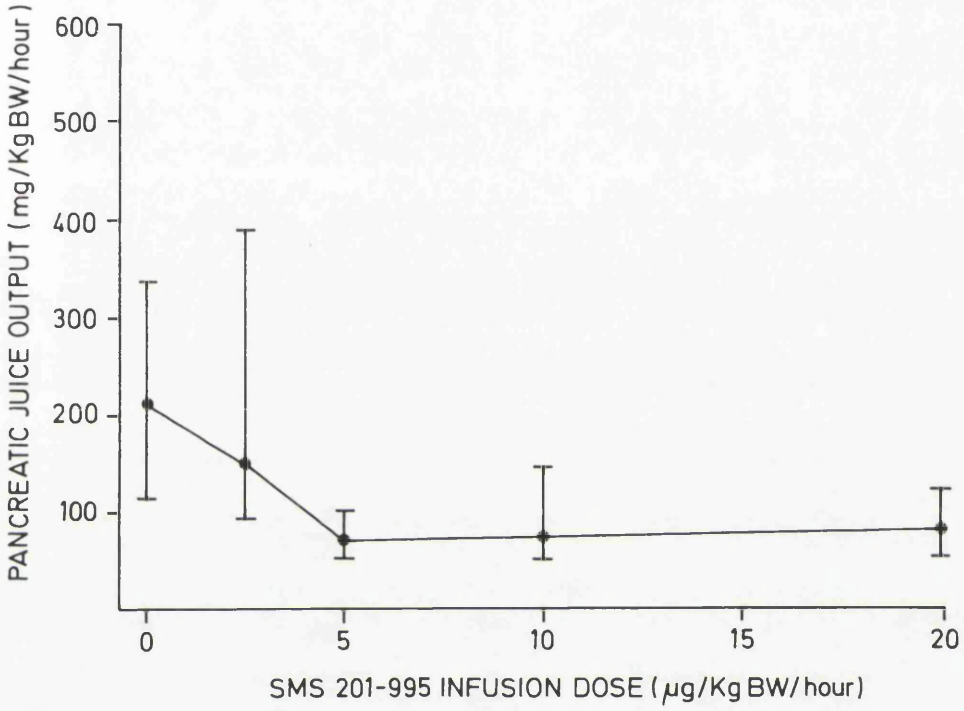

Figure 3.15: Pancreatic juice output (mg/kgBW/hour) in six hamsters during infusion of increasing doses of SMS 201-995 (values expressed as median and interquartile range).

Part 2

3.4 Materials and Methods

The experimental design used was essentially similar to that used in Part 1. In addition to the cannula inserted in the right internal jugular vein, a second cannula was inserted into the left femoral vein. Normal saline was infused through each cannula at a rate of 0.2ml per hour using 5ml glass syringes on an infusion pump. The surgical procedure described in detail in Part 1 was performed in all animals.

Pancreatic juice samples were collected as previously described after an equilibration period. The basal sample was collected over one hour; all subsequent samples were collected over 30 minutes. A 20 minute equilibration period was allowed between doses to allow the new infusate to travel down the intravenous cannula. Both cannulae were cut into lengths calculated to clear fluid infused at 0.2ml/hour in 20 minutes, prior to insertion into the animal.

Infusates were made up as previously described. All substances were infused at a rate of 0.2ml/hour.

A1) Increasing doses of CCK-8 with a background infusion of SMS 201-995

Normal saline was infused for the first hour through both cannulae (basal value), SMS 201-995 was then infused through one cannula in a dose of

5ug/kgBW/hour while normal saline was infused through the second cannula for the next 30 minutes (background value). Thereafter SMS 201-995 was infused through one cannula for the remainder of the experiment in a dose of 5ug/kgBW/hour while step-wise increasing doses of CCK-8 were infused through the second cannula (0.1 - 0.8ug/kgBW/hour).

A2) Increasing doses of SMS 201-995 with a background infusion of CCK-8

Normal saline was infused for the first hour through both cannulae (basal value). CCK-8 was then infused through one cannula in a dose of 0.8ug/kgBW/hour while normal saline was infused through the second cannula for the next 30 minutes (background value). Thereafter CCK-8 was infused through one cannula for the remainder of the experiment in a dose of 0.8ug/kgBW/hour while step-wise increasing doses of SMS 201-995 were infused through the second cannula (2.5 - 20ug/kgBW/hour).

B1) Increasing doses of secretin with a background infusion of SMS 201-995

Normal saline was infused for the first hour through both cannulae (basal value). SMS 201-995 was then infused through one cannula in a dose of 5ug/kgBW/hour while normal saline was infused through the second cannula for the next 30 minutes (background

value). Thereafter SMS 201-995 was infused through one cannula for the remainder of the experiment in a dose of 5ug/kgBW/hour while step-wise increasing doses of secretin were infused through the second cannula (0.04 - 0.32ug/kgBW/hour).

B2) Increasing doses of SMS 201-995 with a background infusion of secretin

Normal saline was infused for the first hour through both cannulae (basal value). Secretin was then infused through one cannula in a dose of 0.3ug/kgBW/hour while normal saline was infused through the second cannula for the next 30 minutes (background value). Thereafter secretin was infused through one cannula for the remainder of the experiment in a dose of 0.3ug/kgBW/hour while step-wise increasing doses of SMS 201-995 were infused through the second cannula (2.5 - 20ug/kgBW/hour).

3.5 Results

A1) Increasing doses of CCK-8 with a background infusion of SMS 201-995

Pancreatic juice output during this infusion experiment in six hamsters (mean body weight 108.9g, range 99.7 - 121.0g) is shown in Figure 3.16. Median basal output during saline infusion only was 374.9mg/kgBW/hour (Q1:Q3 = 280.6:524.5). Median background output during infusion of saline through cannula 1 and SMS 201-995 through cannula 2 was 482.0mg/kgBW/hour (Q1:Q3 = 429.9:527.0). Pancreatic juice output decreased during CCK-8 infusion to a minimum median value of 159.0mg/kgBW/hour (Q1:Q3 = 132.2:290.1) during infusion of CCK-8 0.2ug/kgBW/hour before slowly increasing as the dose of CCK-8 increased.

A2) Increasing doses of SMS 201-995 with a background infusion of CCK-8

Pancreatic juice output during this infusion experiment in six hamsters (mean body weight 114.0g, range 102.8 = 121.8g) is shown in Figure 3.17. Median basal output during saline infusion only was 116.6mg/kgBW/hour (Q1:Q3 = 94.7:142.1). Median background output during infusion of saline through cannula 1 and CCK-8 through cannula 2 was 272.6mg/kgBW/hour (Q1:Q3 = 239.5:431.3). Pancreatic juice output then rose to a maximum of

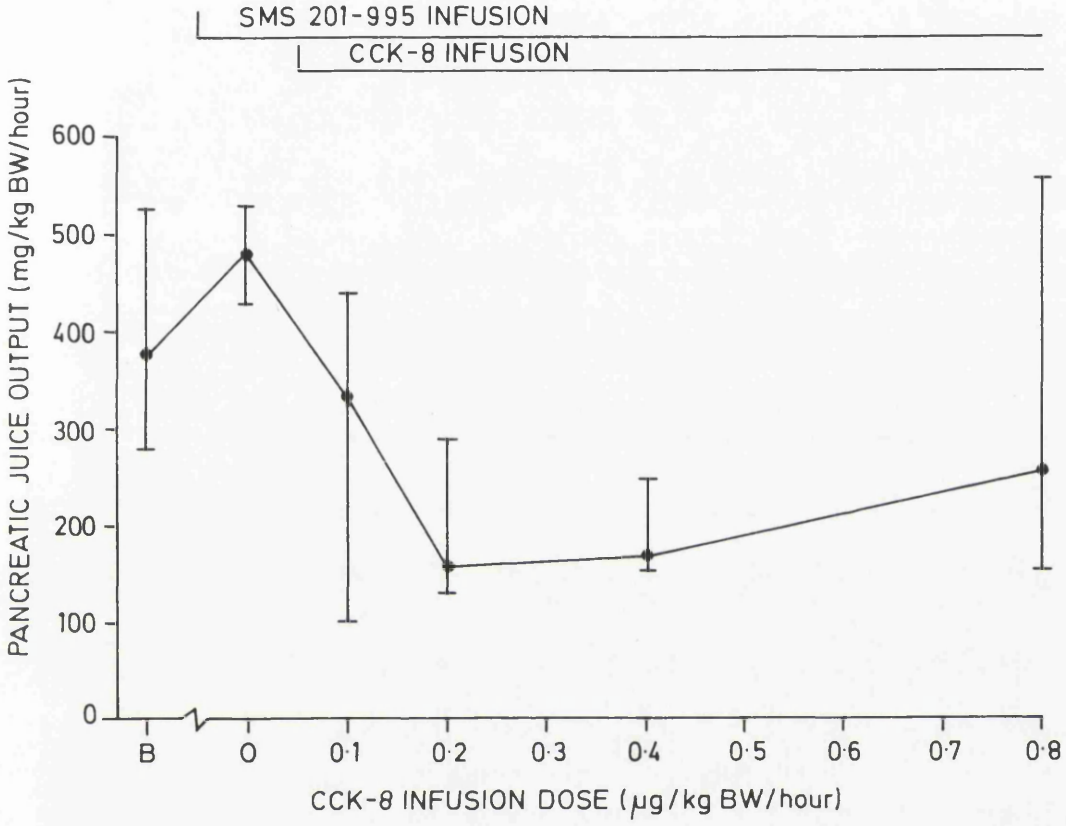

Figure 3.16: Pancreatic juice output (mg/kgBW/hour) in six hamsters during infusion of increasing doses of CCK-8 with a background infusion of SMS 201-995.

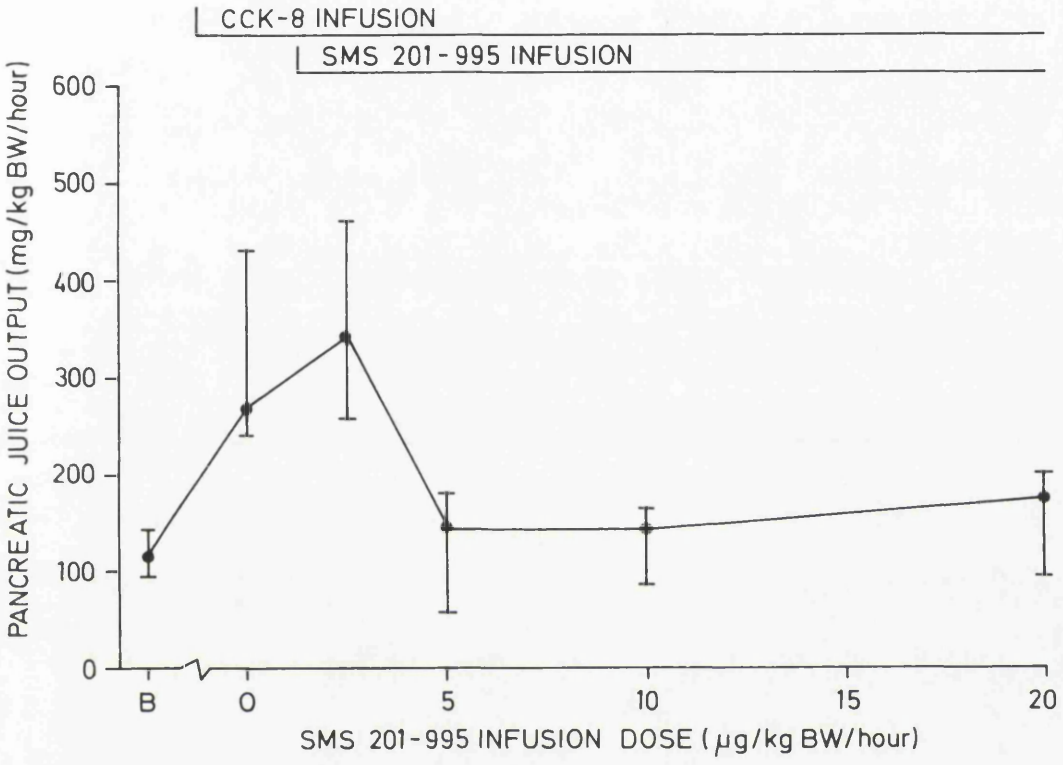

Figure 3.17: Pancreatic juice output (mg/kgBW/hour) in six hamsters during infusion of increasing doses of SMS 201-995 with a background infusion of CCK-8.

343.0mg/kgBW/hour (Q1:Q3 = 256.8:461.4) during infusion of SMS 201-995 at 2.5ug/kgBW/hour before falling to basal values during infusion of SMS 201-995 at 5ug/kgBW/hour. There was a tendency for median pancreatic juice output to increase slowly as the dose of SMS 201-995 was increased.

B1) Increasing doses of secretin with a background infusion of SMS 201-995

Pancreatic juice output during this infusion experiment in four hamsters (mean body weight 123.8, range 85.8 - 146.6g) is shown in Figure 3.18. Median basal output during saline infusion only was 239.8mg/kgBW/hour (Q1:Q3 = 221.8:586.6). Median background output during infusion of saline through cannula 1 and SMS 201-995 through cannula 2 was 444.9mg/kgBW/hour (Q1:Q3 = 412.2:702.4). Pancreatic juice output then fell to a minimum median value of 74.5mg/kgBW/hour (Q1:Q3 = 46.7:115.4) during infusion of secretin 0.04ug/kgBW/hour before slowly increasing as the dose of secretin increased.

B2) Increasing doses of SMS 201-995 with a background infusion of secretin

Pancreatic juice output during this infusion experiment in six hamsters (mean body weight 123.4g, range 95.8 - 142.2g) is shown in Figure 3.19. Median basal output during saline infusion only was

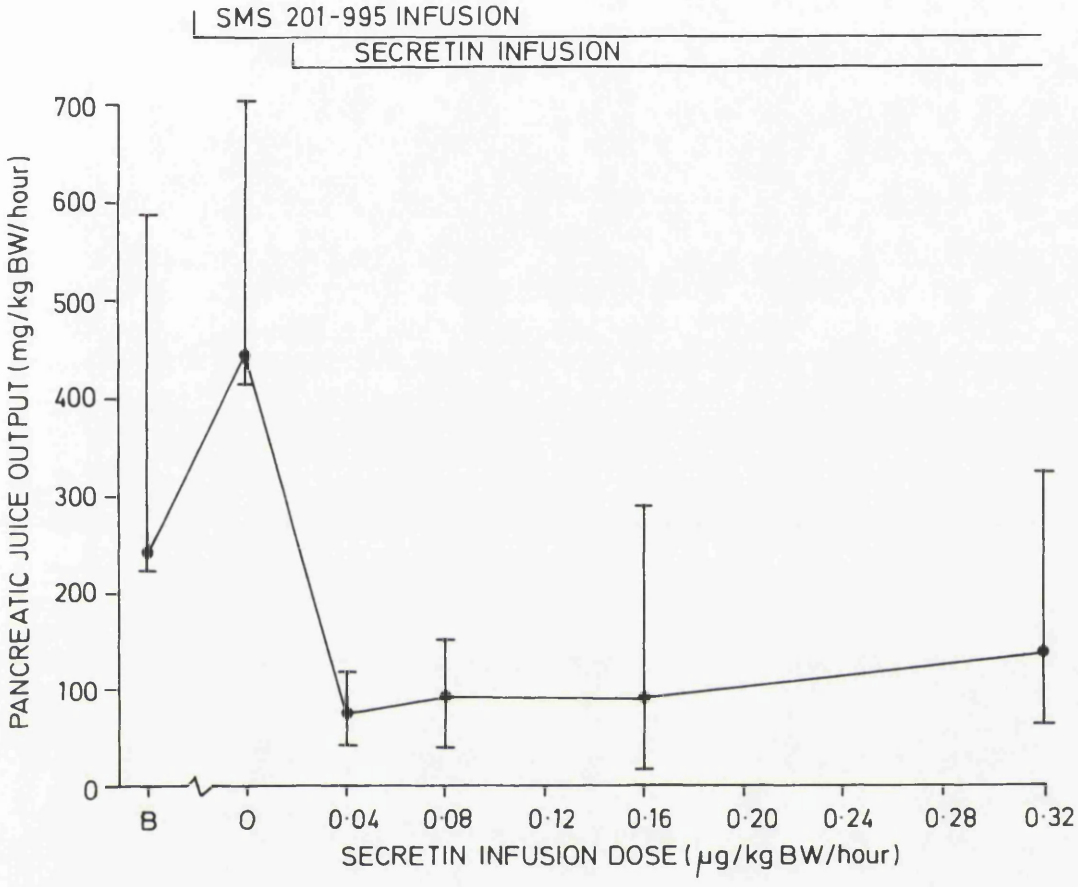

Figure 3.18: Pancreatic juice output (mg/kgBW/hour) in four hamsters during infusion of increasing doses of secretin with a background infusion of SMS 201-995.

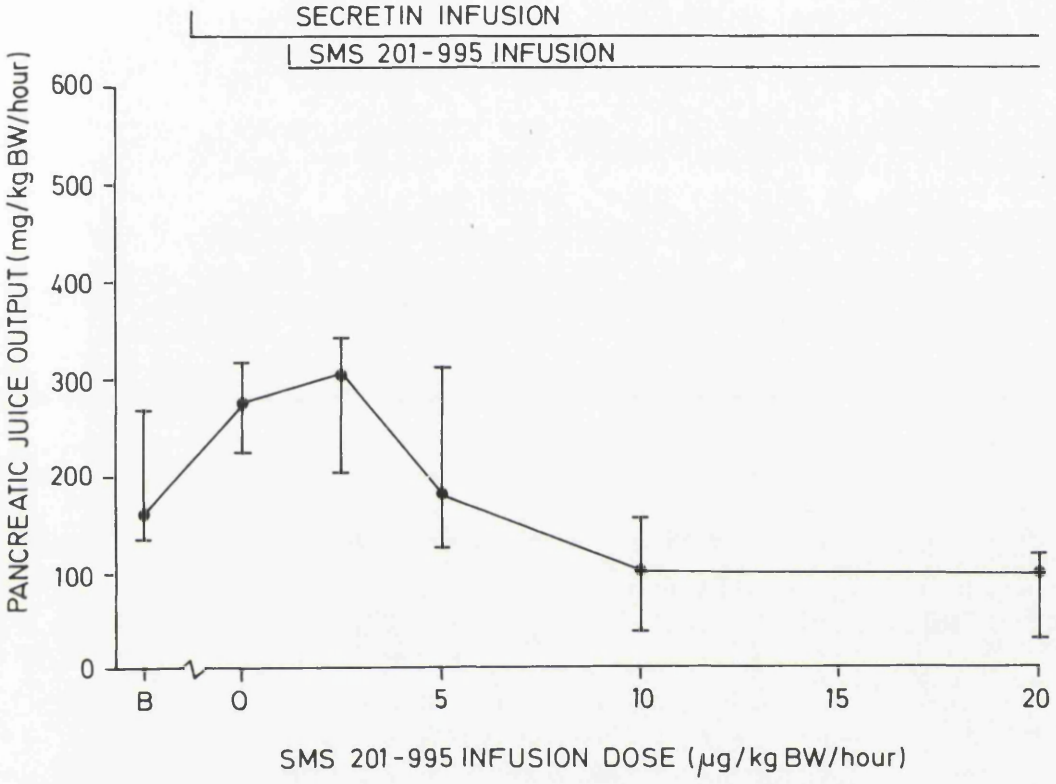

Figure 3.19: Pancreatic juice output (mg/kgBW/hour) in six hamsters during infusion of increasing doses of SMS 201-995 with a background infusion of secretin.

160.2mg/kgBW/hour (Q1:Q3 = 137.4:269.5). Median background output during infusion of saline through cannula 1 and secretin through cannula 2 was 278.8mg/kgBW/hour (Q1:Q3 = 226.4:313.8). Pancreatic juice output increased to a maximum median value of 303.5 mg/kgBW/hour (Q1:Q3 = 206.1:341.0) during infusion of SMS 201-995 at a dose of 2.5ug/kgBW/hour before falling to below basal values during infusion of SMS 201-995 at a dose of 10ug/kgBW/hour.

3.6 Discussion

There have been few studies of the effects of exogenous infusion of gastrointestinal hormones on exocrine pancreatic secretion in the Syrian hamster.

In 1980, Parviz Pour's group published two papers on various aspects of pancreatic secretion in the Syrian hamster (Helgeson et al, 1980a and b). The first of these two papers studied basic unstimulated pancreatic secretion. The authors cannulated the pancreas in a similar way to that used in this study, but recovered the animals after closure of the abdomen. They reported, both in starved and fed animals, that pancreatic juice output increased steadily over the first 10-12 hours (until late evening) after which output levelled off or decreased slowly. Pancreatic juice pH did not change appreciably during the 20 hour study period. Pancreatic juice protein concentration and total pancreatic juice protein output fell steadily over the 20 hours of the study in fed animals; protein concentration in the starved animals, which was on average five times that of the fed animals, fell with time but the total protein output rose during the first 10 hours before subsequently falling.

In the second paper from Pour's group using the same pancreatic duct cannulation technique, secretin (25iu/kgBW) or pancreozymin (20iu/kgBW) were administered as six half-hourly injections at the

start of the sample collection period. Pancreatic juice secretion and composition were measured during the following 20 hours. The pH of pancreatic juice secreted was significantly lower in the secretin-stimulated animals than the pancreozymin-stimulated animals at all measured time points. Pancreatic secretion in the secretin-stimulated animals was, on average, two to three times that of the pancreozymin-stimulated animals during the first three hours in both male and female hamsters and during the second three hour period for males only. Thereafter flow rates were similar for both groups. The protein content of pancreatic juice was higher in the pancreozymin injected hamsters than the secretin injected animals at all time points, averaging at least 40% higher over the 20 hours of study. The total protein output of the two groups was similar in the first six hours of study. The hourly protein output was five to 10 times greater than in the unstimulated hamsters reported in their initial study.

The values for basal pancreatic juice output in the CCK-8 and SMS 201-995 groups in the current study were similar to those reported in Helgeson's initial paper. The values obtained for basal pancreatic juice output in our secretin group were considerably higher, for no apparent reason. Pancreatic juice output during infusion of secretin and CCK-8 in the current study was considerably higher than that reported in

Helgeson's second paper; this was almost certainly due to the different methods of administration of secretin and CCK-8.

Maximal pancreatic secretion in both the secretin and CCK-8 groups compares well with work reported by Howatson and Carter (1985, 1987) using CCK-33 and a different secretin preparation. It would seem therefore that the effects of CCK-8 and secretin seen in the current study represent a true effect of the infused hormone on pancreatic secretion.

In the present study, secretin and CCK-8 both seem to exert a positive effect on pancreatic juice total bicarbonate output, with secretin, not surprisingly exerting a greater effect than CCK-8. Pancreatic juice protein output was increased dramatically by CCK-8 infusion but not by secretin infusion. These observations confirm those made by Ali, Rutishauser and Case (1990). Using a similar technique to that used in the current study, hamster pancreatic juice was collected in response to infusions of secretin (0.01 - 3.0 ug/kg/hour), CCK-8 (0.05 - 2.5 ug/kg/hour) or bombesin (1.0 - 8.0 ug/kg/hour). Maximum rates of pancreatic juice protein secretion in response to CCK-8 were achieved at a dose of 0.5ug/kg/hour (similar to the current study). Similarly, maximal pancreatic juice bicarbonate output occurred in response to a dose of 1ug/kg/hour (higher than the current study). Values

for total pancreatic juice output in Ali's paper are, on average, considerably higher than those recorded in the current study. The reason for this may lie in that Ali's group cleared the pancreatic cannula of bile by injecting each animal with 1.0 U/kg of natural secretin prior to the commencement of each infusion. In addition, there were other small but significant differences in experimental technique with regard to infusion rates (16.7ul/min compared to 6.25ul/min in the current study) and fluids used for infusion.

SMS 201-995 significantly depressed pancreatic juice output during intravenous infusion in the current study. Pancreatic secretion was not completely abolished however suggesting that other positive influences on pancreatic secretion (e.g. neural stimulation via the vagus nerve), were not affected by SMS 201-995. In a recent publication, infusion of low doses of somatostatin-14 suppressed pancreatic secretion in eight human subjects whose pancreatic duct had been cannulated during biliary surgery (Gullo et al, 1988). At a dose of 0.05ug/kg/hour (shown to produce blood levels similar to those measured after a meal), somatostatin-14 did not exert any effect on pancreatic secretion. Higher doses (0.15, 0.45 and 1.35ug/kg/hour) caused a significant and dose-dependent suppression in pancreatic juice, protein and bicarbonate output. The inhibitory effect on protein output was greater at all

doses than the inhibition of bicarbonate output. As in the current study, pancreatic secretion was not completely abolished even at the highest infusion dose.

SMS 201-995 was infused into four dogs in a Japanese study published in the same year (Misumi et al, 1988). Using infusion doses of SMS 201-995 of one to 120 ng/kg/hour, pancreatic juice output (collected from Thomas duodenal cannulae) was measured between and after meals, and after stimulation of pancreatic secretion with intravenous infusions of secretin and CCK-8. SMS 201-995 inhibited pancreatic juice output and the endogenous secretion of CCK, pancreatic polypeptide and motilin in all three experimental situations, in a dose-dependent manner. It was unclear from this study whether the suppression of pancreatic secretion occurred as a direct effect of SMS 201-995 on the pancreas or through suppression of other gastrointestinal hormones.

Contrary results to the above two studies were reported by Muller and coworkers (1988). Using an isolated rat stomach and pancreatic preparation they measured both endocrine and exocrine pancreatic secretion and gastrin secretion from the stomach. Somatostatin-14 was administered by linear gradient or infused intravenously in the presence of caerulein, secretin, electric vagal activity or acetylcholine. No effect on pancreatic exocrine secretion was

detected in any of the experiments although pancreatic endocrine secretion of insulin in response to glucose was significantly suppressed.

Conway and colleagues (1988) demonstrated that subcutaneous injections of SMS 201-995 in dogs significantly reduced pancreatic blood flow without altering systemic haemodynamics. All vessels to the pancreas were divided except the pancreaticoduodenal artery and vein. A flow probe was placed around the pancreaticoduodenal artery. Interestingly, reductions in pancreaticoduodenal blood flow did not seem to depend on the doses of SMS 201-995 administered (0.002mg/kg, 0.02mg/kg, 0.2mg/kg). The absence of systemic haemodynamic effects suggested that SMS 201-995 exerted a local effect on pancreatic blood flow. This observation might explain why Muller's group failed to detect any effect of somatostatin on an isolated perfused preparation and may be of relevance when other aspects of the current study are examined.

After an initial increase in pancreatic juice output, infusion of SMS 201-995 in experiment (A1) reduced pancreatic juice output despite subsequent infusion of CCK-8. During infusion of CCK-8 in a dose of 0.8ug/kgBW/hour the median pancreatic juice output value increased slightly compared to the two previous values, perhaps indicating that further increases in CCK-8 infusion dose would overcome the suppressive effect of SMS 201-995.

Infusion of CCK-8 in experiment (A2) increased pancreatic juice output as expected, although not to the level seen in the CCK-8 infusion study reported earlier in this chapter. CCK-8-stimulated pancreatic juice output was not affected by the initial SMS 201-995 infusion dose (2.5ug/kgBW/hour) but was suppressed to basal values at higher doses. Interestingly, pancreatic juice output under the influence of both CCK-8 and SMS 201-995 together was higher at all dose points above 2.5ug/kgBW/hour SMS 201-995 than that obtained when SMS 201-995 was infused alone in the experiments described above.

It has been suggested that CCK exerts its effect on pancreatic secretion via membrane bound receptors, which, after internalization and activation of phospholipase-C, activate the diacylglycerol intracellular messenger system (Williams & Hootman, 1986; Williams, 1987; Case, 1989 and 1990) or inositol 1,4,5-triphosphate (Case, 1989 and 1990). Somatostatin, on the other hand, is thought to act via the cyclic AMP messenger system negatively coupled to adenylate cyclase through a unique inhibitory guanine nucleotide binding (or G_1) protein (Williams & Hootman, 1986; Williams, 1987). This difference might explain why SMS 201-995 was not able to suppress CCK-8 stimulated pancreatic juice secretion to the levels achieved by infusion of SMS 201-995 alone.

CCK has also been shown in acinar cell preparations to decrease the binding of somatostatin to its cell membrane receptor sites (Matozaki et al, 1986). Whether CCK-8 acts in this way to reduce the efficacy of infused SMS 201-995 is open to speculation.

Recent work has suggested that there are at least two (Matozaki et al, 1989) and possibly three (Yu et al, 1990) distinct receptors for CCK in pancreatic acinar cells; one with high affinity but low capacity for CCK and one with low affinity but high capacity. The third receptor site detected by Yu and colleagues accounted for only 17% of tracer uptake. The functions of each of these receptor sites for CCK are, as yet, not known.

In experiment (B1) secretin-stimulated pancreatic juice output was suppressed to basal values by infusion of SMS 201-995 in a dose of 5ug/kgBW/hour and subsequently suppressed to sub-basal values by higher doses of SMS 201-995. As in the CCK-8 experiment (A1), median pancreatic juice output was not suppressed as much when secretin and SMS 201-995 were infused together as when SMS 201-995 was infused alone.

In experiment (B2) median pancreatic juice output increased during infusion of SMS 201-995 before subsequently falling to sub-basal values following the introduction of the secretin infusion. Infusion of

increasing doses of secretin resulted in only a small increase in pancreatic juice output, not reaching basal values.

Secretin differs from CCK in that it exerts its effects on pancreatic secretion through the same cyclic AMP intracellular messenger system as somatostatin, without the negative coupling of the guanine nucleotide binding protein (Williams & Hootman, 1986; Williams, 1987). The fact that both hormones act via adenylate cyclase, albeit in opposite ways, might explain why SMS was more suppressive to pancreatic juice output in the presence of a secretin infusion compared to a CCK-8 infusion; competitive inhibition of adenylate cyclase activity by SMS 201-995 may be relevant in suppressing secretin-stimulated secretion but be of no relevance in suppressing CCK-8-stimulated secretion.

The role of the endocrine pancreas in exocrine secretion has been the subject of much investigation and speculation. It is generally held that local insulin secretion in the pancreas is necessary for exocrine pancreatic enzyme secretion. Insulin was shown to have a direct regulatory effect on pancreatic acinar cell CCK receptors and CCK-induced acinar cell secretion in a paper by Otsuki and Williams (1983). The role of somatostatin in this regard has been the subject of a recent report by Garry and colleagues (1989). In an isolated rat perfusion model, they

studied the interaction between insulin, somatostatin and amylase secretion. In a complex series of experiments they concluded that somatostatin inhibited the secretion of amylase via its inhibition of the action of insulin. In addition, they concluded that somatostatin may act directly on pancreatic exocrine function by influencing specific acinar somatostatin receptors. Exogenous insulin was shown to potentiate the effect of CCK on amylase secretion, emphasising the complex nature of the relationship between peptide gastrointestinal hormones, insulin and pancreatic function. Whether SMS 201-995 influenced pancreatic secretion and altered the effects of both secretin and CCK-8 in the current study is not known.

CHAPTER 4

The effect of chronic subcutaneous administration of cholecystokinin octapeptide (CCK-8), secretin and somatostatin analogue (SMS 201-995) on the pancreas in the Syrian golden hamster.

4.1 Introduction

In the previous chapter, it was demonstrated that IV infusion of the peptides CCK-8, secretin and SMS 201-995 influenced pancreatic exocrine secretion in the Syrian golden hamster. In addition, the relationship between IV infusion of SMS 201-995 and secretin or CCK-8 in combination with respect to pancreatic secretion was determined.

The next phase of the study was designed to determine whether SC injections of the same peptide hormones, alone and in combination, influenced other aspects of pancreatic morphology and physiology, in particular pancreatic wet weight and DNA content. Several experimental studies have suggested that a number of CCK preparations, and, to a lesser degree, secretin preparations, are trophic to the pancreas in experimental animals. Given the negative effect of SMS 201-995 on pancreatic secretion in the hamster model, this somatostatin analogue might be expected to exert a negative effect on these aspects of pancreatic physiology.

The selection of appropriate doses of peptides to be used in this part of the study proved to be difficult, given the lack of published data on CCK-8 and SMS 201-995 with respect to the pancreas. The work described in the previous chapter identified doses of hormones which, when infused intravenously stimulated or suppressed pancreatic secretion. In

addition, the dose relationships between CCK-8 or secretin and SMS 201-995 with respect to pancreatic secretion were determined.

While it is obvious that a single SC injection of any of the peptides in the doses identified as having an effect when infused intravenously, will result in a total daily hormonal burden to the hamster equivalent to approximately four percent of the intravenous dose (if infused for 24 hours), previous published work from this Department demonstrated that doses so identified were sufficient to result in effects on pancreatic wet weight and DNA content, and subsequently on pancreatic carcinogenesis when the hormones were administered twice daily for three days each week (Howatson & Carter, 1985; Howatson & Carter, 1987). In the first of these two papers cholecystokinin (20% natural CCK, GIH Research Unit, Karolinska Institute) was administered SC in a dose of 30 Ivy Dog Units (IDU)/kg BW, twice daily for three days each week for six weeks. This regimen resulted in a significantly greater ($p<0.001$) mean pancreatic wet weight in the CCK-treated group (466.4 $\pm$ 77mg per 100g body weight) compared to the control group (295.6 $\pm$ 61mg per 100g body weight). There was no difference in total pancreatic DNA content between the two treatment groups. In the second paper, secretin (KabiVitrum, Stockholm, Sweden) was administered in a dose of 20 clinical units (CU)/kg body weight twice

daily for three days each week for six weeks. No significant effect on pancreatic wet weight or total DNA content between treatment and control groups was demonstrated, although in the final part of this set of experiments, secretin (in the same doses described in the trophism experiment) did promote pancreatic carcinogenesis.

Some further support for the use of an SC administered dose of CCK-8 of 0.8ug/kgBW in trophism experiments was provided by Pfeiffer et al (1982). This is the only published paper to provide any data on the trophic effect of CCK-8 on the hamster pancreas; it reported a trophic effect of twice daily SC injection in a dose of 0.5 - 1.5ug/kgBW. The dose of CCK-8 to be used in this chapter of the thesis lies within the dose range used in Pfeiffer's study.

Given that the ultimate aim of this thesis was to determine whether the hormones under investigation could influence pancreatic carcinogenesis, it was not thought necessary to administer the hormone each day for the duration of the experiments. Administration of secretin and CCK for three days in the week with carcinogen injection during this three-day period when the target organ for the carcinogen, namely the pancreas, was most likely to be under the influence of the hormones has been shown to yield positive results in previous work from this laboratory (Howatson & Carter, 1985 & 1987).

4.2 Materials and Methods

All animals used in these experiments were male WO(GD) Syrian golden hamsters (Wrights of Essex) 8-10 weeks of age. Hamsters were kept in standardised animal facility conditions with a twelve hour light-dark cycle, and 21% humidity and caged in groups of five. All animals were fed standard small rodent diet and water ad libitum.

Animals were weighed on day one of each week and injected twice daily at 8am and 4pm on days two, three and four with peptide substances suspended in a 10% gelatin solution to prolong absorption (Petersen et al, 1978).

One hundred and twelve animals were studied. Seven groups containing 16 animals each were sacrificed in subgroups of eight after one and six weeks of treatment. The seven groups of animals studied at each time point were:

a) Control - 10% gelatin solution only, twice daily
b) CCK-8 (0.8ug/kg body weight, twice daily)
c) SMS 201-995 (5ug/kg body weight, twice daily)
d) CCK-8 and SMS 201-995 (0.8 ug/kg body weight and 5 ug/kg body weight respectively, twice daily)
e) Secretin (0.3ug/kg body weight, twice daily)
f) SMS 201-995 (10ug/kg body weight, twice daily)
g) Secretin and SMS 201-995 (0.3 ug/kg body weight 10 ug/kg body weight respectively, twice daily)

Hamsters were sacrificed by intraperitoneal

overdose of pentobarbitone sodium. A full post-mortem examination was performed and the pancreas excised with the duodenum and a piece of spleen to allow identification of the three lobes of the gland. The spleen and duodenum and all extraneous connective tissue fat and lymph nodes were dissected from the pancreas and the organ weighed. A small block was excised from the tail of the splenic lobe of each pancreas and fixed in formal saline solution. These blocks were examined histologically for malignant or pre-malignant lesions after processing and staining with haematoxylin and eosin. The remainder of each gland was snap frozen in liquid nitrogen and stored at -80^{o}C for assay at a later date.

DNA Assay - Hoechst 33258 Reagent (Cesarone, Bolognesi and Santi, 1979; Perkin-Elmer, 1986)

Appendix 1 gives details of the preparation of the reagents for the assay.

Assay Procedure:

1. Tissue Preparation
   (a) Take 3 x 50mg tissue pieces from pancreas
   (b) Add 10ml EDTA/PBS buffer (Solution 6; Appendix 1)
   (c) Homogenise for 30 seconds
   (d) Sonicate for 30 seconds (Sample dilution x 10)
   (e) Take 0.1ml of sample solution and add 1.9ml

EDTA/PBS buffer (Solution 5; Appendix 1) (Sample dilution x 200)

(f) Add 2ml Hoechst 33258 Reagent (1ug/ml) and after one hour analyse DNA content as below

2. Fluorescence Spectrophotometry (Perkin Elmer LS-5 Spectrophotometer)

(a) Switch on machine at wall

(b) Fluorescence lights

(c) FIX SCL lights

(d) Set slits at either side of machine to 10mm

(e) Set excitation to 365nm (Press 3,6,5 then EX then GO TO ^).

(f) Set emission to 458nm (Press 4,5,8 then EM then GO TO ^).

(g) Allow indicator lights to stop flashing

(h) Lift front of turret to place cuvettes containing solutions for analysis

(i) Open shutter by pressing switch on left hand side of instrument (light off)

(j) With reagent blank cell in position allow reading to settle and press AUTOZERO - wait for zero reading

(k) With test cells in position allow reading to settle and press INT - record reading at end of integration period (when light stops flashing)

(l) Compare readings of samples to standard curve

For each pancreas examined, six values for DNA content were obtained (three paired values). Paired-sample assay reproducibility was calculated using analysis of variance. Mean values for each set of six readings were used for comparing the groups. Groups were compared using the Mann-Whitney test for non-parametric, unpaired data.

4.3 Results

There was no evidence of the development of BOP-associated histological changes or any other histological abnormality in any of the pancreatic sections examined.

Figure 4.1 shows the pancreatic wet weights of the seven groups of animals at the end of week 1 of treatment with CCK-8, secretin or SMS 201-995. Appendix 2 gives the raw data for each animal. There were no statistically significant differences between any of the groups.

Figure 4.2 shows the pancreatic wet weights at the end of week 6 of treatment in another seven groups of animals. Appendix 3 gives the raw data for each animal. Again there were no statistically significant differences between any of the groups.

Figure 4.3 shows the DNA contents of the pancreata at the end of week 1 of treatment with the hormones. Appendix 4 gives the raw data for each pancreas analysed. There were no statistically significant differences between any of the groups.

Figure 4.4 shows the DNA content of the pancreata at the end of week 6 of treatment with the hormones. Appendix 5 gives the raw data for each pancreas analysed. There were no statistically significant differences between any of the groups.

The mean analysis of variance for paired samples in 16 DNA assay runs was 2.8% (range 1.7% - 5.3%).

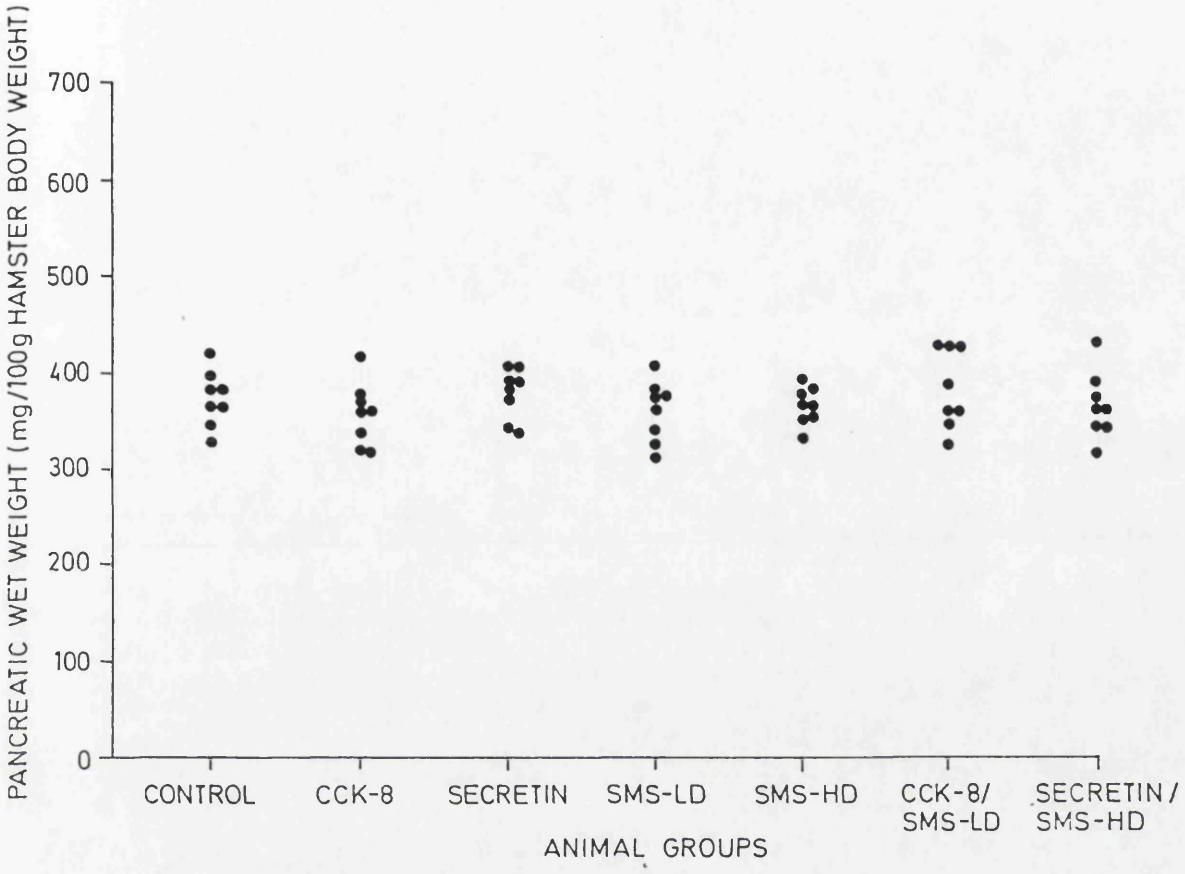

Figure 4.1: Pancreatic wet weights (mg/kgBW) of hamsters injected subcutaneously with CCK-8, secretin or SMS 201-995 in seven groups after one week of treatment.

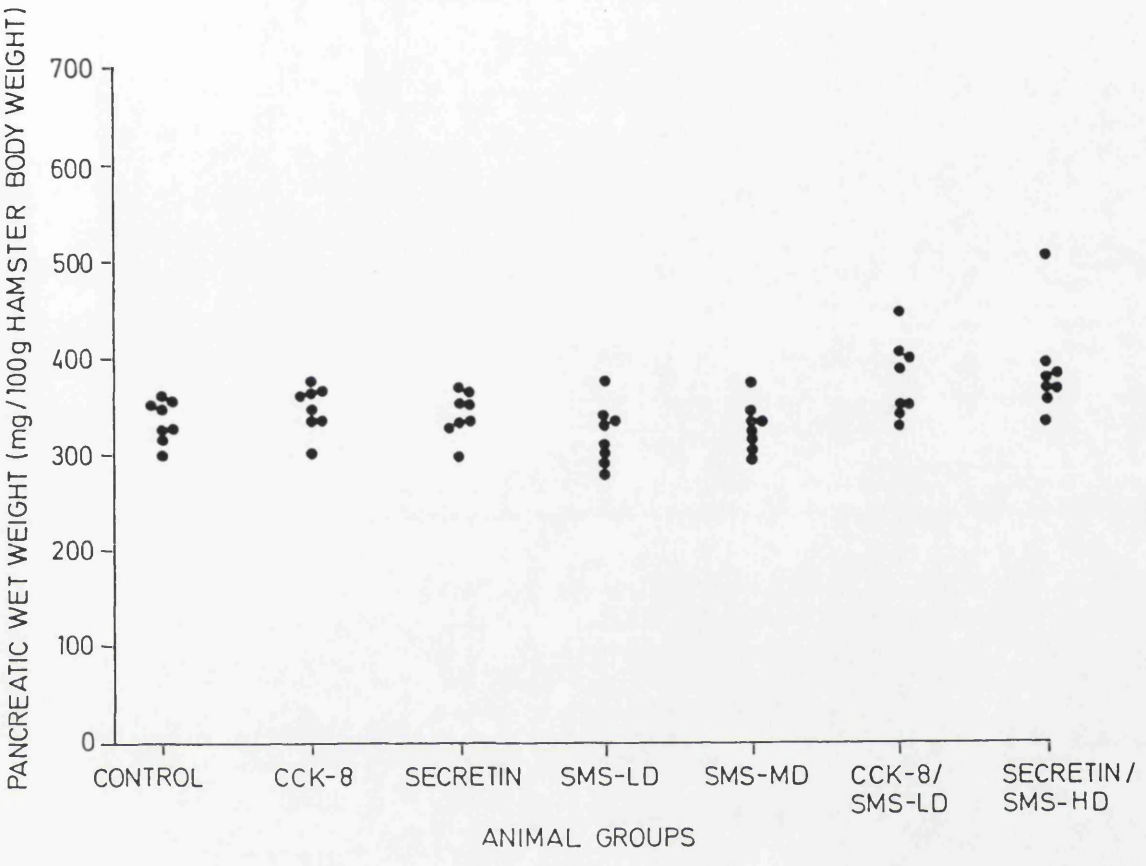

Figure 4.2: Pancreatic wet weights (mg/kgBW) of hamsters injected subcutaneously with CCK-8, secretin or SMS 201-995 in seven groups after six weeks of treatment.

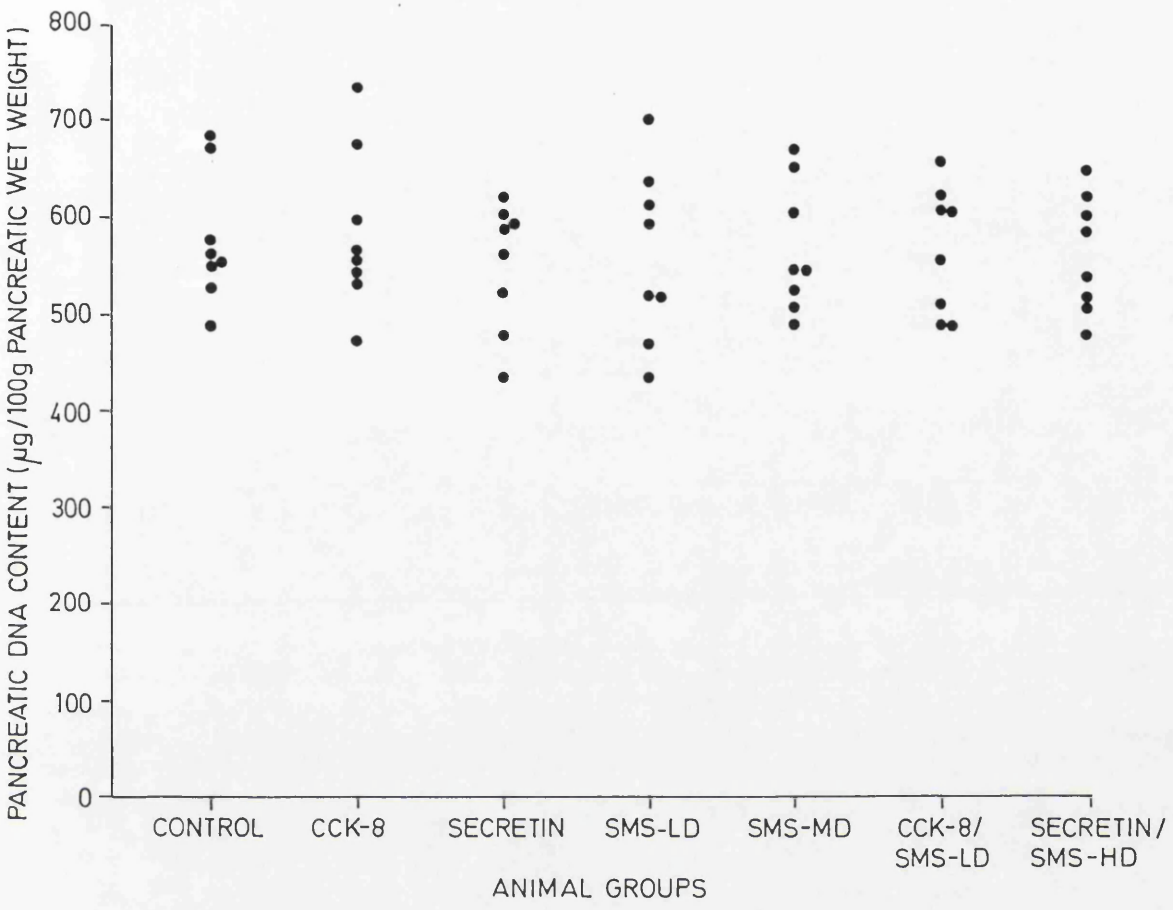

Figure 4.3: Pancreatic DNA content of hamsters injected subcutaneously with CCK-8, secretin or SMS 201-995 in seven groups after one week of treatment.

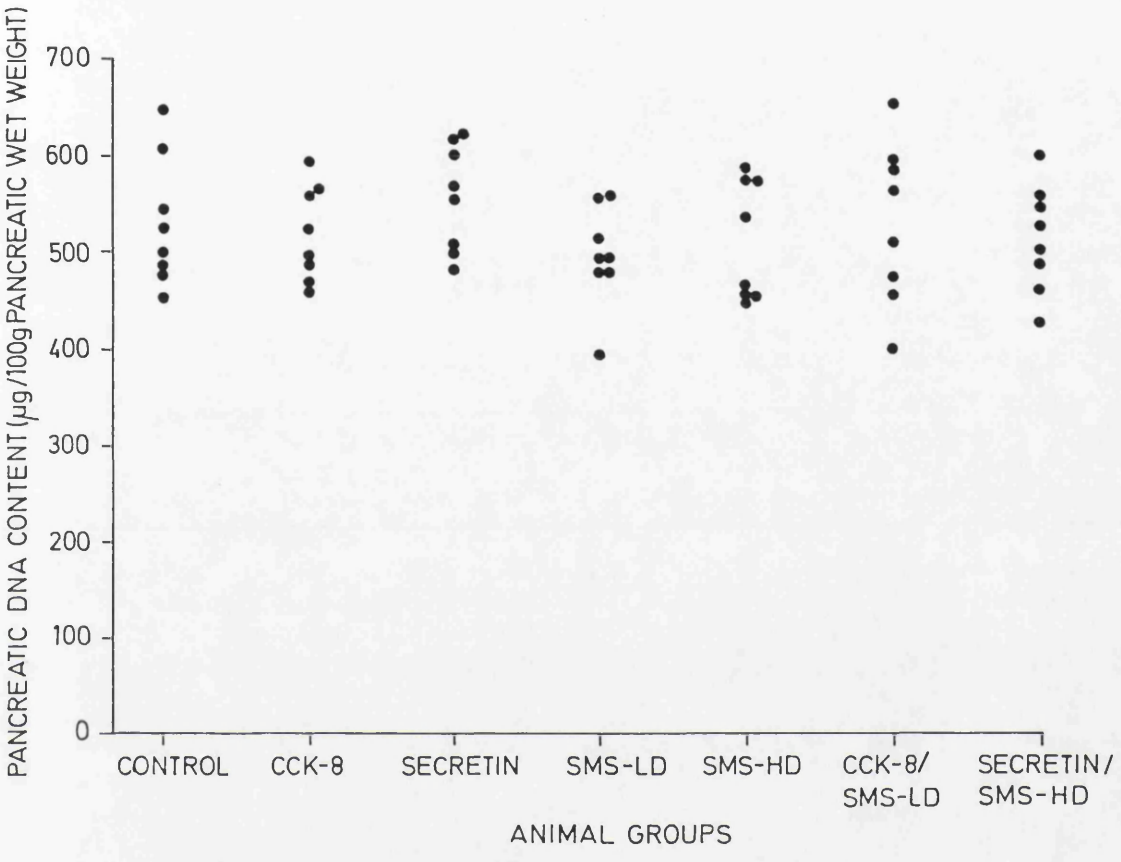

Figure 4.4: Pancreatic DNA content of hamsters injected subcutaneously with CCK-8, secretin or SMS 201-995 in seven groups after six weeks of treatment.

4.4 Discussion

The earliest report of the trophic effect of exogenously administered gastrointestinal hormones on animal pancreas was published by Rothman & Wells in 1967. They demonstrated that pancreatic weights in rats given CCK were significantly greater than in control rats; secretin and methacholine exhibited no such effect on pancreatic weight. This increase in pancreatic weight in response to CCK was confirmed by Mainz, Black and Webster (1973); they also reported an increase in pancreatic protein and DNA content and an increase in the uptake by the pancreas of tritiated thymidine and its incorporation into DNA. Interestingly, another pancreatic secretagogue, bethanechol chloride, an acetylcholine analogue, failed to exert any effect on pancreatic DNA content or synthesis suggesting for the first time that the trophic effects of substances on the pancreas were not necessarily linked to their ability to stimulate pancreatic secretion. In a similar experiment, Barrowman and Mayston (1974) administered 12.5 units of CCK per 100g body weight per day for nine days and demonstrated a slight but significant increase in pancreatic weight in treated rats.

Using a dose of 20% pure CCK which was submaximal for pancreatic secretory stimulation, administered in gelatin three times daily for 15 days, Petersen and co-workers (1978) demonstrated a 57%

increase in pancreatic wet weight in treated rats compared with control animals given the depot gelatin vehicle only; this increase was highly significant. Interestingly, secretin administration in this paper increased pancreatic wet weight in another group of rats by only 9% although this was significant at the 1% level.

In a later paper, Grossman's group demonstrated that caerulein, a decapeptide analogue of CCK derived from frog skin could exert a similar trophic response in rat pancreas (Solomon et al, 1978). When administered thrice daily for five, ten or 15 days in different doses, caerulein exhibited significant dose- and time-dependent increases in pancreatic wet weight and content of DNA, RNA, protein, trypsinogen and amylase. Such increases were only apparent in secretin-treated animals after 15 days of treatment. Administration of caerulein and secretin in combination resulted in increases in all measured parameters of pancreatic trophism, greater than the sum of the effects of the two peptides when administered individually. Similar effects have been demonstrated in the Syrian golden hamster pancreas in response to caerulein and secretin (Townsend et al, 1981).

In 1980, Dembinski and Johnson looked into the relationship between secretin and caerulein with respect to their effects on pancreatic trophism in

more detail. Secretin and caerulein administered eight-hourly resulted in significant increases in pancreatic DNA synthesis and in DNA and RNA content. When both hormones were administered together, DNA and RNA content was greater than the sum of the values for either hormone administered alone. On the basis of these findings they postulated that the important effect of secretin on the pancreas may be in the potentiation of the trophic effects of CCK.

The potentiation of the effect of secretin on a CCK nonapeptide analogue was not confirmed in a later paper by Haarstad et al (1986). This nonapeptide which is almost equipotent with caerulein was infused continuously into rats and induced similar increases in pancreatic wet weight, DNA, RNA and protein content as previous authors have reported for subcutaneous administration of other CCK-like peptides. Secretin infusions produced similar results.

In an attempt to determine whether endogenous secretin and CCK had any effect on pancreatic size, Johnson (1981) infused hydrochloric acid and an amino acid solution into the duodenums of Sprague-Dawley rats for 5 days. He reported an increase in both the secretin- and CCK-treated rats in pancreatic weight, and DNA, RNA and protein content. DNA synthesis, as demonstrated by incorporation of tritiated thymidine into DNA, was only increased in the rats infused with acid. Johnson concluded that endogenous secretin and

CCK both have a trophic effect on the rat pancreas. Unfortunately, Johnson was unable to measure CCK or secretin levels in the animals in response to the infusions of acid and amino acid. It is not possible to be sure therefore that the effects on the rat pancreas demonstrated were mediated by either CCK or secretin and as such, his conclusions with respect to cause and effect were not entirely valid.

In 1982, Pfeiffer and co-workers examined the effect of twice daily subcutaneous injections of CCK octapeptide (CCK-8) for ten days in the Syrian hamster. They reported that pancreatic weight and RNA:DNA ratios were significantly increased over controls over a dose range of CCK-8 of 50-150ng/100g body weight per day. Satake and co-workers (1986) investigated the effects of caerulein in the Syrian hamster. Subcutaneous injections of caerulein twice daily for ten days resulted in increases in pancreatic weight, amylase content, DNA and the pancreatic weight/DNA ratio. Caerulein seemed to have both a hyperplastic and hypertrophic effect on the pancreas. Using autoradiography with tritiated thymidine labelling, DNA synthesis was shown to increase most notably in acinar cells and only slightly in islet and ductal cells.

It came as some surprise that CCK-8 did not seem to exert any effect on the hamster pancreas with respect to pancreatic wet weight (a function of

protein content) and DNA content (a function of cellular proliferation) in our experiment. When similar work was previously undertaken in our Department using a different CCK preparation (and consequently different dose), this was not the case (Howatson & Carter, 1985). Doses of CCK which maximally stimulated pancreatic exocrine secretion, exhibited a trophic effect on the pancreas when administered subcutaneously in a gelatin vehicle. The current secretin results are similar to those reported by Howatson & Carter in their second paper (1987). They reported no difference between control and treatment groups after six weeks with respect to either pancreatic wet weight or total DNA content.

One of the notable features of the published literature on the subject of pancreatic trophism has been the wide variety of trophic substances used, marked variations in dosage administered and striking differences in the dosage schedules used. Tables 4.1 and 4.2 summarize the differences in a number of publications. In addition to the different peptide preparations used in these papers, it is very difficult to compare doses used when they have been expressed in so many different units. In Satake's work the dose of caerulein used was 20ug/kgBW twice daily for ten days compared to a maximum of 5ug/kgBW three times daily for 15 days in Solomon's paper and only 0.75ug/kgBW three times daily for seven days in

Table 4.1: Summary of cholecystokinin doses and preparations used in previously published work investigating the trophic effect of the hormone on animal pancreas

	Cholecystokinin		
Authors	Preparation	Dose	Duration
Petersen et al (1978)	20% natural CCK	37.5 IDU/ kgBW tid	15 d
Solomon et al (1978)	Caerulein	0.2/1.5 ug/kgBW tid	5/10/ 15 d
Dembinski & Johnson (1980)	Caerulein	90/180/ 320/750 ng/ kgBW tid	7 d
Pfeiffer et al (1982)	CCK-8 (Kinevac)	0.5-1.5ug/ kgBW bd	10 d
Howatson & Carter (1985)	20% natural CCK	30 IDU/kg BW bd (3 d each week)	42 d
Satake et al (1986)	Caerulein	20ug/kgBW bd	10 d
Haddock (this work)	CCK-8 (Sigma)	0.8ug/kg BW bd (3 d each week)	7/42 d

(IDU = Ivy Dog Units; tid = three times daily;

bd = twice daily; d = days)

Table 4.2: Summary of secretin doses and preparations used in previously published work investigating the trophic effect of the hormone on animal pancreas

		Secretin	
Authors	Preparation	Dose	Duration
Petersen et al (1978)	not specified	25 ug/kg BW tid	15 d
Solomon et al (1978)	Squibb	25 ug/kg BW tid	5/10/ 15 d
Dembinski & Johnson (1980)	Squibb	50 U/kg BW tid	7 d
Howatson & Carter (1987)	Kabi Vitrum	20 CU/kg BW bd (3 each week)	42 d
Haddock (this work)	Sigma	0.3 ug/kg BW bd (3 each week)	7/42 d

(U = Units; CU = Clinical Units; tid = three times daily; bd = twice daily; d = days)

Dembinski and Johnson's work. The dose of secretin per injection used in the current work was less than 2% of that used by Petersen et al (1978) and Solomon et al (1978).

The majority of authors so far cited have used a continuous dosing schedule when administering the peptides, i.e. the hormones have been administered regularly for a continuous number of days. In the current work, a more intermittent schedule was used after Howatson & Carter (1985, 1987). They were able to demonstrate detectable effects on the pancreas of CCK but not secretin when administered intermittently, although they used different preparations of secretin and CCK than those currently under investigation. Pfeiffer and co-workers used CCK-8 in their investigation in a dose not dissimilar to that under investigation. The peptide was administered for ten consecutive days, unlike the present study where the CCK-8 was administered for only three days out of seven or 18 days out of 42. It is possible that this intermittent method of administration of peptides in very low doses (as in the current work) might have allowed pancreatic cells to recover from the effect of the peptides during the days when not administered, resulting in an overall lack of detectable effect on the hamster pancreata. A paper by Lutcke and colleagues (1987) offers some support for this observation. During a continuous infusion of

caerulein into rats, tritiated thymidine incorporation into DNA increases only after 18 hours of infusion, reaches maximum values at 36 hours and falls to control values after 72 hours.

While there has been some published work on the effect of somatostatin and its analogues on exocrine pancreatic secretion there have been practically no papers on the effects of these substances on cell proliferation and none addressed to such an effect in the pancreas. Lehy and co-workers (1979) demonstrated that somatostatin inhibited incorporation of tritiated thymidine into gastric, duodenal and jejunal mucosa in vitro. It also decreased cell division in gastric mucosa. This observation might lend support to extending somatostatin's inhibitory action with respect to secretion of other gastrointestinal hormones to having a direct negative effect on other cellular functions.

CHAPTER 5

The effect of chronic subcutaneous administration of cholecystokinin octapeptide (CCK-8), secretin and somatostatin analogue (SMS 201-995) and N-nitrosobis(2-oxopropyl)amine (BOP) in the Syrian golden hamster.

5.1 Introduction

In previously published work from this Department, preparations of CCK and secretin have been shown to promote pancreatic carcinogenesis when administered subcutaneously (Howatson & Carter, 1985 & 1987). In the case of CCK, the dose of hormone administered with BOP when administered alone, proved to be trophic to the pancreas in a previous experiment. This was not the case with secretin where an influence on the carcinogenic process was observed without detectable trophism.

Given that BOP is thought to initiate the carcinogenic process at a subcellular level and it is possible that small doses of peptide hormone, administered in doses which do not affect pancreatic wet weight or DNA content (as reported in the preceding chapter of this thesis), might be able to influence the carcinogenic process. For this reason the next phase of the study included administration of the carcinogen BOP in addition to the hormones in the doses administered in the previous chapter.

5.2 Materials and Methods

All animals used in these experiments were male WO(GD) Syrian golden hamsters (Wrights of Essex) 8-10 weeks of age. Hamsters were kept in standardised animal facility conditions with a twelve hour light-dark cycle, and 21% humidity and caged in groups of five. All animals were fed standard small rodent diet and water ad libitum.

Animals were weighed on day one of each week and injected twice daily at 8am and 4pm on days two, three and four with peptide substances suspended in a 10% gelatin solution to prolong absorption. Carcinogen (BOP) was administered subcutaneously between the morning and evening doses of peptide substances on day three, as described in Chapter 4 of this thesis.

Two hundred and ten animals were studied. Seven groups containing 30 animals each were sacrificed in subgroups of 10 at 10, 12.5 and 15 weeks. The seven groups were:

a) Control - 10% gelatin solution only
b) CCK-8 (0.8ug/kg body weight)
c) SMS 201-995 (5ug/kg body weight: SMS-LD)
d) CCK-8 and SMS 201-995 (0.8ug/kgBW and 5ug/kgBW respectively)
e) Secretin (0.3ug/kg body weight)
f) SMS 201-995 (10ug/kg body weight: SMS-HD)
g) Secretin and SMS 201-995 (0.3ug/kgBW and 10ug/kgBW respectively)

Hamsters were sacrificed by intraperitoneal overdose of pentobarbitone sodium. A full post-mortem examination was performed and the pancreas excised with the duodenum and a piece of spleen to allow identification of the three lobes of the gland. Pancreata were cut into blocks prior to fixation; five blocks were cut from each of the splenic and gastric lobes and two from the duodenal lobe. Three blocks from the gastric and splenic lobes and one from the duodenal lobe were fixed in PLPD (paraformaldehyde, lysine, sodium periodate and potassium dichromate). Fixed blocks were washed in tap water for three hours. Three serial sections were cut from each block prior to staining with haematoxylin and eosin. All other blocks were frozen in liquid nitrogen. The large lymph node behind the head of the pancreas was excised separately and fixed in PLPD and stained as other blocks. Any nodules or grossly visible lesions were excised and fixed separately.

All histological sections were examined by the author and assessed for the presence of acinar cell death, acinar-ductular transformation, cystic ductal complexes, ductular carcinoma-in-situ, ductular microcarcinoma, ductal dysplasia, ductal carcinoma-in-situ and frankly invasive adenocarcinoma. The presence of a lesion in any section of any one pancreas was recorded as positive for that pancreas;

no attempt was made to count the number of lesions in each pancreas.

5.3 Results

The histological findings are shown in Tables 5.1 and 5.2 (10 weeks), 5.3 and 5.4 (12.5 weeks) and 5.5 and 5.6 (15 weeks). Results are expressed as the presence or absence of the particular histological abnormality in each animal in Tables 5.1, 5.3 and 5.5. In Tables 5.2, 5.4 and 5.6 the results are expressed as the presence of the particular abnormality in each histological block examined, in an attempt to gauge the extent of histological change in each pancreas. Values in parenthesis at the bottom of each table represent the total number of animals or blocks affected by carcinoma-in-situ, microcarcinoma or adenocarcinoma (no animal or block registering more than one lesion).

After ten weeks of treatment with BOP, the pattern of histological change seen in the control group of hamsters was similar to those reported in the initial studies of carcinogenesis in Chapter 2 of this thesis. In addition to ductal hyperplasia, acinar-ductular transformation and cystic complexes, one control animal exhibited ductal dysplasia. Ductal hyperplastic change was seen more frequently in all six of the treatment groups compared to the control group, although the number of hamsters affected was only significantly greater in the CCK-8, SMS-HD and secretin/SMS-HD groups (Table 5.1). Ductal dysplastic change was significantly more frequent in the secretin

Table 5.1: Histological lesions present in hamsters in each of seven groups after 10 weeks treatment with BOP (results expressed as lesion present or absent in each animal).

	Control (n=10)	CCK-8 (n=10)	Secretin (n=10)	SMS-LD (n=10)	SMS-HD (n=10)	CCK-8+ SMS-LD (n=10)	Secretin + SMS-HD (n=9)
Ductal hyperplasia	3	8*	6	6	9#	5	9+
Ductal dysplasia	1	3	8+	1	4	2	3
Ductal CIS	0	0	1	0	0	0	0
Cystic complexes	2	4	6	0	6	3	2
CIS	0	0	1	1	0	0	0
Microcarcinoma	0	0	0	1	0	0	0
Adenocarcinoma	0	0	0	0	0	0	0

(* = $p<0.05$ versus control, Fisher's Exact Test)
(# = $p<0.01$ versus control, Fisher's Exact Test)
(+ = $p<0.005$ versus control, Fisher's Exact Test)

Table 5.2: Histological lesions present in hamsters in each of seven groups after 10 weeks treatment with BOP (results expressed as lesion present or absent in each histological block).

	Control (n=70)	CCK-8 (n=70)	Secretin (n=70)	SMS-LD (n=70)	SMS-HD (n=70)	CCK-8+ SMS-LD (n=70)	Secretin + SMS-HD (n=63)
Ductal hyperplasia	3	9	8	8	11*	5	9*
Ductal dysplasia	1	5	14^	1	6	3	5
Ductal CIS	0	0	1	0	0	0	0
Cystic complexes	4	4	12*	0	14@	4	3
CIS	0	0	3	1	0	0	0
Microcarcinoma	0	0	0	1	0	0	0
Adenocarcinoma	0	0	0	0	0	0	0
	(0)	(0)	(3)	(2)	(0)	(0)	(0)

(* = $p<0.05$ versus control, Fisher's Exact Test)
(@ = $p<0.02$ versus control, Fisher's Exact Test)
(# = $p<0.01$ versus control, Fisher's Exact Test)
(+ = $p<0.005$ versus control, Fisher's Exact Test)
(^ = $p<0.0005$ versus control, Fisher's Exact Test)

Figures in parenthesis denote the number of sections in each group affected with either CIS, microcarcinoma or adenocarcinoma

Table 5.3: Histological lesions present in hamsters in each of seven groups after 12.5 weeks treatment with BOP (results expressed as lesion present or absent in each animal).

	Control (n=10)	CCK-8 (n=10)	Secretin (n=10)	SMS-LD (n=10)	SMS-HD (n=9)	CCK-8+ SMS-LD (n=9)	Secretin + SMS-HD (n=10)
Ductal hyperplasia	7	2*	5	2*	7	7	7
Ductal dysplasia	8	5	8	3*	6	4	5
Ductal CIS	1	1	4	0	0	2	0
Cystic complexes	9	8	9	10	7	8	9
CIS	3	1	2	3	1	4	1
Microcarcinoma	2	1	1	0	0	1	0
Adenocarcinoma	1	2	1	0	0	0	0
	(5)	(2)	(4)	(3)	(1)	(4)	(1)

(* = $p<0.05$ versus control, Fisher's Exact Test)
(# = $p<0.01$ versus control, Fisher's Exact Test)
(+ = $p<0.005$ versus control, Fisher's Exact Test)

Figures in parenthesis denote the number of animals in each group affected with either CIS, microcarcinoma or adenocarcinoma

Table 5.4: Histological lesions present in hamsters in each of seven groups after 12.5 weeks treatment with BOP (results expressed as lesion present or absent in each histological block).

	Control (n=71)	CCK-8 (n=71)	Secretin (n=70)	SMS-LD (n=70)	SMS-HD (n=64)	CCK-8+ SMS-LD (n=65)	Secretin + SMS-HD (n=70)
Ductal hyperplasia	9	2*	5	2*	10	11	10
Ductal dysplasia	12	15	16	7	15	7	10
Ductal CIS	1	1	6	0	0	2	0
Cystic complexes	27	14@	24	27	16	27	35
CIS	5	2	3	3	2	4	1
Microcarcinoma	3	1	1	0	0	1	0
Adenocarcinoma	1	2	2	0	0	0	0
	(8)	(5)	(6)	(3)	(2)	(5)	(1@)

(* = $p<0.05$ versus control, Fisher's Exact Test)
(@ = $p<0.02$ versus control, Fisher's Exact Test)

Figures in parenthesis denote the number of sections in each group affected with either CIS, microcarcinoma or adenocarcinoma

Table 5.5: Histological lesions present in hamsters in each of seven groups after 15 weeks treatment with BOP (results expressed as lesion present or absent in each animal).

	Control (n=10)	CCK-8 (n=8)	Secretin (n=9)	SMS-LD (n=10)	SMS-HD (n=10)	CCK-8+ SMS-LD (n=9)	Secretin + SMS-HD (n=10)
Ductal hyperplasia	1	2	3	1	8+	2	7#
Ductal dysplasia	9	7	8	10	6	7	5
Ductal CIS	2	2	2	6	0	3	1
Cystic complexes	9	8	9	10	9	9	10
CIS	6	4	4	8	2	7	5
Microcarcinoma	1	0	1	3	1	1	2
Adenocarcinoma	0	1	4*	4*	1	4*	4*
	(6)	(4)	(6)	(8)	(4)	(9)	(7)

(* = $p<0.05$ versus control, Fisher's Exact Test)
(# = $p<0.01$ versus control, Fisher's Exact Test)
(+ = $p<0.005$ versus control, Fisher's Exact Test)

Figures in parenthesis denote the number of animals in each group affected with either CIS, microcarcinoma or adenocarcinoma.

Table 5.6: Histological lesions present in hamsters in each of seven groups after 15 weeks treatment with BOP (results expressed as lesion present or absent in each histological block).

	Control (n=71)	CCK-8 (n=57)	Secre-tin (n=68)	SMS-LD (n=75)	SMS-HD (n=70)	CCK-8+ SMS-LD (n=64)	Secre-tin + SMS-HD (n=75)
Ductal hyper-plasia	1	2	3	1	11+	2	9
Ductal dys-plasia	24	18	19	25	8!	11*	10+
Ductal CIS	2	3	6	13+	0	4	1
Cystic complexes	35	35	44*	35	44	28	48
CIS	8	5	6	14	5	9	7
Micro-carcinoma	1	0	1	4	1	1	3
Adeno-carcinoma	0	2	9!	17!	2	7+	4
	(9)	(7)	(13)	(33!)	(8)	(16)	(14)

(* = p<0.05 versus control, Fisher's Exact Test)
(# = p<0.01 versus control, Fisher's Exact Test)
(+ = p<0.005 versus control, Fisher's Exact Test)
(! = p<0.002 versus control, Fisher's Exact Test)

Figures in parenthesis denote the number of sections in each group affected with either CIS, microcarcinoma or adenocarcinoma

group than in the control group. Significantly more blocks were affected by cystic complexes in the secretin and SMS-HD groups, by ductal hyperplasia in the SMS-HD group and by ductal dysplasia in the secretin group, than in controls (Table 5.2).

No hamsters exhibited adenocarcinoma at this stage in the experiment although two animals in the SMS-LD group (one with carcinoma-in-situ, one with microcarcinoma) and one in the secretin group (with carcinoma-in-situ) exhibited pre-malignant change (Table 5.1).

After 12.5 weeks of treatment hamster pancreata in all groups exhibited the full spectrum of histological changes seen in response to BOP treatment. Significantly fewer of the secretin and CCK-8 animals exhibited ductal hyperplasia than the control group (both $p<0.05$) and fewer animals in the SMS-LD group developed ductal dysplasia ($p<0.05$). There were no other obvious differences between the groups with regard to the frequency of the various histological changes seen (Table 5.3). Fewer blocks were affected by ductal hyperplasia in the CCK-8 ($p<0.02$) and SMS-LD groups than controls (Table 5.4). Adenocarcinoma was demonstrated only in the control, CCK-8 and secretin groups, although animals in all seven groups exhibited carcinoma-in-situ.

At 15 weeks significantly more animals in the SMS-LD and secretin/SMS-HD groups exhibited ductal hyperplasia than the control group (Table 5.5). The only other statistically significant difference between the groups in this table occurred with respect to the development of adenocarcinoma. This lesion affected significantly more animals in four of the groups (secretin, SMS-LD, CCK-8/SMS-LD and secretin/SMS-HD) than in the control group. When the blocks were examined, ductal hyperplasia was significantly more common in the SMS-HD group ($p<0.005$), ductal CIS in the SMS-LD group ($p<0.005$) and cystic complexes in the secretin group ($p<0.05$; Table 5.6). Ductal dysplasia was significantly less common in the SMS-HD, CCK-8/SMS-LD and secretin/SMS HD groups. Adenocarcinoma affected more blocks in the secretin ($p<0.002$), CCK-8/SMS-LD ($p<0.005$) and, most particularly, the SMS-LD ($p<0.002$) groups compared to the controls. When all malignant and pre-malignant lesions (namely CIS, microcarcinoma and adenocarcinoma) were considered together, only the SMS-LD group exhibited a significant increase in lesion frequency over the control group ($p<0.002$).

5.4 Discussion

The pattern of histological changes seen in these experiments in response to subcutaneous injection of BOP is similar to those reported by Parviz Pour in many publications. Despite these publications, Pour and other workers in the field remain unclear as to the significance of the different lesions seen early in the carcinogenic process.

In a recent electron microscopic and immunohistochemical study of the early stages of BOP-induced pancreatic carcinogenesis, Pour (1988) concluded that the pseudoductular structures developed from centroacinar cells. Hypertrophy and hyperplasia of these cells with the formation of small cellular processes which insinuate between acinar cells were the initial changes seen during the neoplastic process. Acinar cells became separated and detached from the glandular lumen and vasculature resulting in their degeneration and replacement by centroacinar cells. It remains unclear from this work whether the tubular and pseudoductular structures which then developed were true precursors of malignant change, or simply represented a parallel change to the more subtle cellular and subcellular effects of the carcinogen. It is interesting to note that a point mutation in codon 12 of the c-Ki-ras oncogene, which has previously been reported in human pancreatic cancer, has recently been reported in BOP induced

hamster pancreatic cancer (Fujii et al, 1990). This subcellular effect of BOP seems more likely to be of relevance in the development of pancreatic cancer than progression from areas of pseudoductular changes.

In the current work it is difficult to detect a pattern of histological change in the different groups during the early part of the carcinogenic process. Given that the significance of many of these changes with respect to the ultimate development of pancreatic adenocarcinoma is unclear, the significance of minor differences between the groups with respect to early carcinogen-induced histological lesions is even less clear. For example, animals in all groups developed cystic changes and although there were some statistically significant differences between the control group and the secretin and SMS-HD groups at 10 weeks (when the number of affected sections were analysed), the relevance of this is uncertain. Similarly, statistically significant differences with respect to the development of ductal hyperplasia and ductal dysplasia at different stages in the experiment, is unclear.

There have been many published studies which suggest that cholecystokinin and its analogues increase the susceptibility of the hamster pancreas to the effects of the carcinogen BOP. It was interesting to note that in the present study no such effect was detected. It may well be that detectable effect was

lacking for the same reasons that CCK-8 failed to stimulate pancreatic hypertrophy or hyperplasia reported in the previous chapter of this thesis, namely that the dose was too small or the intermittent dosing schedule allowed pancreatic cells to recover between injections. This latter possibility seems less likely given that the carcinogen was injected between doses of CCK-8, when any hormone effect would have been expected to be at its greatest. It is of course possible that while the octapeptide analogue is potent with respect to stimulating pancreatic secretion, other parts of the complete CCK molecule are necessary to influence BOP induced pancreatic carcinogenesis.

Despite the above, there have been some conflicting reports of the effect of CCK and its analogues in influencing the susceptibility of the hamster pancreas to BOP and other carcinogens. In 1983, Johnson and colleagues reported that CCK inhibited the development of pancreatic cancer in the hamster model. Another group from Sweden reported that caerulein exerted no effect on the chemical induction of hamster pancreatic cancer (Andren-Sandberg, Dawiskiba and Ihse, 1984). Our own laboratory reported that CCK enhanced the development of hamster pancreatic cancer when a similar dosing schedule to that used in the current study was used (Howatson and Carter, 1985).

In a paper published in 1988, Pour and his colleagues attempted to explain some of these discrepancies. In the first of two experiments they administered CCK-33 to groups of hamsters three hours before the administration of a single injection of BOP (20mg/kgBW, group 1), simultaneously with BOP (group 2), or three hours after BOP (group 3) and sacrificed the animals 46 weeks after BOP injection. CCK-33 was administered in six subcutaneous injections 30 minutes apart to give a total dose of 20 IDU/kgBW per hamster. This dosing schedule had previously been shown to achieve maximal stimulation of pancreatic secretion (Helgeson et al, 1980). One additional group received CCK-33 daily for 15 weeks after the BOP injection (group 4). Control groups received either BOP or CCK alone (groups 5 and 6). In the second experiment a similar treatment schedule was used but CCK-33 and BOP were given weekly for 20 weeks and the BOP dose was reduced (2.5mg/kgBW).

In the first experiment, the incidence of ductular proliferation was similar in each of the groups treated with BOP. Hamsters in group 1 (CCK then BOP) developed significantly fewer of these proliferative lesions than BOP controls, and those in groups 1 (CCK then BOP) and 2 (CCK with BOP), significantly fewer than group 3 (BOP then CCK). The incidence of pancreatic adenocarcinoma was significantly less in groups 1 (CCK then BOP) and 2

(CCK with BOP) than the BOP only control group (group 5). Group 3 hamsters (BOP then CCK) had the highest incidence and multiplicity of malignant lesions comparable to the BOP only group. Pour concluded that the results of experiment 1 supported the concept that CCK administration did interfere with chemical carcinogenesis in the hamster model. His group suggested that CCK administration might increase the elimination of circulating and intracellular BOP resulting in tumour inhibition if the CCK was given before and with BOP, but not if given after BOP. As an alternative explanation, it was suggested that CCK might temporarily stimulate intracellular cytochrome P-450 with resulting N-demethylase, O-dealkylation of carcinogen as has been demonstrated in rats (Fang & Stroebel, 1981). In the second experiment, the incidence of all types of lesion was similar in all BOP treated groups. Pour was unable to satisfactorily explain this observation. A subsequent paper by Meijers and colleagues (1990) supported the findings of Pour et al's 1988 paper. They administered BOP (20mg/kgBW) at six, seven and eight weeks of age to male Syrian golden hamsters and subsequently injected the animals with gelatin vehicle, CCK-8 (2.5ug/kgBW), CR-1409 (a potent CCK-receptor antagonist) or CCK-8 with CR-1409 in a regime similar to that used in this thesis. Although the dose of CCK-8 used was three times that used in this thesis, Meijer's group also

failed to detect any effect of CCK-8 on the incidence of pre-neoplastic and neoplastic pancreatic lesions after 16 weeks of treatment.

It is interesting to note that Johnson et al (1983) administered CCK before or with BOP and reported inhibition of tumour development, whereas Howatson and Carter (1985) and Andren-Sandberg and colleagues (1984) administered the CCK or caerulein after the BOP and reported no such inhibition. In the current study CCK-8 was given both before and after BOP administration making interpretation of our results, in the light of Pour et al's 1988 paper, difficult. It remains possible that the CCK-8 dose administered before the BOP was injected was the only relevant dose of hormone given. Given that the control group in the current study did not develop any frankly invasive adenocarcinomata after 15 weeks of BOP administration it is difficult to assess whether CCK-8 inhibited development of malignant change, as Pour suggested it might.

Unlike the CCK-8 group, the secretin-treated animals in the current experiment did exhibit an increase in the development of pancreatic adenocarcinoma compared to the control group. In another paper published in 1989, Pour & Kazakoff repeated the CCK experiment described above with secretin as the trophic stimulus. They expected greater inhibition of pancreatic carcinogenesis when

secretin was administered before BOP than when CCK was so administered. Not surprisingly this was indeed what they reported. Hamsters injected firstly with secretin and then with BOP developed very few pancreatic carcinomata compared with the control group ($p<0.0005$). Secretin similarly inhibited the development of pancreatic carcinomata when administered with BOP, albeit to a lesser degree, as might be expected from the earlier CCK experiment results.

The most interesting and unexpected finding in the current study was that SMS 201-995 seemed to promote pancreatic carcinogenesis when administered in low dosage. The explanation for this might be as discussed in the previous paragraph. There can be no doubt, however, that both the number of animals in whom malignant change occurred in the pancreas, and the extent of this malignant change was much greater in animals given low dose SMS.

If SMS 201-995 exerts an inhibitory effect on intracellular BOP metabolism in just the opposite way that Pour suggested that CCK and secretin might increase intracellular BOP metabolism, then perhaps this result should not be unexpected. There is no published work on somatostatin and its analogues looking at this particular point.

Somatostatin analogues exhibit species specific effects on pancreatic tumours and varied efficacy

within species. In 1984, Redding and Schally investigated the effects of three somatostatin analogues on transplanted acinar-cell pancreatic cancer DNCP-322 (CA-20948) in Wistar/Lewis rats and on transplanted hamster ductal pancreatic cancer in Syrian golden hamsters. They reported that in rats, one analogue ([L-5-Br-Trp^8]-somatostatin) inhibited the growth of the transplanted tumour, while the other two analogues (cyclo Pro-Phe-D-Trp-Lys-Thr-Phe and somatostatin-28) had no effect. In the hamsters, only the somatostatin-28 had no effect. The behaviour of these transplanted tumours may differ from that of our own experimental pancreatic tumours in situ, and in addition, SMS 201-995 may simply not have the inhibitory effect of other analogues of somatostatin in the hamster.

The cyclical somatostatin octapeptide analogue RC-160 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2) when administered as a microcapsule designed to release 5ug analogue each day, inhibited the progression of established chemically induced hamster pancreatic cancer in hamsters (45 day survival in the treated group 70% vs 35% in the control group) (Paz-Bouza et al, 1987). Continuous release of this analogue might account for tumour inhibition not seen in our study where the dose of analogue was both smaller and intermittent. More recent investigations using microcapsules which released 10ug/day (Zalatnai &

Schally, 1989a), 15ug/day (Szende, Zalatnai & Schally, 1989) and 25ug/day (Zalatnai & Schally, 1989b) of RC-160 have confirmed the initial findings of Paz-Bouza's investigations. In Szende's paper, RC-160 treated tumours exhibited striking regressive changes characteristic of programmed cell death. In another paper from the same group, treatment with RC-160 was shown to result in down-regulation of cellular receptors for insulin-like growth factor and a decrease in binding capacity (Szende et al, 1990).

Upp and colleagues (1988) found that SMS 201-995 inhibited the growth of two human pancreatic cancers transplanted into nude mice. The SMS 201-995 was administered in a dose of 100ug/kg body weight three times daily, a much higher dose than that used in our study. In addition, human tumours may be more susceptible to the effects of SMS 201-995 than hamster tumours. However, it is interesting to note that in a recent publication, none of 12 human pancreatic cancers examined demonstrated the presence of somatostatin receptors (Reubi et al, 1988). It is of course possible that SMS 201-995 exerted its effect indirectly through other hormones rather than directly on the pancreas.

Differences in response to various somatostatin analogues might be explained by the findings of Liebow and colleagues (1989). In a study of the human pancreatic cancer cell line Mia PaCa-2, this group

demonstrated that inhibition of tumour growth was related to cellular tyrosine phosphatase activity affecting the epidermal growth factor receptor. The somatostatin analogue RC-160 caused the greatest stimulation of tyrosine phosphatase activity and the greatest inhibition of tumour cell growth. SMS 201-995 stimulated virtually no tyrosine phosphatase activity and did not inhibit tumour cell growth.

The increase in development of pancreatic adenocarcinoma seen in the CCK-8/SMS-LD and secretin/SMS-HD groups in this thesis is more difficult to explain than any of the other findings. It is possible that in the CCK-8/SMS-LD group the increase in tumour development was due to the effects of the SMS over CCK-8 and similarly, in the secretin/SMS-HD group to the effects of the secretin. This is difficult to accept given that SMS was administered in both groups, although the higher dose of SMS did not seem to exert any effect on carcinogenesis when administered alone.

To date there has been only one published paper on the use of SMS 201-995 as a possible treatment for pancreatic cancer in man (Klijn et al, 1990). Fourteen patients with metastatic pancreatic cancer were injected SC three times daily with 100-200ug of SMS 201-995. Only three patients exhibited stable disease while in the remainder, disease progression was reported. The median survival for the pancreatic

cancer patients was only two months.

Somatostatin is a complex hormone and its many analogues possess different spectra of activity. The effects of SMS 201-995 alone and its interaction with other peptide hormones are obviously complex. Superficially, the current results indicate that in the hamster-nitrosamine model of pancreatic cancer, in certain situations and doses, SMS 201-995 can promote pancreatic carcinogenesis. This effect might be dose dependent and seems to be independent of the effects of other gastrointestinal peptide hormones. Caution must be exercised before extrapolating the results of this thesis to the human situation but at present more evidence for the potential benefits of analogues of somatostatin is necessary before their use in treating pancreatic cancer in man can be justified.

CHAPTER 6

Analysis of the DNA content and cell cycle activity of BOP-treated hamster pancreas using flow cytometry and bromodeoxyuridine immunostaining.

6.1 Introduction

The results of the experiments in the preceding two chapters of this thesis suggest that exogenously administered gastrointestinal hormones, or their analogues, can influence chemically induced pancreatic carcinogenesis when administered in doses which seem to have no detectable effect on the pancreas with respect to total DNA content and pancreatic wet weight. This suggests that, in order to influence carcinogenesis, hormone administration must occur when the pancreatic cells susceptible to the effects of BOP are at their most vulnerable, or alternatively, that BOP has more, or less, of an effect when pancreatic cells are under the influence of the hormones.

In 1988, Lawson and Nagel reported that BOP produced cellular DNA damage in the hamster pancreas; ductal cells were more susceptible to the effects of BOP than acinar cells largely as a result of a slower DNA-repair mechanism. Such BOP-induced damage was also demonstrated in rat pancreas, but in the rat the ability of cellular DNA to undergo rapid repair was much greater than that seen in the hamster, perhaps accounting, at least in part, for the seeming species-specificity of BOP with respect to pancreatic carcinogenicity.

The technique of flow cytometry has been increasingly investigated in recent years. It provides a rapid and objective method of analysing

various cellular substances, such as DNA, using specific fluorochrome stains. The stained particles suspended in fluid are passed across a source of fluorescent light of appropriate wavelength one at a time. This light source is usually a laser. The stain is stimulated to fluoresce by the laser light and the amount of fluorescence emitted by each cell or particle is measured by a sensitive photoelectric cell. Computer storage of the fluorescence values for each particle allows subsequent display of the results in a histogram. The amount of fluorescence generated within each particle is proportional to the amount of DNA or other substance being measured (Koss & Greenbaum, 1986; Quirke & Dyson, 1986).

Tissue is prepared in such a way as to produce suspensions of single cell nuclei. Fresh tissue provides the best and most consistent results due to the fact that there is little background noise generated by cellular fragments. Despite this, meaningful results can be obtained from frozen or paraffin embedded tissue (Hedley et al, 1983; Oud et al, 1986). Normal human cells display a single histogram peak corresponding to the amount of DNA in the normal number of 46 chromosomes (diploid). Many malignant cells have been shown by flow cytometry to exhibit abnormal amounts of DNA in relation to the normal diploid amount (aneuploidy) and this may relate to gene deletions and other genetic changes associated

with tumour progression. In recent years, there has been considerable interest in the DNA content of cells in different forms of cancer. Tumours of many different types have been found to contain aneuploid clones (Friedlander, Hedley & Taylor, 1984). The significance of differences in tumour cell ploidy with respect to outcome in individual patients has been the subject of considerable debate. For example, in colorectal cancer, some authors have indicated that tumour cell ploidy was the single most important determinant of outcome of many pathological and clinical variables studied (Kokal et al, 1986). Others have suggested that cell ploidy status has no prognostic significance, although patients with Duke's stage B aneuploid tumours tended to have a poorer prognosis than those with diploid tumours (Jones, Moore & Schofield, 1988). The situation with respect to cancer of the pancreas in humans is unclear, partly because of the consistently short time course of this disease from presentation to death.

Computer analysis of flow cytometry histograms can also be used to examine the cell cycle. After mitosis (M), a new cell cycle begins. The largest number of cells in any tissue are in the resting phase (G_0). Newly dividing cells then enter the G_1 phase during which the standard diploid complement of DNA is present (2N). Cells can remain in this phase for a variable length of time. Cells which pass through the

G_1 phase then enter the phase of DNA synthesis (S-phase), during which nuclear DNA content is doubled (4N). Cells then enter a relatively short (G_2) phase during which RNA and protein synthesis occur. Mitosis then results in cell division and the production of two daughter cells (Sugarbaker et al, 1979). In a normal flow cytometry histogram (Figure 6.1), the tall narrow peak represents cells in the G_0/G_1 phases of the cell cycle, the second smaller peak represents cells in the G_2 and M phases and the space between these two peaks represents cells in S-phase.

In the nitrosamine-hamster model of pancreatic carcinogenesis, BOP is thought to produce both cell death and sublethal cell injury. This is followed by a period of DNA synthesis reflecting DNA repair and cellular regeneration in cells which survived the lethal effects of BOP (Lawson & Nagel, 1988). Changes in cellular DNA content during the carcinogenic process induced in the hamster pancreas by BOP should be detectable using flow cytometry.

Bromodeoxyuridine (BRDU), a thymidine analogue, is taken up into cells during DNA synthesis. A monoclonal antibody has been developed which has led to the development of an immunoperoxidase technique which will label BRDU. This technique allows the identification of which cells have synthesized DNA, that is have incorporated BRDU into nuclear DNA, during a set time period and would identify which

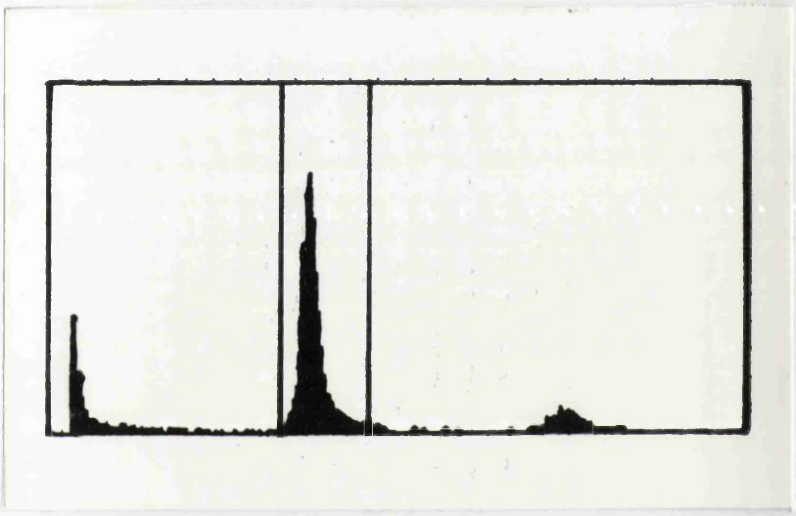

Figure 6.1: Flow cytometry histogram of nuclear DNA in a normal hamster pancreas. The tall narrow peak represents DNA in cells in the G_o/G_1 phases of the cell cycle and accounts for 84.2% of nuclei. The small peak to the right of the G_o/G_1 peak represents DNA in cells in the G_2/M phases of the cell cycle (8.1% of nuclei) and the space between these two peaks, cells in the S phase (7.6% of nuclei).

cells are in or have gone through the S-phase of the cell cycle.

In the light of the results of the preceding chapters of this thesis, it was decided that a final aim should be included in the investigation. Using flow cytometry and BRDU immunostaining, an attempt was made to detect changes in cell cycle activity that occurred in hamster pancreas in response to administration of BOP, which might indicate cellular DNA damage and repair and cellular proliferation. Flow cytometry would identify whether any changes in cell cycle activity occurred in response to BOP and BRDU immunostaining would indicate in which cell types these changes occurred. In addition, an attempt would be made to detect aneuploid cell lines during the carcinogenic process and to determine whether aneuploidy could be ascribed to any particular stage of, or lesion seen during, the carcinogenic process.

6.2 Materials and Methods

The animals used in this study were 8-10 week old male Syrian golden hamsters (WO GD Strain, Wrights of Essex), caged in groups of five in standard animal unit conditions with a 12 hours light/dark cycle and 21% humidity, and fed standard small rodent diet with water ad libitum.

In the first part of the study, 40 hamsters were injected subcutaneously with the carcinogen BOP in a dose of 5mg/kgBW per week. Animals were sacrificed in groups after 5, 10, 15 and 20 weeks and after a full post-mortem examination the pancreas was excised. After trimming of fat and extraneous connective tissue each pancreas was cut into twelve blocks. Five blocks were snap-frozen in liquid nitrogen and the remaining seven blocks fixed in PLPD (paraformaldehyde, lysine, sodium periodate and potassium dichromate) for subsequent staining with haematoxylin and eosin and histological examination. Two further animals were sacrificed prior to the injection of any substance to act as controls for the flow cytometer. Another untreated hamster pancreas was allowed to lie in air over a period of time; pancreatic tissue samples were taken at various time points and analysed to ensure that the results in the BOP treated animals did not simply reflect autolysis of hamster pancreas.

Each frozen tissue block was thawed and processed to produce single nuclear preparations

(Vindelov, Christensen & Nissen, 1983). Nuclei were stained with propidium iodide and passed through an EPICS CS flow cytometer (Coulter, UK). The EPICS Easy-2 programme was used for histogram analysis. Most of this analysis was carried out by Mr. Eric Miller. A frozen section was prepared from each block analysed in this way for staining with haematoxylin and eosin. Sections were examined for the presence of malignant and premalignant lesions. Growth fraction results were compared using a Mann Whitney U test for non-parametric data.

In the second part of the study a different group of hamsters was injected with either BOP (5mg/kgBW per week) or an equivalent volume of the BOP solvent (normal saline). Animals were sacrificed after five and ten weeks of treatment. Twenty hours prior to sacrifice hamsters were injected intraperitoneally with BRDU (200mg/kg body weight; Sigma Chemical Company Ltd., Dorset, UK). At sacrifice, a full post-mortem examination was performed and the pancreas was excised and trimmed. Five blocks were snap-frozen in liquid nitrogen and the remaining seven blocks fixed in methacarn (methacarn fixation allows for better staining with BRDU than PLPD). After fixation, 2um step sections were cut from each block. Each section was stained using monoclonal anti-BRDU (Becton Dickenson, UK). An avidin-biotin-peroxidase (Dako Ltd., UK) method was

used to detect anti-BRDU staining. Histological sections were examined and assessed for anti-BRDU staining. A qualitative assessment was made of the presence of BRDU-positive nuclei in ductular, acinar, inflammatory cells and 'pre-neoplastic' lesions.

6.3 Results

During the first experiment one hamster was sacrificed under the terms of the animal licence due to weight loss in excess of 10% in one week. This animal is not included in any subsequent analysis. Five animals were sacrificed after five weeks of BOP treatment, 10 after 10 weeks, 15 after 15 weeks and nine after 20 weeks. Hamster pancreata exhibited the expected histological changes throughout the 20 week experimental period, described in Chapter 2 of this thesis.

The flow cytometry results are shown in Table 6.1. In total, 210 tissue blocks from 42 animals (excluding the pancreas left to stand over time) were analysed using flow cytometry. Control pancreas exhibited a growth fraction (G_2+M plus S-phase fractions) of 8.4 $\pm$ 2.1% (mean $\pm$ standard deviation). This increased to 17.5 $\pm$ 2% ($p<0.02$ compared to five week group) by 10 weeks and remained high in the 15 and 20 weeks groups (20.0 $\pm$ 4.6% and 21.2 $\pm$ 3.1%; both $p<0.01$ compared to the five week group). The S-phase fraction increased over control values after 5 weeks of BOP treatment whereas the G2 + M fraction did not show any obvious increase over the controls until after 10 weeks of BOP treatment. The half peak coefficient of variation was less than 3% for all flow cytometry histograms.

Table 6.1: Flow cytometry analysis of BOP treated hamster pancreas

Duration of treatment (weeks)	S-phase	G2 + M	GF	n
0	3.6 ± 0.9	4.9 ± 0.2	8.4 ± 1.1	2
5	8.9 ± 1.9	4.1 ± 1.0	12.9 ± 2.7	6
10	10.1 ± 1.9	7.4 ± 1.4	17.5 ± 2.0	10
15	8.6 ± 2.3	11.3 ± 3.8	20.0 ± 4.6	15
20	12.0 ± 1.8	9.2 ± 2.1	21.2 ± 3.1	9

(Excludes 7 aneuploids with DNA indexes <1.3)

(S-phase = S-phase fraction (%);
G2+M = G2+M fractions (%);
GF = growth fraction (%) (S-phase + G2+M);
n = number of hamster pancreata)

The pancreas left to stand in air and analysed at various time intervals in a 24 hour period did not show any changes in cell cycle activity with time.

In only seven of the 210 samples analysed were aneuploid peaks identified. These had DNA indices <1.3 and represented only approximately 10% of nuclei in each case which is at the limit of detection for the flow cytometer used. These peaks were seen from 5 weeks BOP treatment and there was no relationship to histological appearances as assessed by frozen section histology. Their significance is uncertain.

In the second phase of this study using BRDU immunostaining, 20 hamster pancreata were examined; five in each group after five weeks of treatment and five after 10 weeks. As expected, sections with pre-malignant lesions exhibited a marked inflammatory response. Some inflammatory cells were BRDU labelled. In general there was only infrequent positivity in cystic complexes (Figure 6.2). More nuclei were BRDU positive in areas of ductal hyperplasia (Figure 6.3) and ductal dysplasia (Figure 6.4).

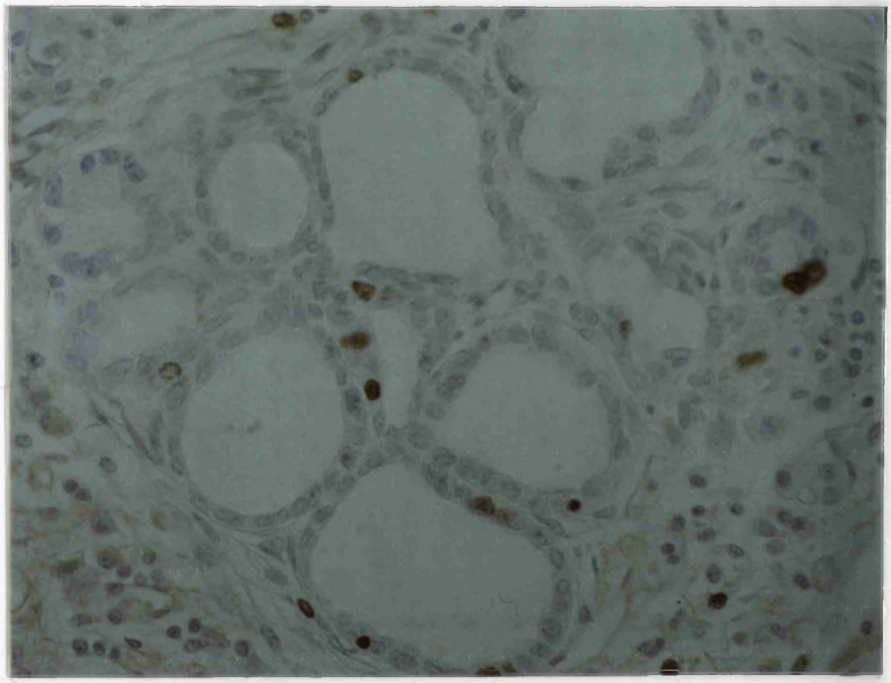

Figure 6.2: Bromodeoxyuridine immunostaining in a cystic complex in hamster pancreas after 10 weeks of treatment with BOP. Labelled cells stain brown. (x140 magnification).

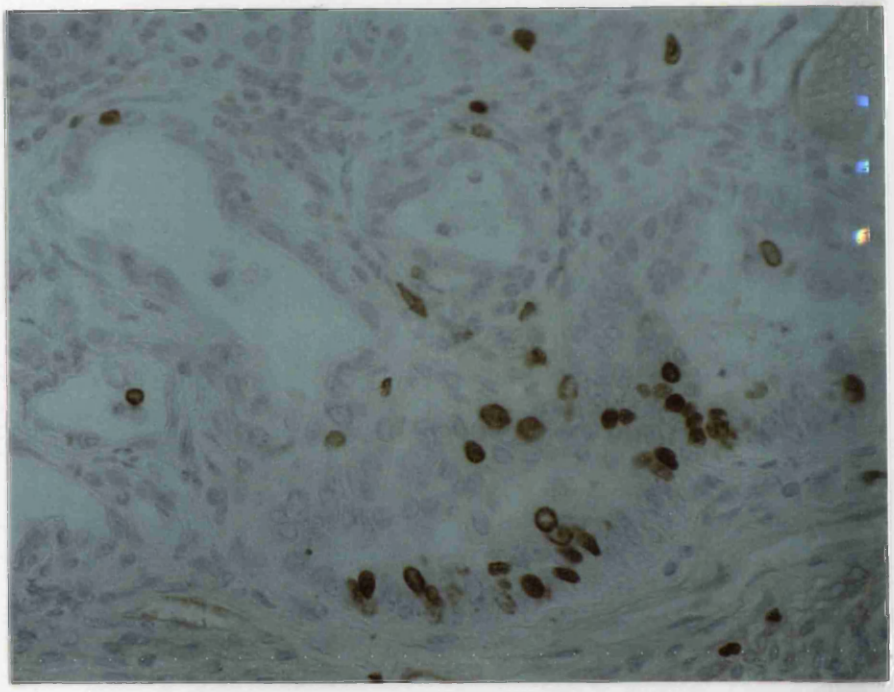

Figure 6.3: Bromodeoxyuridine immunostaining in an area of ductal hyperplasia adjacent to a small cystic complex, in hamster pancreas after 10 weeks of treatment with BOP. Labelled cells stain brown. (x140 magnification).

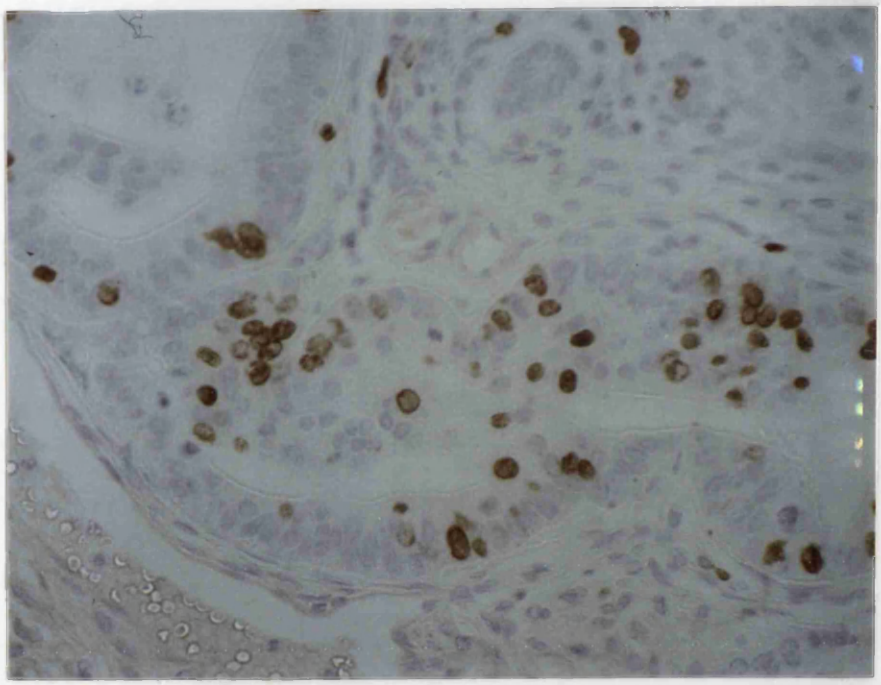

Figure 6.4: Bromodeoxyuridine immunostaining in an area of ductal dysplasia in hamster pancreas after 10 weeks of treatment with BOP. Labelled cells stain brown. (x140 magnification).

6.4 Discussion

The flow cytometry results in this study indicate that BOP produces changes which result in an increase in cell cycle activity in the hamster pancreas. Proliferation occurred in duct, acinar and inflammatory cells and was not necessarily related to 'pre-neoplastic' histological changes. Furthermore, maximal proliferation was established by 5 weeks, before any recognisable pre-neoplastic lesions were identifiable.

In 1979, Ames, interpreting the results of a number of investigations into the effects of carcinogens on bacteria, strongly suggested that carcinogens were mutagenic, i.e. that carcinogens resulted in increased cell division or proliferation which resulted ultimately in the development of malignant tumour. Unfortunately, it is now generally believed that this observation oversimplifies the situation in mammalian cells. Carcinogen-induced mutagenesis is considerably more difficult to achieve in mammalian cells and may be an important rate limiting step in malignant progression. The multistage theory of cancer causation suggests that malignant change requires the influence of an initiator or initiators, and a promotor or promotors, which stimulate proliferation of the altered cell(s) (Weinstein, 1991). In the hamster model, it seems reasonable to conclude, on the basis of the results of

this thesis and many other publications that BOP is the initiator of the carcinogenic process. It also acts as a promotor to some extent although some of the hormones used in experiments in previous chapters of this thesis may be more important tumour promotors even under physiological conditions. The absence of any effect of these hormones on total pancreatic DNA content (Chapter 4) would suggest that any promotional effect is subtle and need not result in gross changes in pancreatic composition or increase in tissue mass.

The flow cytometry results in this chapter are interesting. BOP produces pancreatic cell death and this is followed by regeneration of surviving tissue resulting in a proliferating population of cells. Many of these cells are acinar or inflammatory and therefore are probably not related to tumorigenesis. However, given that proliferating cells are prone to undergo spontaneous mutation and that ductal cells may have less effective DNA repair mechanisms, it is possible that mutagenic injury has occurred leading to tumour initiation. The cytometer may be detecting the whole population of proliferating cells and cannot distinguish between simple regeneration/proliferation and potentially tumorigenic proliferation. Aneuploidy might have been uncommon because neoplastic changes were still at an early stage. Interestingly, recent flow cytometric analyses of human pancreatic cancers have indicated that the majority of such tumours

analysed exhibit aneuploidy, and that the tumours with aneuploid nuclear DNA had the worst prognoses (Alanen et al, 1990; Wido et al, 1990; Weger et al, 1991).

In a recent publication looking at diethylnitrosamine-induced hepatic carcinogensis in Sprague-Dawley rats, aneuploidy occurred in altered hepatic foci before any histological evidence of malignancy could be detected (Wang et al, 1990). This observation indicated that, in rat liver at least, aneuploidy may be an early phenomenon resulting from sublethal cell injury, DNA repair and subsequent proliferation of altered cells. The failure to detect significant aneuploid cell lines in the current study may be due to the inability of the flow cytometer to distinguish small foci of aneuploid cells in the overwhelming number of diploid cells. Certainly, Wang's results in rat liver were obtained by the technique of microspectrophotometric measurement which allowed DNA analysis of tiny areas of liver tissue which had undergone histologically detectable changes in appearance, generally accepted as being pre-malignant (altered hepatic foci).

It seems likely, therefore, that cell proliferation is an important factor in BOP-induced neoplasia. However, using conventional techniques we have not been able to distinguish between repair/regeneration particularly in acinar cells where there is not thought to be a risk of neoplasia in the

hamster, and neoplastic proliferation in ductal cells which represents the early stages of tumour initiation and promotion. To address this problem further will require the use of molecular techniques to study the control and regulation of specific genes thought to be involved in the development of neoplasia.

CHAPTER 7

Conclusions

The exact aetiology of pancreatic cancer in man remains obscure. Little progress has been made in elucidating the causes of this dismal disease in the last five years although further epidemiological evidence in support of some of the previously discussed risk factors has been forthcoming.

Some early studies, cited in Chapter 1 of this thesis, suggested that diet played an important part in increasing susceptibility to the development of pancreatic cancer. More recently, there have been a number of studies published which have added to the evidence in support of this assertion. In an epidemiological study of 247 patients with pancreatic cancer from Northern Italy, LaVecchia and colleagues (1990) noted that there was a tendency to a decreased risk of pancreatic cancer with increasing consumption of fruit. Another group from the Netherlands noted that risk was diminished with total cooked vegetable consumption (especially cruciferous vegetables) and fresh vegetable consumption (Bueno de Mesquita et al, 1991). Consumption of eggs and fish increased risk in this study. In a study of 249 patients with pancreatic cancer, Howe, Jain and Miller (1990) reported that pancreatic cancer risk was positively associated with total caloric intake, particularly in relation to carbohydrate consumption. Fibre intake from fruit, vegetables and cereals was inversely associated with risk in this study.

Exactly how diet influences susceptibility to the development of pancreatic cancer remains unclear. A high dietary intake of fresh fruit and vegetables seems to reduce risk. Given that nitrosamines can induce pancreatic cancer in experimental animal models, it seems reasonable to suggest that this protective effect might come about through a reduction in, or reversal of, gastric nitrosation reactions caused by high levels of vitamins C and E in fruit and vegetables. Whether dietary fibre intake can be implicated in a similar way is not clear.

Similarly, it remains open to speculation whether dietary factors implicated in increasing risk act via their effect on gastrointestinal hormone release. A recent publication has certainly suggested a role for gastrin and perhaps CCK in increasing susceptibility to pancreatic cancer (Borch et al, 1988). A group of 361 patients with pernicious anaemia were followed up over a seven year period. Patients in the study group exhibited an increased incidence of both gastric and pancreatic cancer when compared to age and sex-specific incidence rates in the general population. Hypergastrinaemia resulting in both gastric and pancreatic hypertrophy was suggested as a possible explanation for these findings although the authors also speculate that increased nitrosation in the hypochlorhydric stomach might be an alternative, and perhaps more reasonable, explanation.

Interestingly, in an addendum to the paper, the authors reported that serum CCK levels in 68 of their pernicious anaemia patients were significantly increased compared to a small group of control patients (although caution has been expressed regarding possible crossreactivity with gastrin in the assay; personal communication, I. Ihse).

Coffee consumption has been shown to confer no increase in risk of pancreatic cancer in three recent studies (Farrow & Davis, 1990; Ghadirian, Simard & Baillargeon, 1991; Jain et al, 1991). Similarly, alcohol has recently been shown to confer no increase in risk (Bouchardy et al, 1990; Farrow & Davis, 1990; Jain et al, 1991). Indeed Farrow's group suggested that daily white wine consumption was protective and Ghadirian et al (1991) suggested that those who consumed alcohol were generally at lower risk than non-drinking controls. Cuzick and Babiker (1989) noted in a study of 216 pancreatic cancer patients that cases drank significantly more beer than controls and that there was evidence of a positive trend in risk with total alcohol consumption.

Further investigations into genetic changes which occur in pancreatic cancer have been reported in the last five years. The initial findings of Hirai and colleagues (1985), demonstrating activated c-Kirsten-ras (c-K-ras) oncogene in a pancreatic cancer cell line, and Yamada et al (1986) reporting the

finding of a point mutation at codon 12 in the same oncogene have been supplemented by other similar publications. In 1988, a group of investigators from New York and California reported that 21 of 22 human pancreatic cancers analysed exhibited a point mutation at codon 12 of the c-K-ras oncogene (Almoguera et al, 1988). In seven of the tumours analysed, the reported codon 12 mutation was noted in both the primary tumour and in metastases, suggesting that early c-K-ras mutational activation was an early event in the development of exocrine pancreatic cancer. Smit and colleagues (1988) from the Netherlands reported similar findings in 28 of 30 human pancreatic cancers analysed. They reported that the mutations were predominantly guanine-thymidine transversions, unlike colon cancer where similar mutations have been reported as predominantly guanine-adenine transitions. Aqueous extracts of cigarette tar have been shown to cause single strand breaks in DNA due to a reduction of oxygen to superoxide and hydroxyl radicals (Borish et al, 1987). It is possible that such DNA damage could be the initial step in altering the pancreatic cellular genome to produce mutations in c-K-ras.

Cigarette smoking certainly remains the only convincing aetiological factor for the development of pancreatic cancer. A group from Toronto in Canada reported that cigarette smoking increased the relative risk of dying from the disease in a dose-dependent

manner (Howe et al, 1991). Cessation of smoking resulted in the increased risk falling to that of a non-smoking population in 10 to 15 years. Cigarette smoke contains many different nitrosamines. It is interesting to note that until recently none of these nitrosamines could induce pancreatic cancer in animal models. In 1988, a group of investigators from New York reported that the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a constituent of cigarette smoke, induced both benign and malignant neoplasms in rat pancreas (Rivenson et al, 1988). The malignant tumours were duct-like in morphology, including, in one animal, areas of epidermoid, keratin-generating tissue. The major metabolite of NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) also produced pancreatic tumours in the rat when administered subcutaneously, and the authors suggested that this substance might be the proximate carcinogen affecting the pancreas.

The observation that a nitrosamine derived from cigarette smoke can induce pancreatic cancer in a rodent model underlines the importance of Parviz Pour's BOP-hamster model of pancreatic cancer. In this model, a nitrosamine administered in regular low dosage to the Syrian golden hamster, induces pancreatic cancer almost exclusively. Clinically, the chemically induced tumour has similar effects in the hamster to pancreatic cancer in man. Histologically,

the induced tumour has a ductal or ductular morphology and resembles the tumour seen in man; it also possesses the ability to metastasize.

The first aim of this thesis was to successfully establish Pour's model of pancreatic carcinogenesis in our laboratory. This was achieved without undue difficulty. The histological appearances of the pancreata after different durations of BOP injection, were similar to those described by Pour and other workers in many previous publications. Concern over possible excessive morbidity in the hamsters due to repeated subcutaneous injections of BOP prompted a comparison of the effects of SC and IP BOP. The only morbidity experienced was in the IP group where a number of animals were sacrificed due to accidental injection of carcinogen into testes which had been retracted into the abdomen. The pattern of histological changes in the hamster pancreata in both groups was similar, although after 20 weeks of treatment, changes in the pancreata of IP injected hamsters were generally less extensive than those in the SC group, particularly with respect to the development of the premalignant lesions, ductular carcinoma-in-situ and microcarcinoma, and adenocarcinoma itself. The reason for these discrepancies might lie in differences in the predominantly hepatic metabolism of BOP; SC injection would result in chemical appearing at the liver in the

systemic circulation, whereas IP injection might result in a higher proportion of chemical appearing in the portal circulation with subsequent differences in cellular metabolism.

Previous publications have demonstrated that pharmacological doses of gastrointestinal hormones can influence pancreatic exocrine secretion both in man and in experimental animal models. The second aim of this thesis was to investigate the effects of intravenously infused physiological doses of secretin, CCK-8 and SMS 201-995 on pancreatic juice output and, where possible, composition, in the Syrian golden hamster. Intravenous infusions of increasing doses of secretin and CCK-8 increased pancreatic juice output and altered pancreatic juice composition with respect to protein and bicarbonate content. SMS 201-995 suppressed pancreatic juice output: the small volumes of pancreatic juice thus obtained made meaningful analysis of composition unfeasible.

Pancreatic juice output was suppressed during infusion of SMS 201-995 and remained suppressed during subsequent infusion of CCK-8. Increased pancreatic juice output stimulated by infusion of CCK-8 was suppressed by subsequent infusion of SMS 201-995. These results suggest that SMS 201-995 could overcome the stimulatory effects of CCK-8. The exact mechanism whereby SMS 201-995 achieves this result is not clear but may involve diminution in pancreatic blood flow

(Conway, Djurlein & Prinz, 1988), reduction in pancreatic insulin secretion which is thought to play an important role in regulating pancreatic exocrine enzyme production and secretion (Muller et al, 1988), through inhibition of secretion of other gastrointestinal hormones, by direct interference with cellular membrane bound CCK receptor sites or by stimulation of inhibitory intracellular messenger systems. The interaction between infused secretin and SMS 201-995 was similar to that seen with CCK-8 and SMS 201-995, although higher doses of SMS 201-995 were required to overcome the stimulatory effects of secretin. Secretin and somatostatin are thought to share the same intracellular messenger system, cyclic AMP, whereas CCK is thought to act via the diacylglycerol intracellular pathway. This might explain the slight differences in the results seen in this part of the study.

Subcutaneous administration of CCK-8, secretin, SMS 201-995 and combinations of SMS 201-995 with the other two hormones did not influence pancreatic wet weight or pancreatic DNA content after one or six weeks of treatment. In the light of previously published work, particularly with respect to secretin and cholecystokinin and analogues, this seemed initially to be a surprising finding. Unfortunately, considerable variations in the doses and preparations of hormones used in these papers makes direct

comparison of results difficult. It is conceivable that the doses of hormones used were too small to have an effect on pancreatic trophism. Similarly, the intermittent dosing schedule used could have allowed pancreatic cells to recover from the effects of the hormone prior to sacrifice of the hamsters and analysis of the pancreas.

The final initial aim of this thesis was to determine the effects of subcutaneous injections of the hormones, in the doses used in the earlier experiments, on BOP carcinogenesis. CCK-8 failed to promote pancreatic carcinogenesis as had been expected from earlier published work with CCK and other CCK analogues. Subsequent publications have reported conflicting results with a number of CCK preparations; some have promoted carcinogenesis while others have not, and in some cases have in fact inhibited carcinogenesis. The timing of carcinogen injection in relation to administration of the hormone has been thought to be critical in explaining these conflicting results.

The most interesting finding of the experiment reported in Chapter 5 of this thesis was that at the lowest dose regime used, SMS 201-995 administration increased the number of animals affected by pancreatic adenocarcinoma and the premalignant lesions, ductular carcinoma-in-situ and microcarcinoma. In addition, the extent of the malignant change seemed to be more

extensive in this group compared to all the other experimental groups. These results conflict, to a degree, with a number of papers which have reported that some somatostatin analogues can inhibit the growth of established pancreatic cancers (Paz-Bouza et al, 1987; Zalatnai & Schally, 1989a & 1989b; Szende, Zalatnai & Schally, 1990). These reports have used continuous release preparations of somatostatin analogues in pharmacological doses; as a consequence the results reported might be expected. Other groups have reported successful inhibition of the growth of transplanted human pancreatic cancers in nude mice using SMS 201-995 (Upp et al, 1988). In this study thrice daily injections of very large doses of analogue were used. Although the experiment in this part of the thesis was designed to look not at cancer treatment but at the early stages of cancer initiation/promotion, the results should alert investigators to the possibility that intraperson variation in response to treatment of human pancreatic cancer might, in the unlucky few, result in tumour promotion rather than inhibition. Our knowledge of the actions of somatostatin and its analogues is still grossly inadequate to suggest that this substance should be used to treat human pancreatic cancer.

It was interesting to note that alterations in carcinogenesis could be induced by doses of hormones which did not affect total pancreatic DNA content or

pancreatic wet weight. This prompted an additional experiment to look at the effects of BOP on pancreatic cell cycle activity using flow cytometry and BRDU immunostaining. BOP significantly increased cell cycle activity during the 20 week experiment. The percentage S-phase fraction increased after 5 weeks of BOP treatment whereas the percentage G_2/M fraction did not increase until 10 weeks. Added together, the percentage growth fraction exhibited a steady increase over 20 weeks of BOP treatment.

The increase in cell cycle activity demonstrated by flow cytometry indicated that cellular proliferation in the pancreas occurred in response to BOP administration. BRDU immunostaining indicated that this occurred predominantly in ductal and ductular cells. It remains unclear whether cellular proliferation occurred as a consequence of regeneration of cells which had survived the lethal effects of BOP or of replication of cells sublethally damaged by BOP which would become malignant lesions. Further investigation into the subcellular effects of carcinogens like BOP might help to explain why exogenously administered hormones can affect carcinogenesis without altering other aspects of cellular function.

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APPENDIX 1

DNA Assay - Preparation of Reagents

1. Phosphate Saline Buffer (PBS)

(a) Dissolve 35.814g of $Na_2HPO_4.12H_2O$ (MW 358.14g) into 1 litre distilled water (Solution 1).

(b) Dissolve 15.601g of $NaH_2PO_4.H_2O$ (MW 156.01g) into 1 litre distilled water (Solution 2).

(c) Take 810mls of Solution 1 and 190mls Solution 2 and make up to 2 litres with distilled water (Solution 3).

This provides 2 litres of 0.05M $NaPO_4$ Buffer solution.

(d) Add 4 moles of NaCl to Solution 3 (Solution 4).

(e) Adjust pH of Solution 4 to 7.4 with 4N NaOH solution.

(f) Take 1 litre of Solution 4 and add 0.744g EDTA (Solution 5).

(g) Readjust pH of Solution 5 to 7.4.

Keep Solution 4 (Reagent Buffer) and Solution 5 (Sample Buffer); Solutions 1 and 2 can be retained for later use.

2. Stock DNA Solution

(a) Dissolve purified calf thymus DNA in distilled water to final concentration of 1mg/ml.

(b) Make up DNA standards in the following concentrations:

500ng/ml	1ug/ml	1.5ug/ml	2ug/ml
2.5ug/ml	3ug/ml	3.5ug/ml	4ug/ml

3. Hoechst 33258 Reagent

(a) Dilute stock solution (200ug/ml) in distilled water to give final reagent concentration of 1ug/ml.

APPENDIX 2

Pancreatic wet weights in one-week treated animals.

Results are expressed as mg/100g hamster body weight.

Groups	Animals							
Control	366	385	365	383	398	421	328	345
CCK-8	321	362	418	366	320	371	379	340
Secretin	385	408	377	404	394	391	343	339
SMS-LD	315	367	374	384	409	379	328	341
SMS-HD	380	333	370	369	352	386	357	394
CCK/SMS-LD	430	348	431	429	362	362	389	328
Sec/SMS-HD	347	363	432	391	316	344	375	365

APPENDIX 3

Pancreatic wet weights in six-week treated animals. Results are expressed as mg/100g hamster body weight.

Groups	Animals							
Control	299	346	323	359	356	350	316	326
CCK-8	335	364	301	367	334	376	360	346
Secretin	369	363	352	297	332	351	333	327
SMS-LD	290	303	331	280	375	310	339	333
SMS-HD	342	317	373	325	302	295	336	334
CCK/SMS-LD	403	400	348	331	342	389	445	352
Sec/SMS-HD	380	372	357	367	382	504	392	332

APPENDIX 4

DNA assay results in one-week treated animals. DNA content values are shown for each pancreatic section analysed - three sections per animal pancreas, each sample paired. Results are expressed as ug DNA/100g pancreatic wet weight

	Run 1	Run 2	Run 3	Run 4
Control	588 619	586 612	652 628	529 564
	484 558	527 568	753 715	601 565
	513 531	538 578	671 609	514 537
CCK-8	544 571	693 700	752 671	477 564
	505 540	663 655	758 720	560 571
	574 530	656 692	743 752	508 522
Secretin	441 452	541 566	630 626	601 594
	530 514	560 581	525 501	550 554
	468 468	733 737	541 557	604 626
SMS-LD	411 428	627 600	693 738	474 509
	378 384	601 627	534 539	554 525
	435 572	568 556	597 568	521 532
SMS-HD	497 516	517 543	722 704	497 517
	536 524	502 511	571 559	500 575
	476 496	574 629	748 705	579 596
CCK/SMS-LD	576 587	578 599	589 578	549 538
	553 564	569 585	624 628	404 409
	522 528	646 675	667 635	504 512
Sec/SMS-HD	520 526	616 626	588 592	437 452
	543 543	653 667	586 558	559 575
	437 482	665 640	570 642	615 598

APPENDIX 4 (Continued)

	Run 5	Run 6	Run 7	Run 8
Control	541 549	694 740	457 493	552 561
	510 531	662 703	472 476	578 591
	521 513	622 665	502 526	581 588
CCK-8	542 545	594 603	499 515	608 618
	607 621	540 548	488 514	579 579
	500 524	547 568	411 427	603 611
Secretin	486 514	585 608	401 416	586 602
	- -	578 605	458 465	597 579
	536 548	612 638	437 437	604 588
SMS-LD	507 520	732 762	490 513	580 659
	502 514	685 693	477 492	612 638
	515 551	651 684	418 432	682 654
SMS-HD	524 571	552 567	495 495	568 586
	567 578	600 615	529 557	752 790
	456 445	642 651	436 436	580 635
CCK/SMS-LD	550 570	686 721	549 569	564 568
	505 522	639 648	453 468	672 681
	468 456	612 637	428 456	562 579
Sec/SMS-HD	540 540	660 666	435 465	608 624
	529 560	586 612	486 482	582 616
	438 492	598 617	498 505	590 590

APPENDIX 5

DNA assay results in six-week treated animals. DNA content values are shown for each pancreatic section analysed - three sections per animal pancreas, each sample paired. Results are expressed as ug DNA/100g pancreatic wet weight.

	Run 9	Run 10	Run 11	Run 12
Control	656 679	495 542	682 705	493 512
	678 703	418 430	568 577	443 459
	578 578	489 533	545 566	441 530
CCK-8	562 592	520 520	545 530	417 385
	650 650	519 547	609 618	502 468
	553 562	364 352	530 530	494 517
Secretin	589 631	525 559	606 602	493 507
	655 647	650 606	560 579	460 465
	578 601	518 545	630 630	489 482
SMS-LD	648 656	503 503	563 582	383 420
	438 433	495 466	540 551	378 394
	572 603	496 513	551 570	389 409
SMS-HD	493 488	535 520	608 583	495 472
	605 595	442 451	534 561	444 447
	637 650	398 402	580 597	440 432
CCK/SMS-LD	584 584	573 565	615 615	373 380
	580 567	572 549	684 696	418 434
	620 583	567 560	668 647	387 423
Sec/SMS-HD	524 419	582 591	601 620	420 444
	584 584	534 534	576 580	396 435
	610 624	522 533	602 624	441 445

APPENDIX 5 (Continued)

	Run 13	Run 14	Run 15	Run 16
Control	456 486	548 568	453 407	462 483
	587 604	502 524	532 532	459 442
	555 575	500 517	528 543	438 438
CCK-8	540 544	417 442	502 537	466 490
	586 610	536 521	511 498	512 500
	547 571	510 505	525 577	501 501
Secretin	504 518	659 672	522 522	499 518
	488 492	566 589	596 567	512 486
	524 524	607 636	580 560	476 509
SMS-LD	520 488	464 500	509 523	457 476
	489 496	522 548	503 503	499 513
	440 468	522 540	474 474	478 498
SMS-HD	535 543	427 415	565 578	474 452
	582 623	492 504	539 539	422 439
	624 620	466 497	498 514	448 469
CCK/SMS-LD	613 628	491 507	499 484	466 463
	576 608	458 470	538 553	425 457
	566 574	452 468	497 497	466 479
Sec/SMS-HD	509 522	370 340	548 528	507 516
	466 501	455 478	502 524	509 522
	460 484	560 574	537 544	492 476

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