JOHANNA LAUKKARINEN

The Effect of Thyroxine on Biliary Motility

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ACADEMIC DISSERTATION

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IV Laukkarinen J, Kööbi P, Kalliovalkama J, Sand J, Mattila J, Turjanmaa V, Pörsti I, Nordback I. Bile flow to duodenum is reduced in hypothyreosis and enhanced in hyperthyreosis. Neurogastroenterol Mot, in press.

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ABBREVIATIONS

ACAT	acylcholesterol acyl transferase
ACh	acetylcholine
ALP	alkaline phosphatase
ALT	alanine aminotransferase
Bil	total bilirubin
Ca	calcium
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium concentration
CBD	common bile duct
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
Crea	creatinine
DMSO	dimethyl sulfoxide
EDTA	ethylene diamine tetra-acetic acid
ERCP	endoscopic retrograde cholangiopancreatography
FT_4	free thyroxine
HDL	high-density lipoprotein
HDL-C	HDL-cholesterol
HDT	hilum-duodenum transit time
HIDA	diethyliminodiacetic acid
Hist	histamine
HMG-CoA	hydroxymethyl glutaryl-coenzyme A
i.v.	intravenous
K^+	potassium
KCl	potassium chloride
LDL	low-density lipoprotein
LDL-C	LDL-cholesterol
L-NAME	N_g-nitro-L-argine methyl ester

LRP	low-density lipoprotein receptor-related protein
MLCK	myosin light chain kinase
MMC	migrating myoelectric complex
NANC	non-adrenergic, non-cholinergic
NO	nitric oxide
NPY	neuropeptide Y
NS	not significant
P	phosphorus
PBS	phosphate buffered saline
PHI	peptide histide-isoleucine
PP	pancreatic polypeptide
PYY	peptide YY
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
SO	sphincter of Oddi
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
SR-BI	scavenger receptor BI
TBST	tris buffered saline + 0.05% tween 20
T_3	triiodothyronine
T_4	thyroxine
T-Chol	total cholesterol
TG	triglycerides
TH	thyroid hormone
TR	thyroid hormone receptor
TSH	thyroid-stimulating hormone i.e. thyrotropin
VIP	vasoactive intestinal polypeptide

INTRODUCTION

In western countries, 10 to 12 % of adults develop gallstones (Diehl 1991, Heaton et al. 1991, Kratzer et al. 1998). The prevalence of common bile duct (CBD) stones in patients with gallbladder stones varies from 8 to 16 % (Applemann et al. 1964, Jordan 1982). The pathogenesis of gallstones is a complex process involving factors affecting bile content and bile flow. A crucial factor in the forming of bile duct stones is biliary stasis (Thistle 1998), which can be caused by sphincter of Oddi (SO) stenosis, SO dyskinesia or bile duct strictures (Geenen et al. 1980, Osnes et al. 1981, Sandstad et al. 1994, Thistle 1998). Since Sandblom (1935) first demonstrated the hormonal action of cholecystokinin (CCK) on the SO in 1935, several other hormones have been shown to affect SO activity. Of the steroid hormone family, it has been suggested that oestrogen affects the motility of the SO in a prairie dog model (Tierney et al. 1993). However, the effects on SO contractility of thyroid hormones (TH), or of steroid hormones other than oestrogen, have not been studied.

In the course of clinical practice it was observed that a number of patients with CBD stones had hypothyroidism in their medical history. Because of the lack of previous studies on this particular topic, the possible relation between diagnosed hypothyroidism and CBD stones was investigated in a case-controlled retrospective study. A positive correlation was found which raised further questions as to the direct effect of thyroxine (T_4) on the SO, and a series of studies was therefore undertaken to further investigate the possible reasons for this clinical association.

REVIEW OF THE LITERATURE

1 Biliary anatomy

The hepatic ducts, cystic duct, gallbladder, CBD and SO constitute the biliary tract. Bile is secreted from the hepatocytes into canaliculi, which communicate with numerous interlobular ducts to drain into two main hepatic ducts. These main right and left hepatic ducts fuse into the common hepatic duct. The cystic duct from the gallbladder usually joins the common hepatic duct to form the CBD (Dowdy et al. 1962). As the CBD descends posterior to the first portion of the duodenum, lying in a groove either within or posterior to the head of the pancreas (Lytle 1959), it is joined by the pancreatic duct. The CBD and the pancreatic duct either merge into a common channel 3-8 mm in length (Sterling 1953) before entering the papilla of Vater (68-86%), or join without a common duct at the orifice of the papilla (6-22%), or have separate openings into the duodenum (8-10%) (Mann et al. 1920, Millbourn 1950, Allescher 1989). The human papilla of Vater is a small protrusion from the pancreatic border of the duodenum, usually at the junction of its second and third portions, and rarely exceeding 1cm in diameter (Becker 1993)

Smooth-muscle fibers surrounding the choledochoduodenal junction constitute the SO (Oddi 1887, Hendrickson 1898), which is divided into four sections: choledochal, pancreatic and ampullar sphincter and intermediate fibers (Boyden 1937 and 1957) (Fig. 1). In humans, the median intramural length of the choledochal sphincter is 14 mm and the median extramural length 5 mm (Teilum 1991). The lumen of the SO is characterized by a complex system of mucosal folds, which are packed so closely that they almost cover the whole lumen of the SO and produce an effective valve-like mechanism (Holle 1960, Allescher 1989). The SO is richly innervated by cholinergic, adrenergic and peptidergic neurons (Toouli and Baker 1991, Sand et al. 1993b and 1994). In addition, neural connections exist between the SO, gallbladder and proximal gastrointestinal tract (Müller et al. 1984, Grace and Pitt 1987, Padbury et al. 1993).

The principal parasympathetic and sympathetic extrinsic nervous pathway to the human SO is the nerve plexus surrounding the CBD. Parasympathetic connections at the choledochoduodenal junction are derived from both vagus nerves (Alexander 1940) and sympathetic connections from the left and right coeliac ganglia, and from the superior mesenteric ganglia, from which the nerve fibers continue as periarterial nerve plexuses to the SO. In addition, the SO area has nerve connections to the gastroduodenal nerve (Kyösola 1976).

The SO possesses a characteristic intrinsic innervation. A dense network of adrenergic nerve fibers supplies the SO muscle and can be observed around blood vessels (Mori et al. 1971, Kyösola and Rechardt 1973, Cai and Gabella 1983). A dense cholinergic innervation of the smooth muscle of the SO has also been described (Kyösola 1974 and 1979, Cai and Gabella 1983). Immunohistochemical techniques have demonstrated various peptide-containing nerves innervating the muscle of the SO. Nerves immunoreactive to vasoactive intestinal polypeptide (VIP) (Alumets et al. 1979, Cai et al. 1983, Sand et al. 1993b), substance P (Cai et al. 1983, Goehler 1988), somatostatin (Cai et al. 1983), calcitonin gene-related peptide (CGRP) (Goehler et al. 1988, Sand et al. 1993b), enkephalin (Thune et al. 1992), galanin (Harling et al. 1991, Parodi et al. 1992, Becker 1993, Sand et al. 1993b), neuropeptide Y (NPY) (Sand et al. 1993b), bombesin (Cai et al. 1983, Sand et al. 1993b) and peptide histidine-isoleucine (PHI) (Sand et al. 1993b) have been demonstrated.

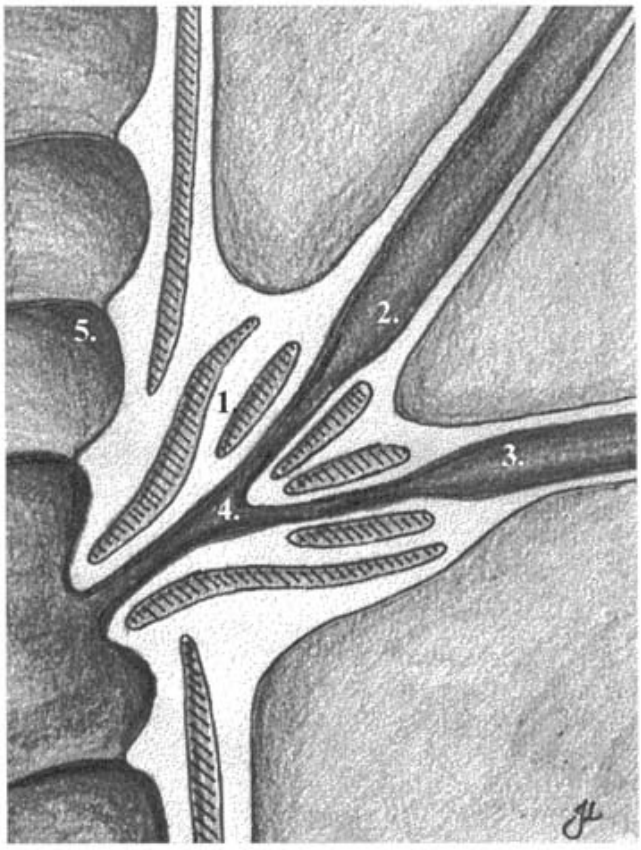

Figure 1. *The human sphincter of Oddi (SO) and choledocho-duodenal junction. 1. SO with ampullary, biliary and pancreatic sphincters, 2. common bile duct, 3. pancreatic duct, 4. ampulla of Vater, and 5. duodenum.*

2 Biliary motility

2.1 Physiology

Bile flows through the cystic duct to fill and empty the gallbladder. The two physiologic mechanisms identified in the gallbladder are mucosal absorption of water and electrolytes, which concentrates the stored hepatic bile, and smooth-muscle contraction, which discharges gallbladder contents into the upper small intestine (Ivy and Bergh 1934). Motility of the gallbladder occurs in the absence of food (interdigestive period) and in response to meals (digestive period), the latter being divided into four phases depending on the site of origin of the stimulus: cephalic, gastric, intestinal and ileocolonic (Niebergall-Roth et al. 1997). The SO plays a key role in directing the bile flow into the gallbladder or the duodenum, and preventing reflux of duodenal contents into the biliary tree (Ono et al. 1968, Tierney et al. 1993).

During the interdigestive period, SO contractility is characterized by prominent phasic contractions (Geenen et al. 1980, Pitt et al. 1982, Müller et al. 1984) which are superimposed on a low basal pressure and coupled to the antroduodenal migrating myoelectric complex (MMC) (Honda et al. 1982, Toouli et al. 1983, Coelho et al. 1985). Spike potential bursts give rise to the phasic contractions of the SO. The frequency of the spike potentials in the SO and duodenum increases gradually from MMC phase I to a maximum frequency in phase III, decreasing then rapidly during phase IV to initiate a new MMC cycle (Keane et al. 1980, Coelho and Wiederkehr 1996). The basal pressure of the SO is usually 5 to 15 mm Hg higher than that of bile and pancreatic duct. Phasic contractions of 50 to 150 mm Hg in amplitude occur 3 to 8 times per minute (Coelho and Wiederkehr 1996). The pressure gradient at the SO inhibits the flow of bile into the duodenum and favours its entry into the gallbladder (Tierney et al. 1993). However, bile flow into the duodenum occurs not only during feeding but also during fasting, correlating with the MMC phases. This flow may have the function of preventing stasis and accumulation of biliary microcrystals and debris during interdigestive periods (Coelho and Wiederkehr 1996).

Gallbladder filling during the interdigestive period is determined by the rate of bile secretion from the liver and the resistance to bile flow through the lower end of the bile duct produced by the SO (Svanvik et al. 1984, Rådberg et al. 1993). The interdigestive gallbladder motility is coupled with phase III of the MMC (DiMagno et al. 1979, Ura et al. 1992, Stolk et al. 1993). During fasting these periodic contractions empty concentrated viscous bile and enable the gallbladder to refill with unconcentrated hepatic bile (Takahashi et al. 1982a, Toouli et al. 1986).

During the digestive period, ingestion of food causes an increase in hepatic bile flow and gallbladder contraction, and a reduction in the pressure of the SO. These events promote bile flow into the duodenum (Hong et al. 1956, Debas and Yamagishi 1979, Takahashi et al. 1982a). The amplitude of the phasic contractions of the SO decreases and the basal pressure is reduced, thus promoting passive bile flow across the SO (Worthley et al. 1989). The delivery of bile into the duodenum is the result of a complex interaction between neural, humoral and possibly paracrine factors which affect the motor activity of the gallbladder and the SO (Niebergall-Roth et al. 1997).

2.2 Control of gallbladder

Gallbladder motility is controlled by humoral and neural mechanisms. Many peptides function both as hormones and as neurotransmitters or neuromodulators in different circumstances. The innervation of the gallbladder plays mainly a facilitating role in the effect of the gastrointestinal hormones and peptides, which are the prime regulators of gallbladder motility (Tierney et al. 1993).

Endogenous CCK is the main driving force behind gallbladder emptying (Fried et al. 1983). CCK causes gallbladder contraction as measured by an increase in gallbladder pressure and emptying in vivo (Ivy and Oldberg 1928, Mutt and Jorpes 1968, Shaffer et al. 1980, Lilja et al. 1982), and produces a dose-dependent contraction of gallbladder muscle strips in vitro (Yau et al. 1973). Several forms of the CCK peptide are released from the duodenal mucosa in response to luminal acid and nutrients, in particular fat and

amino acids (Thompson et al. 1975). The action of CCK on the gallbladder is mediated by direct binding with a specific receptor on the smooth-muscle cells of the gallbladder wall (Steigerwalt et al. 1984). The response of the gallbladder to CCK is calcium (Ca^{2+}) - dependent (Crochelt and Peikin 1987). Gallbladder contraction in response to CCK is also mediated by cholinergic vagal neurones (Liedberg 1969, Inberg and Vuorio 1969, Traynor et al. 1987). Release of CCK is inhibited by the presence of pancreatic enzymes, particularly trypsin (Owyang et al. 1986, Fölsch et al. 1987), and also, in a negative feedback fashion, by the presence of bile salts in the duodenal lumen (Gomez et al. 1986).

Other gastrointestinal peptides and neuropeptides exert either cholecystokinetic (direct and/or CCK-potentiating) or cholecystostatic (direct and/or CCK-inhibiting) actions (Grace et al. 1990). Gastrin causes gallbladder muscle contraction in some species (Valenzuela et al. 1976), but not in humans (Cantor et al. 1986). Secretin potentiates the action of CCK on the gallbladder (Ryan and Cohen 1976), and abolishes the net water absorption from bile in the gallbladder (Jansson and Svanvik 1977), but on its own has no effect on the gallbladder muscle (Chowdhury et al. 1975, Jansson and Svanvik 1977). Substance P is a direct stimulant of gallbladder contraction, but weaker than CCK, in some species (Lonovics et al. 1985). Motilin is weakly cholecystokinetic, the effect being limited to the interdigestive period (Takahashi et al. 1982b). Neurotensin causes indirect gallbladder contraction in the dog (Fujimura et al. 1984), and relaxation in humans (Walker et al. 1985). Histamine H_1-receptor stimulation causes contraction and H_2-receptor stimulation relaxation of the gallbladder muscle (Waldman et al. 1977).

Pancreatic polypeptide (PP) causes relaxation of the gallbladder and a decrease in intraluminal pressure (Adrian et al. 1982), which facilitates gallbladder refilling after contraction (Conter et al. 1987a). VIP inhibits the contractile response of the gallbladder to CCK (Ryan et al. 1977, Lonovics et al. 1979, Strah et al. 1986) and reduces resting gallbladder pressure, eliminating spontaneous contractile activity (Ryan et al. 1977). VIP mediates the inhibitory effect of the vagus nerve, while the stimulatory effect is mediated by acetylcholine (ACh) and CCK (Strah et al. 1986). Somatostatin is a potent inhibitor of

gallbladder emptying (Lonovics et al. 1979, Fisher et al. 1987), and it diminishes the hepatic secretion of bile (Ricci and Fevery 1981). Peptide YY (PYY) potentiates gallbladder relaxation and refilling after CCK-induced contraction (Conter et al. 1987b). CGRP (Hashimoto et al. 1988b) and pancreastatin (Hashimoto et al. 1988a) have been shown to inhibit CCK-induced gallbladder contraction in the guinea pig. In addition, immunoreactivity for galanin and peptide histide-isoleucine (PHI) has been shown in the pig gallbladder (Sand et al. 1993b), but their effect is not yet known.

In the presence of progesterone (Davis and Ryan 1986), or with progesterone treatment prior to cholecystectomy (Ryan and Pellechia 1982), the contractile response of isolated gallbladder strips to cholinergic or hormonal stimuli is reduced. Progesterone also diminishes resting activity and the response of guinea pig gallbladder muscle strips to electrical and hormonal stimulation (Davis and Ryan 1986). Both oestrogen and progesterone receptors have been detected in the gallbladder (Daignault et al. 1988). Pronounced changes in biliary motility are seen in women during pregnancy, most of them being attributed to progesterone (Tierney et al. 1993): resting gallbladder volume is increased (Braverman et al. 1980), and emptying of the organ in response to a fatty meal is impaired (Mann and Higgins 1927, Braverman et al. 1980, Everson et al. 1982b).

Also prostaglandins have potent effects on gallbladder motility. Human gallbladder strips in vitro contract in a dose-dependent fashion in response to several different prostaglandins (Kotwall et al. 1984). Arachinodic acid promotes in vitro contraction of the guinea pig gallbladder, which is inhibited by indometacin (Wood and Stamford 1977). Prostaglandins also produce in vivo contraction of the gallbladder in some species (Wood et al. 1980). (Table 1)

Table 1. *The effects (direct and/or potentiating) of different neurohumoral substances on gallbladder contractility. See text for more detailed effects in different species and circumstances (* in the dog, ** in humans).*

Stimulators	**Inhibitors**
CCK	PP
Gastrin	VIP
Secretin	Somatostatin
Substance P	PYY
Motilin	Pancreastatin
Neurotensin*	Neurotensin**
Stimulation of H_1-receptors	Stimulation of H_2-receptors
Prostaglandins	Progesterone
Arachinodic acid	

2.3 Control of the sphincter of Oddi

2.3.1 Neural control

The control of SO motility takes place via an interplay of extrinsic and intrinsic nerves and gastrointestinal hormones (Toouli and Baker 1991). The SO is regulated by excitatory and inhibitory neural pathways (Behar and Biancani 1984). The role of parasympathetic and sympathetic extrinsic nerves in the control of SO motility has been widely studied, but the results are ambiguous (Toouli and Baker 1991).

ACh contracts the SO in the cat and the calf (Crema and Berte 1963, Persson 1971). Parasympathetic stimulation increases SO activity in the dog (Funch-Jensen et al. 1981) and the opossum (Toouli et al. 1983). However, in the dog vagotomy has been observed to reduce (Schein et al. 1969), increase (Williams and Huang 1969), or to have no effect on (Tansy et al. 1974, Funch-Jensen et al. 1981) SO activity.

Exogenous sympathetic block has no effect on human SO motility (Schein et al. 1970). However, stimulation of the SO adrenergic β-receptors causes relaxation of the SO in

humans and the cat (Dahlstrand et al. 1991). In addition to parasympathetic and sympathetic innervation, non-adrenergic, non-cholinergic (NANC) neurones, by which the effect of CCK is at least partly mediated, have been reported to exist in the SO (Behar and Biancani 1980, Helm et al. 1989).

Many neuropeptides affect SO motility. In addition to their effect via neural pathways, some of them have also humoral actions (Toouli and Baker 1991). Somatostatin (Adami et al. 1986) and the somatostatin analogue octreotide (Ahrendt et al. 1992) inhibit SO motility in the rabbit and in the prairie-dog, whereas in humans octreotide increases the basal pressure and the frequency of phasic contractions of SO (Binmoeller et al. 1992). VIP induces a dose-dependent relaxation of the SO in the cat (Dahlstrand et al. 1989a and 1989b). In patients with SO dysfunction, but not in healthy subjects, an increase in plasma VIP concentration induced by transcutaneous nerve stimulation causes a decrease in SO pressure (Guelrud et al. 1991). PYY inhibits CCK-stimulated SO activity in the prairie dog, and may play a role in modulating biliary motility in the interdigestive period by inhibiting bile flow into the duodenum (Grace et al. 1988). NPY increases SO activity in the prairie dog and may thus regulate bile flow (Lillemoe et al. 1988). Galanin has a direct inhibitory effect on SO motility in the pig (Harling et al. 1991), and it may also inhibit the release of VIP from nerve endings (Fox-Threlkeld et al. 1991). Substance P has a stimulatory effect on the SO in the opossum (Parodi et al. 1989 and 1990), the dog (Guo et al. 1989) and the cat (Dahlstrand et al. 1988). Bombesin may cause CCK-mediated relaxation in the canine SO (Sievert et al. 1988). Serotonine (Behar and Biancani 1983) and enkephalins (Behar and Biancani 1984) induce SO contraction, which in the cat is followed by a prolonged relaxation. Immunoreactivity for PHI and CGRP has been observed in the nerves of the pig SO (Sand et al. 1993b), but their possible effect on the SO is not known.

Histamine increases SO contractility in the guinea-pig in vitro (Pauletzki et al. 1993), and in the pig both in vivo and in vitro, this effect being mediated by H_1-receptors (Sand et al. 2000), while it reduces SO contractility in the opossum in vivo (Toouli et al. 1981). In the pig, nitric oxide (NO), a potent smooth-muscle relaxant produced by the enteric

nerves, reduces SO contractility and electrical activity. Inhibition of endogenous NO production, again, enhances SO contractility (Sand et al. 1997).

2.3.2 Humoral control

CCK is an important regulator of SO motility (Grace et al. 1990). It causes SO relaxation by reducing the phasic activity and the baseline pressure of the SO in humans (Geenen et al. 1980), primates (LaMorte et al. 1980) and cats (Behar and Biancani 1980), and this relaxation facilitates the passive bile flow from the CBD into the duodenum. However, in the rabbit (Sarles et al. 1976), the opossum (Becker et al. 1982) and the prairie dog (Doty et al. 1981) CCK increases the phasic wave activity of the SO without affecting baseline pressure, and thus the increased SO activity propels bile actively into the duodenum (Grace et al. 1990). In addition to the direct effect of CCK on the smooth muscle of the SO, its effect is also mediated by NANC neurones (Behar and Biancani 1980). Caerulein (Bertaccini et al. 1968, Lin and Spray 1969, Agosti et al. 1971) and gastrin (Sandblom et al. 1935) have identical carboxyterminal pentapeptide to CCK, and are inhibitors of SO activity, gastrin being less potent compared to CCK and caerulein (Agosti et al. 1971).

Since Sandblom (1935) first demonstrated the hormonal action of CCK on the SO in 1935, several other hormones have been shown to affect the SO activity. Secretin reduces SO resistance in the dog, probably indirectly by potentiating the effect of CCK (Nebel 1975a, Carr-Locke et al. 1985). Glucagon reduces the SO resistance in the dog (Lin and Spray 1969) and humans (Nebel 1975a). Motilin increases SO contractility in the cat (Müller et al. 1987, Behar and Biancani 1988), possibly by an intramural excitatory pathway via opiate, serotonergic and cholinergic neurones, and its physiological role on the SO may be to regulate its cyclical activity during the interdigestive period (Behar and Biancani 1988, Grace et al. 1990). Of the steroid hormone family, it has been suggested that oestrogen affects the motility of the SO in a prairie dog model (Tierney et al. 1993). However, the effect of THs or of steroid hormones other than oestrogen, on SO contractility has not been studied yet.

Table 2. *The effects (direct and/or potentiating) of different neurohumoral substances on sphincter of Oddi contractility. See text for more detailed effects in different species and circumstances (* in humans; ** in the prairie dog; *** in the guinea-pig, pig; **** in the opossum; $^{+}$ in the rabbit, opossum, prairie-dog, $^{++}$ in humans, the primates, cat).*

Neural substances		**Humoral substances**	
Stimulators	**Inhibitors**	**Stimulators**	**Inhibitors**
ACh		CCK^{+}	CCK^{++}
Parasymph. stim.	Adrenergic stim.	Motilin	Caerulein
	Somatostatin		Gastrin
Octreotide *	Octreotide**		Secretin
NPY	VIP		Glucagon
Substance P	PYY		
Serotonine	Galanin		
Enkephalins	Bombesin		
Histamine***	Histamine****		

2.3.3 Pharmacological control

Various pharmacological agents influence SO function. The SO is highly sensitive to opioids, which is of clinical importance. Even low doses of morphine, petidine (less potent), and other opioids, with the exception of buprenorphine, cause marked contractions of the SO, increasing the resistance to bile flow (Watts and Dunphy 1966, Economou and Ward-McQuaid 1971, Persson and Ekman 1972, Staritz et al. 1985, Helm et al. 1988), and are thus contraindicated in the management of biliary pain. Fentanyl can cause SO spasm (Chessick et al. 1975). This stimulatory effect of opioids seems to be mediated via intramural serotonergic nerves (Behar and Biancani 1984).

Bentsodiazepines have no effect on human SO motility (Nebel 1975b, Staritz et al. 1986). Barbiturates increase bile flow through the SO (Wyatt 1967). Ketamine affects biliary tract motility in the prairie dog (Ryan et al. 1982, Grace et al. 1987b). Alcohol, when given intravenously or into the duodenum, causes a moderate decrease in the baseline pressure of SO without affecting motility (Staritz 1988).

Glyseryl trinitrate causes relaxation of the SO (Staritz et al. 1985). The Ca^{2+} channel blocker nifedipine reduces SO baseline pressure and the frequency, amplitude and duration of the SO phasic contractions in humans (Guelrud et al. 1988). Nifedipine improves bile flow and can be used as an analgesic in patients with SO dyskinesia (Sand et al. 1993a). The muscarinic ACh receptor blocker butylscopolamine reduces SO motility without affecting baseline pressure (Staritz 1988), while pirenzepine reduces the basal pressure and the amplitude and frequency of phasic contractions of the human SO (Garrigues et al. 1986). The β_2-adrenoceptor agonists isoprenalin and terbutalin cause relaxation of the human SO (Dahlstrand et al. 1991).

2.3.4 Physical and intraluminal control

A high temperature of 40°C stimulates and low temperature inhibits SO activity in the rabbit (Sarles et al. 1975). Arterial hypertension raises and hypotension diminishes the SO opening pressure in the dog (Tansy et al. 1974). SO activity is influenced by the degree of gallbladder filling; gallbladder distension increases and gallbladder emptying reduces SO activity (Wyatt 1967, Doty et al. 1981, Müller et al. 1984). This cholecysto-SO reflex may be neurally mediated (Webb et al. 1988). Intraduodenal infusions of acidified saline (Grace et al. 1987a) and sodium oleate (Grace and Pitt 1987) lower SO activity, whereas protein infusions (Webb et al. 1987) stimulate it in the prairie dog. The SO motility response to duodenally administered sodium oleate and intravenous CCK is altered after cholecystectomy. Thus, as mentioned above, the gallbladder may be involved in SO regulation by a neural pathway (Grace and Pitt 1987). SO phasic contractions correlate with MMC (Honda et al. 1982, Coelho et al. 1985).

3 Pathogenesis of gallstones

3.1 Classification of gallstones

In western countries, 10 to 12 % of adults have gallstones (Diehl 1991, Heaton et al. 1991, Kratzer et al. 1999). On the basis of their location, gallstones can be divided into gallbladder stones and bile duct stones. The incidence of bile duct stones in patients with gallbladder stones varies from 8 to 16 % (Applemann et al. 1964, Jordan 1982). Bile duct stones are usually classified either as primary stones, formed de novo in the bile ducts, or as secondary, having passed out of the gallbladder but retained in the bile ducts (Thistle 1998).

Depending on their pathogenesis and composition, gallstones can be divided into cholesterol stones, which constitute 70 to 80 % of gallstones encountered in the western world (Busch and Matern 1991), and brown and black pigment stones (Donovan 1999). Some 40 % of bile duct stones are pigment stones (Way and Sleisenger 1989), mostly brown, whereas black pigment stones are rarely located in the bile ducts (Leuschner et al. 1994). Cholesterol and black pigment stone formation is characterized by abnormalities in cholesterol and bilirubin metabolism, but brown pigment stone formation occurs as a result of bacterial infection of the biliary tree, typically in the presence of bile stasis (Donovan 1999).

Cholesterol gallstones are composed predominantly of cholesterol monohydrate crystals agglomerated by a mucin glycoprotein matrix, and of minor components such as unconjugated bilirubin and small amounts of Ca^{2+} phosphate (Trotman et al. 1974). Black pigment stones contain Ca^{2+} salts of unconjugated bilirubin in a polymeried matrix, and the additional presence of Ca^{2+} phosphate in the mucin matrix results in opacity on radiographs (Malet et al. 1984). Brown pigment stones, in contrast, include bacterial degradation products of biliary lipids, Ca^{2+} salts of fatty acids and unconjugated bilirubin, in addition to precipitated cholesterol (Cetta 1986).

The pathogenesis of gallstones is a complex process involving factors affecting bile content and bile flow.

3.2 The role of bile content

3.2.1 Biliary lipid secretion

Hepatic cholesterol is derived either from preformed cholesterol from serum lipoproteins (via the low-density lipoprotein (LDL) receptor, the low-density lipoprotein receptor-related protein (LRP) and the scavenger receptor BI (SR-BI)), or from endogenous cholesterol synthesis within the hapatocyte, the latter via the rate-limiting enzyme hydroxymethyl glutaryl-coenzyme A (HMG-CoA). Free cholesterol may be exported into bile directly, used for bile salt synthesis via the rate-limiting enzyme cholesterol-7-α-hydroxylase, or converted into cholesterol esters after esterification by acylcholesterol acyl transferase (ACAT) (Donovan 1999). An alternative pathway, initiated by the peripheral conversion of cholesterol by sterol 27-hydraxylase to 27-hydroxycholesterol, which is then transported to the hepatocyte, appears to be the initial step in a substantial fraction of bile salt synthesis (Cali and Russell 1991, Donovan 1999). Cholesterol-7-α-hydroxylase is regulated both by end products and by hormones, such as thyroxine and glucocorticoids (Stravitz et al. 1993). Cholesterol and phospholipids are secreted from the liver as unilamellar vesicles. With addition of bile acids, there is a dynamic interchange of vesicles and mixed micelles (Ko and Lee 1999).

3.2.2 The mechanism of cholesterol gallstone formation

The genesis of cholesterol gallstones involves cholesterol saturation of bile, formation and growth of cholesterol monohydrate crystals, and the absorptive, secretory, and motor functions of the gallbladder (Ko and Lee 1999).

During transit through the biliary tract vesicles may fuse and aggregate, which process is crucial for nucleation (Ko and Lee 1999). Vesicles with increased ratios of cholesterol to

phospholipid aggregate, fuse and nucleate more easily (Halpern et al. 1986). Bile supersaturation with cholesterol is necessary for cholesterol gallstone formation, but not all individuals with supersaturated bile form gallstones, and additional factors must be present (Ko and Lee 1999). Whether bile is supersaturated with cholesterol, is largely determined at the moment it is secreted into the canalicus, although some modification may occur as a result of lipid absorbtion in the gallbladder (Strasberg 1998).

Supersaturation appears usually to be due to cholestererol hypersecretion. This can be produced by multiple mechanisms, since there are many points in the intermediary metabolism of cholesterol at which a slight increase or decrease in enzymatic activity or receptor expression can result in cholesterol supersaturation. Many factors affecting these mechanisms have been shown to be risk factors for cholesterol stone formation (Strasberg 1998).

Supersaturation of bile increases with age due to increased cholesterol secretion (Valdivieso et al. 1978, Einarsson et al. 1985), possibly because of decreased utilization of hepatic cholesterol for bile salt synthesis (Bertolotti et al. 1993), the underlying cellular mechanisms not being completely known (Strasberg 1998). The linkage of cholesterol gallstones and obesity is widely known, (Bennion and Grundy 1975), and excessive cholesterol synthesis is thought to be the main defect in this (Ahmed et al. 1995, Stahlberg et al. 1997). Rapid weight loss in the obese patient is associated with a sharp increase in cholesterol secretion into bile and contributes to gallstone formation (Bennion and Grundy 1975), altered gallbladder motility and accelerated crystallization rates being other possible contributors (Andersen 1992). Oestrogen promotes secretion of cholesterol into bile (Bennion and Mott 1980), possibly due to increased cholesterol synthesis rates (Kern et al. 1981), and, in addition, it slows gallbladder emptying (Braverman et al. 1980, Everson et al. 1982a). A high-cholesterol diet plays an important role in cholesterol supersaturation. Persons who develop gallstones respond inappropriately to high dietary cholesterol by not diverting it into the bile salt synthesis pathway, but instead to bile, a process in which genetic mechanisms may also be involved (Kern 1994, Khanuja et al. 1995). One factor inducing supersaturation seems to

be enrichment of the bile salt pool with the secondary bile salt deoxycholate, the underlying mechanism suspected to be increased cholic acid 7 α-dehydroxylation activity of the intestinal microflora (Pomare and Heaton 1973, Berr et al. 1996). Paradoxically, however, also colectomy has been shown to result in bile supersaturation (Harvey et al. 1991).

As mentioned above, supersaturation is needed for stone formation, but it does not guarantee stone formation. The important factors leading to stone formation from the supersaturated bile are a defect in gallbladder motility (see below) and a defect in kinetics, the latter resulting in more rapid maturation, aggregation and so forth in the crystallization pathway (Strasberg 1998). Nucleation may be promoted by several factors present in saturated bile, e.g. glycoproteins, Ca^{2+}, IgA and IgM, fibronectin, phopholipase C, and apolipoprotein E-4, and inhibited by apolipoproteins A-1 and A-2 and several glycoproteins (LaMont and Carey 1992). A direct consequence of cholesterol-supersaturated bile is gallbladder hypomotility and mucin hypersecretion (Donovan 1999). Formation of a viscoelastic gel developing as a result of mucin hypersecretion by the gallbladder and bile duct epithelium to the gallbladder wall, may create an environment in which cholesterol crystal nucleation first occurs (Schoenfield et al. 1989, Smith 1990, Ko and Lee 1999).

3.2.3 The mechanism of pigment gallstone formation

The formation of black pigment stones is a complex process. Supersaturation of bile with water-insoluble, unconjugated bilirubin, Ca^{2+} and Ca^{2+} bilirubinate is the most crucial element in their development. Additional factors are a shift in pH to an alkaline milieu, and a decrease in Ca^{2+} binding by bile salts and phospholipid-cholesterol vesicles. In addition, overproduction of organic matrix as a result of cholecystitis or biliary bacterial infection seems to accelerate nidus formation. In the uninfected gallbladder, the mucin of the bladder crypts gives rise to black stone nuclei development (Trotman et al. 1980, Leuschner et al. 1994).

Brown pigment stones are found in infected bile during cholestasis. The bacteria have a high β-glucuronidase activity. The interaction of this bacterial enzyme and its inhibitors, such as glucaro-1,4-lactone, and bile acids seems to be important. The steps leading to brown bilirubinate stone formation are bile stasis and infection, mucin hypersecretion and enzymatic deconjugation and hence precipitation of previously water-soluble compounds (Leuschner et al. 1994).

3.3 The role of bile flow

Gallbladder hypomotility is a direct consequence of cholesterol-supersaturated bile (Donovan 1999). This condition directly depresses gallbladder contractility (Behar et al. 1989), obviously as a result of the increased cholesterol content of the cellular membranes (Xu and Shaffer 1996, Yu et al. 1996). It has also been proposed that gallbladder stones correlate with a reduced number of gallbladder CCK receptors, impairing its contractility (Thompson et al. 1982, Upp et al. 1987, Poston et al. 1988b and 1992). Gallbladder filling is impaired, with a substantial decrease in the flux of bile into the gallbladder (Jazrawi et al. 1995). Further, the viscous nature of the mucin gel physically impairs clearance of precipitates by gallbladder contraction. Depressed motility gives rise to a prolonged residence of bile in the gallbladder. This may contribute to retention of cholesterol crystals in the organ, thereby allowing sufficient time for nucleation and continual growth into mature gallstones. In addition, impairment of gallbladder contractility, mucosal absorption and bile concentration leads to a greater fraction of newly secreted bile to be diverted directly into the intestine, where it undergoes bacterial metabolism (Donovan 1999).

Reduced motility of the gallbladder and increased resistance of SO may contribute to gallstone formation after truncal vagotomy (Pitt et al. 1982 and 1983). Patients receiving prolonged total parenteral nutrition are also at risk of rapid gallstone formation because of biliary stasis (Roslyn et al. 1983, Holzbach 1983). Reductions in bowel motility, CCK release and vagal activity possibly leading to gallbladder hypotonicity and biliary stasis after major abdominal operations can also predispose to gallstones (Roslyn et al. 1983).

The rate of gallbladder emptying decreases with age, obviously by reason of the reduced sensitivity of gallbladder muscle to CCK (Khalil et al. 1985, Poston et al. 1988a). Somatostatin analogue octreotide therapy, e.g. in acromegaly, gastroenteropancreatic endocrine tumors and secretory diarrhea, entails a risk of gallstone formation in a mechanism involving factors such as inhibition of gallbladder emptying, SO motility, hepatic bile secretion and modification of bile composition (Ewins et al. 1991, Bigg-Wither et al. 1992, Catnach et al. 1993, Redfern and Fortuner 1995).

Brown pigment stones form secondary to biliary stasis (Thistle 1998), which is the major factor leading to anaerobic bacterial degradation and precipitation of biliary lipids (Carey 1993, Donovan 1999). Mechanical obstruction of the biliary tract leading to biliary stasis may be caused by bile duct strictures, SO stenosis or SO dyskinesia (Osnes et al. 1981, Sandstad et al. 1994, Thistle 1998). Brown pigment stones may also occur around a nidus of black or cholesterol stones or foreign material, which partially obstruct the CBD. Once initiated, the pathogenetic mechanism of stasis and bacterial overgrowth is difficult to reverse. The incidence of brown stones increases with age, which phenomenon may be associated with deterioration of the SO function (Thistle 1998).

Based upon endoscopic SO manometry, SO dysfunction may be subdivided into SO stenosis, characterized by an abnormally elevated SO basal pressure, and SO dyskinesia, characterized by an alteration in SO activity. The latter may evince one or more of the following patterns: rapid phasic contraction activity, SO hypertonocity/spasm, SO propagation abnormality, and paradoxical response to CCK (Geenen et al. 1980). The pathological basis for many of the abnormalities of the SO has not been established. The paradoxical response to CCK may be due to damage of SO intrinsic NANC-neurons (Toouli et al. 1982). An elevated SO basal pressure indicating SO stenosis may be due to fibrotic stenosis of the SO, hypertrophy of smooth muscle or local inflammatory response (Toouli and Baker 1991).

4 Thyroid function

4.1 Hyperthyroidism

Hyperthyroidism is a common disorder, especially in women, the incidence being about 80/100 000 women yearly (Vanderpump et al. 1995). In young patients its cause is usually Graves' disease, whereas in elderly patients toxic nodular goitre is also a common cause (Woeber 2000). In this disorder the basal metabolism is elevated, and synthesis and breakdown of proteins, carbohydrates and fat are accelerated (Wuttke 1989). Its clinical symptoms include nervousness, heat intolerance and sweating, palpitations, tremulousness, weight loss with good appetite, muscle weakness, emotional lability, and hyperdefecation. Possible clinical signs of hyperthyroidism are thyroid enlargement, eye stare and lid lag, warm and smooth skin, fine tremor, brisk reflexes, onycholysis, tachycardia or atrial fibrillation, and in Graves' disease proptosis of the eyes and ophthalmoplegia, and pretibial myxoedema (Feingold et al. 1993).

Measurement of serum thyroid-stimulating hormone, i.e. thyrotropin (TSH), is the most sensitive test in screening for hyperthyroidism, an undetectable value being a hallmark. Confirmation can be made by measuring serum free T_4 (FT_4). In a patient without ophthalmopathy, measurement of thyroid radioiodine (^{131}I) uptake is performed to establish the cause of thyrotoxicosis. Hyperthyroidism may be treated with antithyroid drugs, ^{131}I, or subtotal thyroidectomy (Woeber 2000).

4.2 Hypothyroidism

Hypothyroidism is likewise a common disorder, again especially in women, the incidence being about 350/100 000 women yearly (Vanderpump et al. 1995). The prevalence of subclinical hypothyroidism among women over 60 years of age is as high as 20% (Dickey and Feld 2000). Hypothyroidism is defined as in any state which results in a deficiency of TH, including hypothalamic or pituitary diseases, generalized tissue resistance to TH, and disorders directly affecting the thyroid gland (Woeber 2000).

Thyroid deficiency in adults is characterized by a slowing of all metabolic processes (Wuttke 1989). The possible clinical symptoms of the condition include weakness, lethargy and fatigue, memory impairment, dementia, cold intolerance, weight gain, constipation, loss of hair, hoarseness, deafness, dyspnea, myalgia and arthralgia, paraesthesias, precordial pain and menstrual irregularity (Feingold et al. 1993). When THs are lacking in early childhood, the result is severe bodily and mental retardation (Wuttke 1989). Clinical signs of hypothyroidism may be dry, coarse and cold skin, periorbital and peripheral oedema, coarse and thin hair, pallor of skin, thick tongue, slow speech, decreased reflexes, hypertension, bradycardia, pleural and pericardial effusions, ascites and vitiligo. In laboratory tests, e.g. hypercholesterolaemia and anaemia are associated findings (Feingold et al. 1993).

The laboratory hallmark of primary hypothyroidism and the most sensitive test for detecting early thyroid failure is an increased serum TSH concentration. The serum FT_4 level is decreased in clinical hypothyroidism (Woeber 2000). In the subclinical form an increased serum TSH level is accompanied by a normal serum FT_4 level, and the patient is asymptomatic (Woeber 1997). The presence of thyroperoxidase antibody confirms chronic autoimmune thyroiditis as the cause of hypothyroidism. The treatment of hypothyroidism with levothyroxine is usually lifelong (Woeber 2000).

4.3 The effects of thyroid hormones

The thyroid gland secretes both T_4 and triiodothyronine (T_3) into the circulation. In extrathyroidal tissues, T_4 is converted to T_3, assumed to be the major active TH (Polikar et al. 1993, Surks and Sievert 1995). Characteristic of THs is the multiplicity of the cellular functions they regulate in virtually every type of vertebrate tissue. The diverse responses to TH can be divided into two major categories: (1) regulation of metabolic activity, energy consumption and muscular activity in adult mammals, and (2) regulation of postembryonic or perinatal growth and development (Chatterjee and Tata 1992).

Most actions of THs can be explained by their interaction with nuclear receptors (Glass and Holloway 1990). THs bind to specific intranuclear TH receptors (TR), $TR\alpha_1$, $TR\beta_1$ or $TR\beta_2$. This ligand-receptor complex binds to TH response elements in the target genes to regulate the rate of synthesis of specific messenger RNAs. This results in a change in the amount or activity of the cognate proteins, which in turn alter the rate of the metabolic process. The TRs are expressed in a tissue- and development-stage-specific fashion (Lazar and Chin 1990, Chatterjee and Tata 1992, Lazar 1993, Chin 1994). For example, $TR\alpha_1$ is known to be highly expressed in brain, muscle and fat, and has been identified in frog, chicken, rat, mouse and humans; $TR\beta_1$ is highly expressed in liver and kidney, and has been demonstrated in frog, chicken, rat, mouse and humans. $TR\beta_2$, again, is highly expressed in the pituitary, and has been demonstrated in rat and mouse (Chatterjee and Tata 1992). The expression of TRs in the SO has not been studied.

The genomic effects of THs necessarily require a finite period of time for protein synthesis and for the biological response. Acute response of a cell to THs is unlikely to involve a transcriptional mechanism but is rather a result of nongenomic mechanisms involving extranuclear sites of action (Salter et al. 1992). Extranuclear sites of TH action include the cell membrane (Segal 1989), the cytoskeleton (Siegrist-Kaiser et al. 1990), the sarcoplasmic reticulum (Warnick et al. 1993), the cytoplasm (Lawrence et al. 1989), the mitochondria (Sterling 1986), and in vascular smooth-muscle cells presumably the contractile elements (Ishikawa et al. 1989, Ojamaa et al. 1993). For example, THs mediate sugar uptake (Segal 1989), adenylate cyclase (Limas and Limas 1987), and Ca^{2+}-ATPase activity (Davis et al. 1983, Rudinger et al. 1984) directly at the level of the plasma membrane of various tissues. The second messengers associated with the extranuclear actions of the THs are not yet known (Yoneda 1998).

4.4 Thyroid hormones and cholesterol metabolism

The elevation of serum cholesterol levels is a clinically important accompaniment of hypothyroidism (Dickey and Feld 2000). Patients with overt hypothyroidism have approximately 50% higher serum cholesterol levels than euthyroid patients (Kutty et al.

1978, Elder et al. 1990); 90% of all hypothyroid patients have elevated cholesterol levels, triglyceride levels, or both (Dickey and Feld 2000). This is primarily due to elevations in low-density lipoprotein (LDL) rather than high-density lipoprotein (HDL) levels (Kuusi et al. 1988, Packard et al 1993). Treatment of hypothyroid patients who also have hyperlipidaemia will have beneficial effects on serum cholesterol levels (Elder et al. 1990). The aetiology of the hypercholesterolaemia in hypothyroid patients is probably multifactorial (Field et al. 1986).

THs have been shown to have a number of effects on cholesterol metabolism (Andreini et al. 1994). LDL receptor activity is increased (Ness et al. 1990) because of increased expression of the LDL receptor gene, and the expression may be decreased in hypothyroidism, leading to reduced removal of cholesterol from the serum (Scarabottolo et al. 1986, Ness et al. 1990). THs increase the synthesis of cholesterol (Day et al. 1989, Ness et al. 1990) by regulating the expression of HMG-CoA reductase, and the regulation is reduced in hypothyroid patients, leading to decreased cholesterol synthesis (Day et al. 1989). Also the synthesis of bile salts is increased by THs by the effect on cholesterol-7-α-hydroxylase (Stravitz et al. 1993), and a decrease in biliary bile salt concentration in hypothyroidism has been reported (Strand 1962). Absorbtion of cholesterol is decreased by THs (Mathe and Chevallier 1976). Hypothyroidism lowers biliary cholesterol secretion in the rat, while T_4 replacement in hypothyroid animals markedly increases cholesterol secretion (Gebhard et al. 1992, Gebhard and Prigge 1992). However, in the cholesterol-fed hypothyroid rat, biliary cholesterol content is significantly increased and the rate of bile secretion decreased (Field et al. 1986).

Biliary secretion of cholesterol is reduced in hypothyroidism compared to euthyroidism. However, when serum cholesterol values rise, bile may also become supersaturated with cholesterol and thus result in gallstone formation. An association has been reported between cholesterol gallstones and treated hypothyroidism in women (Honore 1981). It has also been reported that gallbladder stones may have been dissolved after T_4 treatment (Vassilakis and Nikolopoulos 1981).

4.5 Thyroid hormones and smooth-muscle

4.5.1 Smooth-muscle contraction

Neuronal or hormonal stimulation of the smooth-muscle cell results in an increase in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. Ca^{2+} enters the sarcoplasm (1) from the extracellular space via voltage-gated or receptor-operated Ca^{2+} channels, or (2) from the sarcoplasmic reticulum (SR) via inositol 1,4,5-triphosphate receptor/Ca^{2+} -release or ryanodine receptor/Ca^{2+} -release channels. As a consequence of its elevated concentration, Ca^{2+} binds to calmodulin (CaM), inducing a conformation change which exposes hydrophobic sites for interaction with a number of target proteins, including myosin light-chain kinase (MLCK). The resultant active form of MLCK, $(Ca^{2+})_4$-CaM-MLCK, phosphorylates Ser19 of each of the two 20 kDa light chains of myosin. This phosphorylation reaction triggers the cycling of myosin crossbridges along the actin filaments, with a development of force or contraction of the smooth muscle (Allen and Walsh 1994, Horowitz et al. 1996).

Relaxation of the smooth muscle follows the restoration of resting $[Ca^{2+}]_i$ by (1) extrusion of Ca^{2+} from the cell by a sarcolemmal Ca^{2+} pump or Na^{2+}- Ca^{2+} exchanger, or (2) pumping of Ca^{2+} into the SR by a Ca^{2+} pump in the SR membrane. MLCK is then rapidly inactivated by the dissociation of CaM, and myosin is dephosphorylated by myosin light-chain phosphatases (Allen and Walsh 1994).

The most obvious means of controlling smooth-muscle contraction is by regulation of $[Ca^{2+}]_i$. α_1-adrenergic agonists and membrane-depolarising neurotransmitters cause an increase in $[Ca^{2+}]_i$, eliciting the contractile response. β-adrenergic agonists, atrial natriuretic factor and NO induce smooth-muscle relaxation via a decrease in $[Ca^{2+}]_i$ (Allen and Walsh 1994).

4.5.2 The effect of thyroid hormones on smooth-muscle

The effect of THs on smooth-muscle contraction depends on the smooth muscle type and the species studied. THs have a direct effect on vascular smooth muscle contractility. T_3 acts directly, presumably without a transcriptional mechanism, on vascular smooth-muscle cells of the rat aorta, causing cellular relaxation, the effect not following the administration of T_4 (Ojamaa et al. 1996). Changes in the contractility response of the rat abdominal aorta vascular rings in relation to altered TH status are not endothelium-dependent (McAllister et al. 2000). In the rat coronary arteries, T_3 and T_4 are equally effective in inducing an immediate, dose-dependent, transient vasodilatation and a decrease in the perfusion pressure, the effect not being related to NO synthesis. The rapidity of this effect suggests a mechanism other than TR-mediated, i.e. a direct, nongenomic effect of THs. THs may thus play a role in preventing myocardial ischaemia by inducing coronary artery vasodilatation (Yoneda et al. 1998).

The administration of THs in patients undergoing cardiopulmonary bypass surgery increases cardiac output and lowers systemic vascular resistance (Klemperer et al. 1995a). Significant decreases in systemic (Kapitola and Vilimovska 1981, DiPierro et al. 1996) and coronary (Klemperer et al. 1995b) vascular resistance are seen within 30 minutes of T_3 administration in animal models, resulting in improvement of cardiac contractile function. Isolated perfused kidneys of hyperthyroid rats show an increased vascular reactivity to vasoconstrictors, which may play a role in the maintenance of elevated blood pressure in these animals (Sabio et al. 1994).

T_3 relaxes isolated mesenteric arteries in a dose-dependent fashion (Ishikawa et al. 1989). T_4-induced relaxation of the rat mesenteric resistance arteries seems to be mediated (1) by an indirect effect via the endothelium, T_4-induced relaxation being impaired by the inhibitor of endothelium-produced NO, L-NAME, and (2) by a direct effect on vascular smooth-muscle cells, possibly by influencing Ca^{2+} fluxes. However, since vascular relaxation of the rat mesenteric resistance arteries is established at supraphysiologic

concentrations of TH, it may not be relevant for an in vivo situation (Zwaveling et al. 1997).

In both large arteries and resistance vessels, sensitivity to vasoconstrictors is markedly decreased in hypothyroid rats. This may play a role in the lowered blood pressure in these animals (Sabio et al. 1994). The number of β-adrenergic receptors decreases in hypothyroidism and increases in hyperthyroidism (Williams et al. 1977, Lefkowitz et al. 1984). In pulmonary artery preparations from hyperthyroid rats, β_1-adrenoceptors are functionally predominant, whereas in euthyroid rats β_2-adrenoceptors are functionally predominant (O'Donnell and Wanstall 1986). The magnitude of the relaxant response of the rat pulmonary artery to β-adrenoceptor agonists is reduced in hypothyroidism and increased in hyperthyroidism (O'Donnel et al. 1987).

The regulatory effects of THs on vascular smooth muscle are mediated by intranuclear binding of TH to the TR (Dillmann 1990, Ojamaa et al. 1992, Brent 1994), and also partly by nongenomic mechanisms involving extranuclear sites of action (Salter et al. 1992). The potassium (K^+) -channel blocker glibenclamide attenuates T_3-induced vasodilatation in rat skeletal muscle arteries, and T_3-induced vasodilatation might thus be mediated by ATP-sensitive K^+-channels (Park et al. 1997). T_3 has a stimulatory effect on prostaglandin production by the rat aortic smooth-muscle cells (Nakao et al. 1981, Noguchi et al. 1985). Thus, THs might play a role in the protection of arteries from atherosclerotic changes by stimulating production of prostacyclin, a potent vasodilator and endogenous inhibitor of platelet aggregation (Nakao et al. 1981).

The existence of gastrointestinal hypoactivity in hypothyroidism is well known (Johansson 1966, Middleton 1971, Duret and Bastenie 1971, Kowalewski and Kolodej 1977, Miller et al. 1978, Shafer et al. 1984, Goto et al. 1992). Hypothyroid rats show relatively low anal canal pressure, and a decreased frequency of rhythmic colonic activity, which deficiency is improved by T_4 replacement therapy. In hypothyroid cats, a fall in lower esophageal sphincter (LES) pressure has been documented. In clinical observations thyroid replacement therapy is associated with a return of esophageal

peristalsis and LES pressure to normal (Eastwood et al. 1982). In addition to gastrointestinal effects, THs also have effects on the urinary tract. Hyperthyroidism stimulates and hypothyroidism inhibits ACh and KCl -induced contractile responses of the rat urinary bladder strip (Adeniyi et al. 1994). The effect of THs on biliary motility or SO contractility has not this far been studied.

AIMS OF THE STUDY

The aim of the present study was to investigate the effect of thyroid gland function on the formation of CBD stones, biliary dynamics and the contractility of the SO.

The specific objectives were to study:

1 whether there is any association between treated hypothyroidism and the occurrence of CBD stones (study I).
2 the effect of T_4 on the pig SO (study II)
3 the effect of T_4 on the human SO (study III),
4 the specificity and the mechanism of action of T_4 on the SO (studies II-III).
5 the bile flow in rats in relation to experimentally altered thyroid gland function (study IV).
6 the bile flow in humans in relation to altered thyroid gland function (study V).

EXPERIMENTAL ANIMALS, PATIENTS AND STUDY DESIGN

1 Study I. Association between common bile duct stones and treated hypothyroidism

The prevalence of hypothyroidism was studied retrospectively in CBD stone patients (group I), control patients (group II), and gallbladder stone patients (group III). Group I (n = 86, 30 men, median age 73 (range 22-92) years) consisted of all patients verified to have CBD stones in endoscopic retrograde cholangiopancreatography (ERCP) in Tampere University Hospital in 1995. Group II was adjusted for age, sex and hospital admission, but patients with diagnosed gallstone disease were excluded. In groups I and II diagnosed hypothyroidism was found only in the subgroup of patients over 60 years of age (n = 66, 23 men), and thus group III (n = 36, 11 men) comprised patients over 60 years, who were cholecystectomized due to gallbladder stones between January 1995 and April 1996, and in whom the presence of CBD stones was considered very unlikely judging by liver chemistry plus ultrasonography and/or magnet cholangiography. Medical records of all patients were reviewed for all diagnosed diseases and treatments.

2 Studies II-III. Direct effect of thyroxine on the sphincter of Oddi contractility in the pig (II) and in humans (III) and mechanism of the prorelaxing effect of thyroxine on the sphincter of Oddi (II-III)

SO rings. For animal experiments, pigs (weight 20 to 30 kg) were anaesthetised with midazolam 5 mg and ketamin 300 mg. Laparotomy and duodenotomy were performed and the SO removed. For experiments with human SO, the SOs were received from four patients who underwent a Whipple resection. The SO was removed immediately from the surgical specimen. In these cases the malignancy necessitating resection was located in the head of the pancreas away from the SO. In all cases the SO was dissected free from the covering mucosa and three successive 1-1.5 mm rings were prepared from each SO.

SO responses in vitro. All the SO rings, either human or pig, were initially treated in like fashion: they were placed between two stainless steel hooks and suspended in an organ bath chamber in oxygenated salt solution (pH 7,4; composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2; gassed with 95% O_2 + 5% CO_2 mixture), and the force of SO contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FT 03 transducer & Model 7 E Polygraph; Grass Instrument Co., Quincy, MA) (Fig. 2). The function of the rings was confirmed by a contraction response to 125 mM potassium chloride (KCl). Only the responding rings were included in the study. After the first KCl test, the SO rings were stabilized for 30 minutes, whereafter they were maximally and ”unspecifically” stimulated with 125 mM KCl. When the maximal responses had developed, the rings were repeatedly rinsed with the salt solution and once the resting tension was restored, further test stimulations were performed with ACh (10 μM or 100 μM) to study muscarinic receptor-mediated contraction, and with histamine (Hist; 10 μM or 100 μM) to study H_1-receptor-mediated contraction.

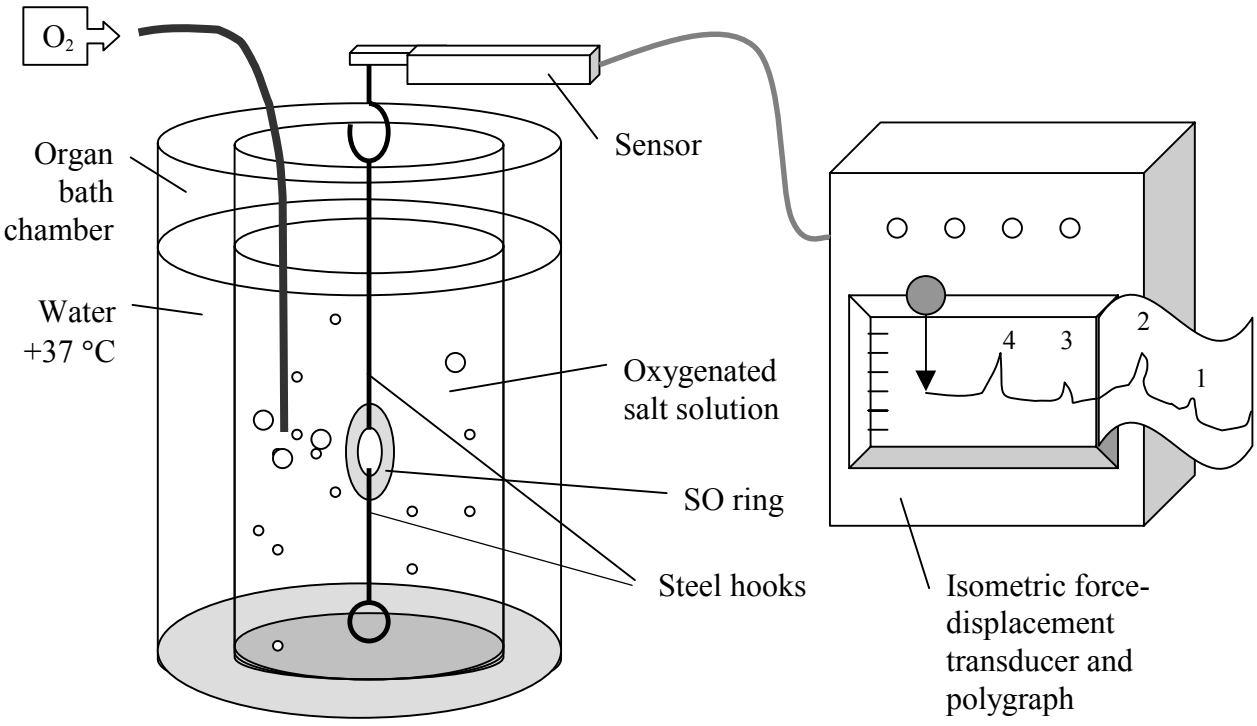

Figure 2. *The equipment for studies of the SO contractility ex vivo.*

Pig SO studies. To study the effect of T_4 on SO contraction, the following studies were undertaken. Group 1 - T_4 (0.1 nM 20 SO rings and 10 nM 34 SO rings) incubation for 30 minutes, whereafter KCl, ACh, and Hist responses were again elicited.

To study the specificity of the T_4 effect, the following procedures were performed: group 2 (5 SO rings) - T_3 (1 nM and 0.1 μM) incubation for 30 minutes, whereafter KCl, ACh, and Hist responses were again elicited. Group 3 (6 SO rings) - progesterone (0.1 μM) incubation for 30 minutes, whereafter KCl, ACh, and Hist responses were again elicited; group 4 (4 SO rings) - cortisone (1 μM) incubation for 30 minutes, whereafter KCl, ACh, and Hist responses were again elicited; group 5 (4 SO rings) - oestrogen (1 μM) incubation for 30 minutes, whereafter KCl, ACh, and Hist responses were again elicited; group 6 (4 SO rings) - testosterone (1 nM) incubation for 30 minutes, whereafter KCl, ACh, and Hist responses were again elicited.

The mechanism of action of the T_4 effect was elucidated as follows: dose dependence of the T_4 effect was studied by the following experiments: group 7 (7 SO rings): Incubation with T_4 concentrations of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM and 10 nM for 30 min, whereafter KCl, ACh, Hist responses were again elicited. The time course of the T_4 effect was studied as follows: group 8 (60 SO rings) - T_4 (10 nM) incubation for 2, 15, 30 and 120 min, whereafter ACh, Hist responses were again elicited.

To study the role of adrenergic and NO-ergic innervation on the T_4 effect the following experiments were performed: group 9 (7 SO rings) - propranolol (10 μM) and phentolamine (10 μM) together with T_4 (10 nM) incubation for 30 minutes, whereafter ACh and Hist responses were again elicited; group 10 (7 SO rings) - l-NAME (10 μM) together with T_4 (10 nM) incubation for 30 minutes, whereafter ACh and Hist responses were again elicited. To study whether the T_4 effect is neurally mediated or acting directly on smooth muscle, the role of the blockade of nerve-endings was studied by the following procedures: group 11 (7 SO rings) - tetrodotoxin (10 μM) together with T_4 (10 nM) incubation for 30 min, whereafter ACh, Hist responses were again elicited.

The role of new protein synthesis in the transcriptional or translational level and the need for prostaglandin synthesis were studied in the following experiments: group 12 (3 SO rings) - actinomycin D (10 μM) together with T_4 (10 nM) incubation for 30 minutes, whereafter ACh and Hist responses were again elicited; group 13 (8 SO rings) - cyclophosphamide (10 μM) together with T_4 (10 nM) incubation for 30 minutes, whereafter ACh and Hist responses were again elicited; group 14 (5 SO rings) - diclofenac (10 μM) together with T_4 (10 nM) incubation for 30 minutes, whereafter ACh, and Hist responses were again elicited.

The role of the K^+-channels was studied by the following experiments: group 15 (7 SO rings) - glibenclamide (10 μM) together with T_4 (10 nM) incubation for 30 minutes, whereafter ACh and Hist responses were again elicited; group 16 (3 SO rings) - glibenclamide (10 μM) incubation alone for 30 minutes, whereafter ACh, Hist responses were again elicited.

Human SO studies. To study the effect of T_4 on the human SO (8 SO rings), the SO rings were incubated with T_4 (10 nM) for 30 minutes, after which KCl and ACh responses were restudied.

Concentrations and dissolvents of the solutions. The concentrations of the KCl, ACh and Hist substances used had been selected previously (Sand et al. 1997 and 1998). Since to our knowledge ex vivo experiments with receptor antagonists and prostaglandin and protein synthesis inhibitors on SO contraction have not hitherto been made, sufficiently large concentrations of these substances were determined in preliminary procedures. The stock solutions were dissolved in dimethyl sulfoxide (DMSO) (T_4, T_3, glibenclamide), methanol (progesterone, cortisone, oestrogen, testosterone, cyclophosphamide, actinomycin D) or water (phentolamine, propranolol, l-NAME, diclofenac). All solutions were freshly prepared and protected from light. The effects of equivalent volumes of vehicles alone were also studied, and they were found to have no effect of their own on SO contraction.

Western blotting. The presence of TRs in the human SO was studied in Western blotting. The human cervical adenocarcinoma cell line (HeLa) lacks TRs, and was used as a negative control. Total cell lysates from HeLa cells and from human SO were prepared in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 30 μg/ml aprotinin, 1 mM sodium orthovanadate). HeLa cells were disrupted by passage through a 21 gauge needle and sphincter tissue by dounce homogenisation. Proteins were separated on 9% SDS-PAGE and transferred to nitro-cellulose membrane, blocked with 1 × TBS, 0.05% Tween 20, (TBST), 5% non-fat milk, and blotted with rabbit polyclonal anti-TRα antibody, which recognizes TRα, $TR\beta_1$ and $TR\beta_2$ (sc-772, Santa Cruz Biotechnology, Santa Cruz, CA; 1 μg/ml). The blotting was done in TBST followed by biotinylated goat anti-rabbit secondary antibodies (Dako A/S, Glostrup, Denmark), and streptavidin-biotin horseradish peroxidase-conjugate (Amersham Pharmacia Biotech, Buckinghamshire, UK). The detection of specific signal was performed using enhanced chemiluminescence.

3 Study IV. Bile flow to the duodenum is reduced in hypothyreosis and enhanced in hyperthyreosis in the rat

Animals. Eighty-one Sprague Dawley Hannover outbred male rats (age 13 weeks, weight 437±4 g (mean ± SEM), M&B A/S, Denmark) were divided into three groups. Euthyroid rats (n = 27, group 1) were kept on normal rat chow. Hypothyroid rats (n = 27, group 2) received thiouracil (about 37 mg/100 g body weight/day) in chow and water for four weeks to induce hypothyroidism. Hyperthyroid rats (n = 27, group 3) received thiouracil like those in group 2 plus T_4 (about 0.46 mg/100 g body weight/day) in chow for four weeks. Body weight and indirect blood pressure from the tail were measured before and after the four-week treatments.

Experiments. After the four-week diet the rats were anaesthetised with intraperitoneal 15% urethane (1 ml/100 g body weight). Blood pressure was measured directly from the carotid artery with a manometry catheter at the beginning of the experiment.

^{99m}Technetium (Tc) HIDA (0.7 ml, radioactivity about 0.2 μCi/ml) was injected into the jugular vein. Nine rats from each experimental group were exsanguinated at 15, 45, or 60 minutes after the ^{99m}Tc HIDA injection. Liver and intestine without mesenterium, from the superior part of the duodenum down to the rectum, were dissected off. A blood sample was taken. One ml of blood was collected in tubes containing ethylene diamine tetra-acetic acid (EDTA), and the rest, about 7 ml, was centrifuged at 3000 rpm for 10 minutes to obtain serum.

Measuring bile flow. The ^{99m}Tc-HIDA activity of both liver and intestine was measured for 60 seconds with an automatic gamma counter (CliniGamma 1272, LKB-Wallac, Turku, Finland) made for *in vitro* diagnostic purposes. Corrected counts per minute were used in calculations. The tissue activities were related to the weight of intestine and liver, respectively, in each animal. The amount of bile flow to the intestine was determined by counting the relative intestine versus liver radioactivity in each animal.

Liver histology. Liver histology was examined by light microscope from formalin-fixed, paraffin-embedded liver specimens by a liver pathologist without knowledge of the study group. The following stains were used: haematoxylin and eosin, Rhodanide for copper, and the Prussian blue method for iron.

Blood and serum determinations. Blood haemoglobin (Hb) was determined by electronic counter. The following serum determinations were made: FT_4 (immunofluorometric three-phase determination), liver function tests (alanine aminotransferase (ALT; method of the European committee for clinical laboratory standards), alkaline phosphatase (ALP; Scandinavian method) and total bilirubin (Bil; diatzo reaction method)), lipids (total cholesterol (T-Chol; enzymatic method), HDL-cholesterol (HDL-C; method of precipitation with phosphotungestane and magnesium chloride), LDL-cholesterol (LDL-C; Friedewald formula method) and triglycerides (TG; method of enzymatic determination of glycerol liberated by lipase)), calcium (Ca; o-Cresolphthalein method) and creatinine (Crea; Jaffe reaction method).

4 Study V. Is bile flow reduced in patients with hypothyroidism?

Patients. Eight female patients, median age 49 (range 29-66) years, with diagnosed untreated hypothyroidism (one patient) or total thyroidectomy performed due to papillary thyroid cancer (seven patients) were taken into the study. None had diagnosed gallbladder stones, and all had gallbladder in situ. Medical records of the patients were reviewed to study for all diagnosed diseases and treatments including medications and operations.

Methods. The patients were first examined in the hypothyroid stage, confirmed by serum TH values. In thyroid cancer patients this was 14-28 days after total thyroidectomy, prior to initiation of T_4 replacement therapy. The patients were examined again after euthyroidism had been achieved, as diagnosed by serum TH levels, a minimum of two months after the commencement of T_4 replacement therapy. Each patient thus served as her own control in the two stages of the study. Quantitative ^{99m}Tc HIDA cholescintigraphy, biliary ultrasonography and serum values (see below) were examined in each patient in the two stages of the study, the hypothyroid and the euthyroid stage.

Cholescintigraphy. Quantitative ^{99m}Tc HIDA cholescintigraphy was performed after overnight fasting. At the beginning of the examination, 150 MBq of ^{99m}Tc HIDA was injected into the antebrachial vein. Serial analogue images were obtained for 90 minutes at one-minute intervals with a gamma camera (Orbiter, model 6601, Siemens, Chicago, IL) made for clinical diagnostic purposes. After 20 minutes from the ^{99m}Tc HIDA injection, four decilitres of energy-rich drink (total 300 kcal, 26g fat, 71.6g carbohydrate; Nutridrink, N.V. Nutricia, Stockholm, Sweden) was taken p.o. to promote gallbladder emptying, as described in a previous study (Mäkinen et al. 1997). Appearance of radioactivity in the large bile ducts at the hilum, hepatic maximal uptake, hepatic clearance at 45 and 60 minutes, and the hilum-duodenum transit time (HDT) were measured.

Biliary ultrasonography. In biliary ultrasonography the size of the gallbladder, the width of the gallbladder wall and CBD, and the existence of stones and/or sludge in the gallbladder and/or CBD were studied.

Serum determinations. The following serum determinations were made by the same methods as mentioned above in study IV: S-FT_4, S-TSH, liver function tests (S-ALT, S-ALP), lipids (fS-T-Chol, fS-HDL-C, fS-LDL-C, fS-TG), fS-Ca, and, in addition, phosphorus (fS-P; phosphomolybdate, colorimetric method).

5 Statistics

Data are given as mean ± SEM when normally distributed, and as median and range when showing skew distribution. Fisher's exact test (study I), paired-samples t-test (studies II and III) and independent samples t-test (study IV) with two-tailed significance, Wilcoxon nonparametric test for related samples (study V), and one-way analysis of variance (studies II and III) were used to calculate the statistical significance of differences between the groups. Multivariate analysis (log-linear model) was performed to study the independent associations between the variables, which were significant in the univariate analysis (study I). Differences of $p < 0.05$ were considered significant. The statistic programs used in the analysis of results included BMDP (version 1990) on a SUN/UNIX mainframe and SPSS/Win (version 5.1).

6 Ethical aspects

The studies were conducted in accordance with the Helsinki Declaration. The study protocols were approved by the Ethical Council of Tampere University Hospital (studies I, III and V), the Animal Experimentation Committee of the University of Tampere (studies II and III), and the Animal Committee of the Social and Health Department of the Provincial Government of Western Finland (study IV).

RESULTS

1 Study I. Association between common bile duct stones and treated hypothyroidism

In group I (CBD stones) seven patients, two men and five women, out of the total 86 (8.1%) in the group were diagnosed to have hypothyroidism, whereas in the control group II only one female patient had diagnosed hypothyroidism (1.2%) (p=0.01). All these patients were on T_4 supplementation therapy. In both groups all patients with diagnosed hypothyroidism were over 60 years of age. In the patients of over 60 years of age the prevalence of hypothyroidism was 7/66 (11%) in group I and 1/66 (2%) in group II (p=0.01) (Study I, Table 2; Fig. 3). In Group I the highest prevalence of diagnosed hypothyroidism was in the age group 71 to 80 years, where five out of the 31 patients (16%) had diagnosed hypothyroidism (Study I, Table 1). In group III (gallbladder stone patients) only two of the 36 (6%) patients over 60 years of age had diagnosed hypothyroidism, that is, about half of the number in group I patients and about three-fold when compared to that among control group II patients of that age (Fig. 3). In group I eight of the 86 patients (9 %), all over 60 years of age, had been cholecystectomized a median 9 (2-18, range) years before the current diagnosis of CBD stones. Two of them had hypothyroidism, the diagnosis being made in both cases before the cholecystectomy.

In group I significantly more diagnosed biliary acute pancreatitis, hepatic diseases and gastrointestinal diseases other than hepatopancreaticobiliary were found, compared to group II (Study I, Table 3). In multivariate analysis CBD stone disease was independently associated with both hypothyroidism and biliary acute pancreatitis (p=0.001), but not with hepatic diseases or gastrointestinal diseases other than hepatopancreaticobiliary. In the frequency of other thyroid gland diseases apart from hypothyroidism, or in the frequency of any other diseases than those mentioned above, no significant difference was found between groups I and II.

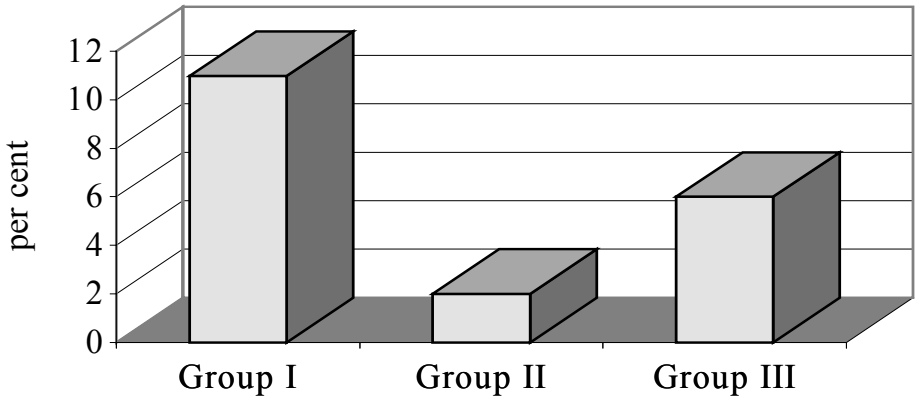

Figure 3. *The prevalence of hypothyroidism in CBD stone (group I; n=66), control (group II; n=66) and gallbladder stone (group III; n=36) patients over 60 years of age.*

2 Study II. The effect of thyroxine on pig sphincter of Oddi contractility

KCl, ACh and Hist induced strong contractions in the pig SO rings. The addition of T_4 with an incubation time of 30 minutes (group 1) did not influence the KCl-induced contractions, but the ACh- and Hist-induced contractions decreased by a mean 37%-44% and 54%-56%, respectively, as compared to contractions without T_4 (Fig. 4). Similar to T_4, also T_3 (group 2) had an inhibitory effect on the SO (Study II, Table 2 and Fig. 2).

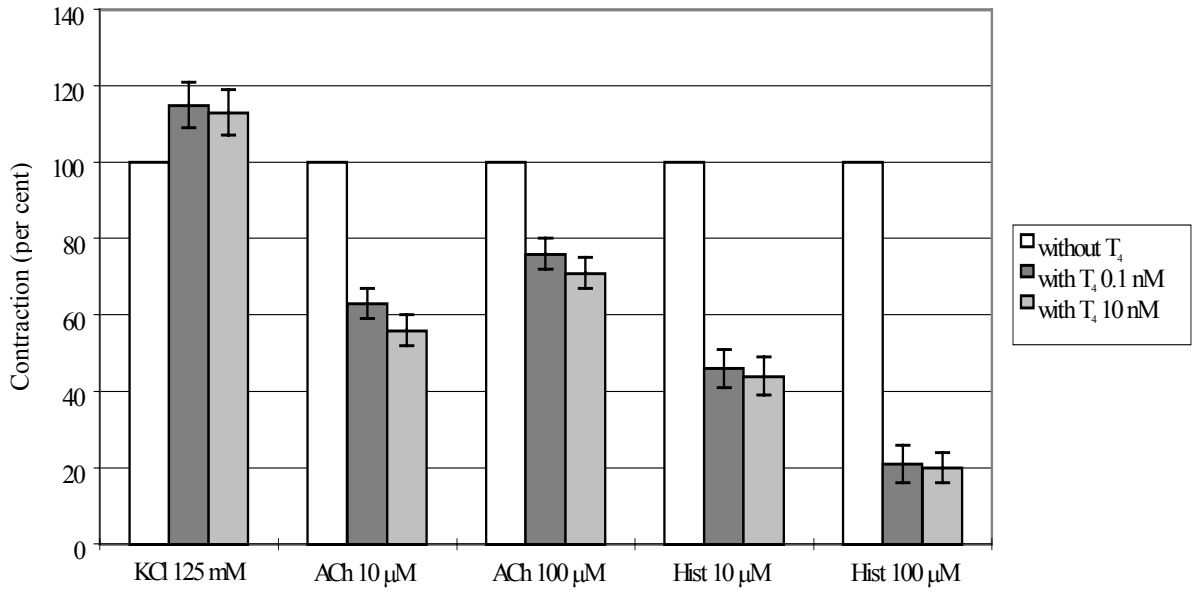

Figure 4. *The effect of T_4 (0.1 nM and 10 nM) on SO contraction (%; mean±SEM) compared to the control contractions (without T_4) in pig ex vivo. With T_4, KCl-induced contractions did not change significantly. ACh 10 µM induced contractions decreased from 726±64 mg to 376±43 mg (T_4 concentration 0.1 nM) and to 378±46 mg (10 nM). ACh 100 µM induced contractions decreased from 1410±105 mg to 913±89 mg (0.1 nM) and to 943±96 mg (10 nM). Hist 10 µM induced contractions decreased from 773±67 mg to 270±25 mg (0.1 nM) and to 297±46 mg (10 nM). Hist 100 µM induced contractions decreased from 1562±116 mg to 646±61 mg (0.1 nM) and to 799±96 mg (10 nM) (mean±SEM). Significance with regard to control contractions in all ACh and Hist induced contractions: $p<0.001$.*

3 Study III. The effect of thyroxine on human sphincter of Oddi contractility

In the human SO rings KCl and ACh induced strong contractions. The addition of T_4 (10 nM) with an incubation time of 30 minutes affected neither the resting SO tension nor the KCl-induced contractions, but the ACh-induced contractions decreased by a mean 49 % ($p<0.05$), as compared to those without T_4 (Study III, Fig. 1).

4 Studies II-III. The specificity and mechanism of action of thyroxine on sphincter of Oddi contractility

Of the steroid hormones studied, only progesterone (group 3) significantly reduced the KCl-, ACh- and Hist-induced SO contractions (34 %, 39-61% and 40-60 %, respectively) (Study II, Fig. 3). Cortisone (group 4), oestrogen (group 5) or testosterone (group 6) did not affect the contractions.

The dose-response studies with a T_4 (0.001 nM, 0.01 nM, 0.1 nM, 1 nM, or 10 nM) incubation of 30 minutes (group 7) showed a concentration of 0.1 nM to be the lowest to reach the maximal T_4 effect, smaller concentrations also having significant effects (Fig. 5).

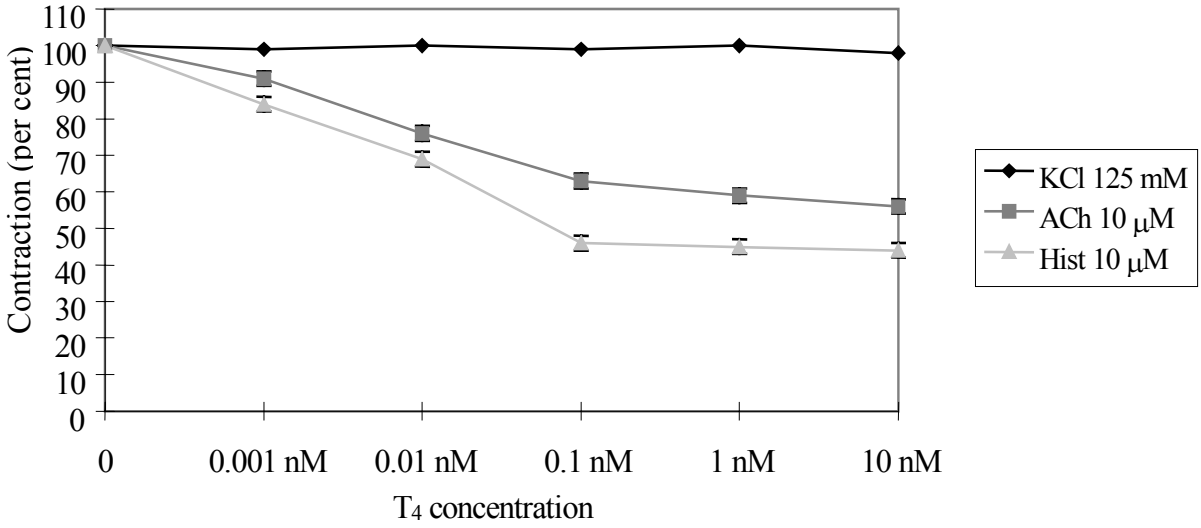

Figure 5. *The effect of different T_4 concentrations on SO contraction (%; mean±SEM) induced by various stimuli in pig ex vivo. With T_4, KCl-induced contractions did not change significantly. ACh-induced contractions decreased from 726±64 mg to 675±58 mg, $p<0.05$ (T_4 concentration 0.001 nM); to 551±49 mg, $p<0.01$ (0.01 nM); to 457±59 mg, $p<0.001$ (0.1 nM); to 428±60 mg, $p<0.001$ (1 nM); and to 407±42 mg, $p<0.001$ (10 nM) (mean±SEM). Hist-induced contractions decreased from 773±67 mg to 649±59 mg, $p<0.05$ (0.001 nM); to 533±52 mg, $p<0.01$ (0.01 nM); to 348±41 mg, $p<0.001$ (0.1 nM); to 340±46 mg, $p<0.001$ (1 nM); and to 337±39 mg, $p<0.001$ (10 nM) (mean±SEM).*

With an incubation time of 2 or 15 minutes T_4 (group 8) had no effect on SO contractility. The addition of T_4 with an incubation time of 30 minutes did not significantly influence the KCl-induced contractions, but the ACh- and Hist-induced contractions decreased by a mean 44% ($p<0.001$) and 52% ($p<0.001$), respectively, as compared to those without T_4. With a longer incubation time of 120 minutes the prorelaxing effect of T_4 on the ACh- and Hist-induced contractions was similar to that found with the 30 minutes incubation time. (Study III, Fig. 3)

The addition of the α-adrenergic receptor antagonist phentolamine, the β-adrenergic receptor antagonist propranolol (group 9) or the NO-synthesis inhibitor l-NAME (group 10) did not affect the T_4-induced inhibition of contraction. Blockade of the nerve endings with tetrodotoxin (group 11) was also without effect on the T_4-induced inhibition of contraction.

The addition of either transcription inhibitor actinomycin D (group 12) or translation inhibitor cyclophosphamide (group 13) partially reversed the T_4-induced inhibition of contraction (Study III, Figure IV). The addition of cyclo-oxygenase inhibitor diclofenac (group 14) had no effect in this regard. The addition of the K^+-channel blocker glibenclamide (group 15) totally reversed the T_4-induced inhibition of contraction (Fig. 6). Without T_4 glibenclamide (group 16) did not alter the SO contractility response to ACh and Hist.

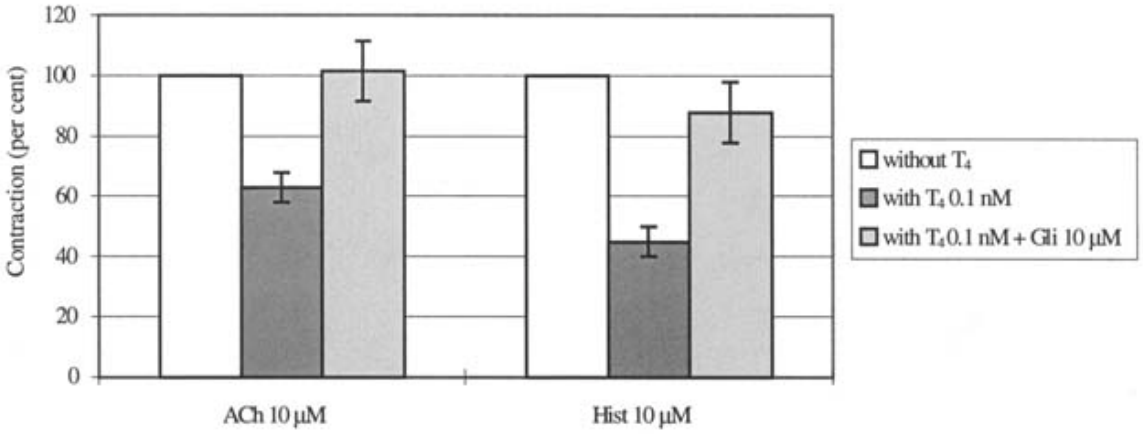

Figure 6. *The effect of T_4 (10 nM) and T_4 and glibenclamide (10 µM) on SO contraction (%; mean±SEM) induced by various stimuli in pig ex vivo. ACh induced contraction (726±64 mg) decreased with T_4 incubation (457±59 mg, $p<0.001$), and increased to the control level when incubated with T_4 and glibenclamide (737±141 mg). Hist induced contraction (773±67 mg) decreased with T_4 incubation (348±41 mg, $p<0.001$), and increased near to the control level when incubated with T_4 and glibenclamide (680±127 mg) (mean±SEM).*

Analysis of variance showed that there was difference in the SO contractions in group 1 (the effect of T_4), group 2 (the effect of T_3), group 3 (the effect of progesterone), group 7 (the effect of different T_4 concentrations), group 8 (the effect of different T_4 incubation times), group 12 (the effect of actinomycin D and T_4), group 13 (the effect of cyclophosphamide and T_4), group 15 (the effect of glibenclamide and T_4) and in the study with human SO rings ($p<0.001$).

In Western Blotting, the antibody recognized 53 kDa and 58 kDa proteins in the human SO tissue but not in the negative control HeLa cells (Study III, Fig. 6). The molecular weights of the proteins corresponded to the β_1 and β_2 isoforms of TR, respectively. α_1 isoform of TR with the molecular weight of 47 kDa was not recognised.

5 Study IV. Bile flow to the duodenum is reduced in hypothyreosis and enhanced in hyperthyreosis in the rat

In group I (euthyroid), group II (hypothyroid) and group III (hyperthyroid) rats blood pressure before the diet was similar (157±4 mmHg; mean±SEM). After the treatment, S-FT_4 was 47.6±1.6, 4.8±0.2 and >80 pmol/l in groups I, II and III, respectively (mean±SEM). After the four-week diet, indirectly measured blood pressure in group 2 was 6% lower and in group 3 13% higher than in group 1 (group 2 131±4, group 3 159±3, group 1 140±4 mmHg (mean±SEM; p=0.001 group 1 v. group 3). After induction of anaesthesia, blood pressures measured directly from the carotid artery did not differ between the groups (125±5 mmHg; mean±SEM).

After the treatment there was no difference in the weight of hyperthyroid (group 3) and euthyroid (group 1) rats (482±5 g), both gaining about 46 g during the study. Hypothyroid (group 2) rats maintained their weights throughout the four-week study period and weighed 430±4 g after the treatment (mean±SEM, p=0.001 group 2 v. group 1 and group 3). However, there was no difference between the three study groups in the weight of the liver (66±2 g), or intestine (67±2 g).

The highest ^{99m}Tc HIDA activity in the liver was measured at 15 minutes in each group, and it did not differ between the groups (Fig. 7). Relative activity intestine v. liver was 44 % lower in hypothyroid (group 2) rats compared to euthyroid (group 1) rats at 45 minutes (p=0.004 group 2 v. group 1). At 60 minutes the relative activity intestine v. liver was increased by 73 % in hyperthyroid (group 3) compared to euthyroid (group 1) rats (p = 0.002 group 3 v. group 1) and by 107 % compared to hypothyroid (group 2) rats (p = 0.001 group 3 v. group 2) (Fig. 7).

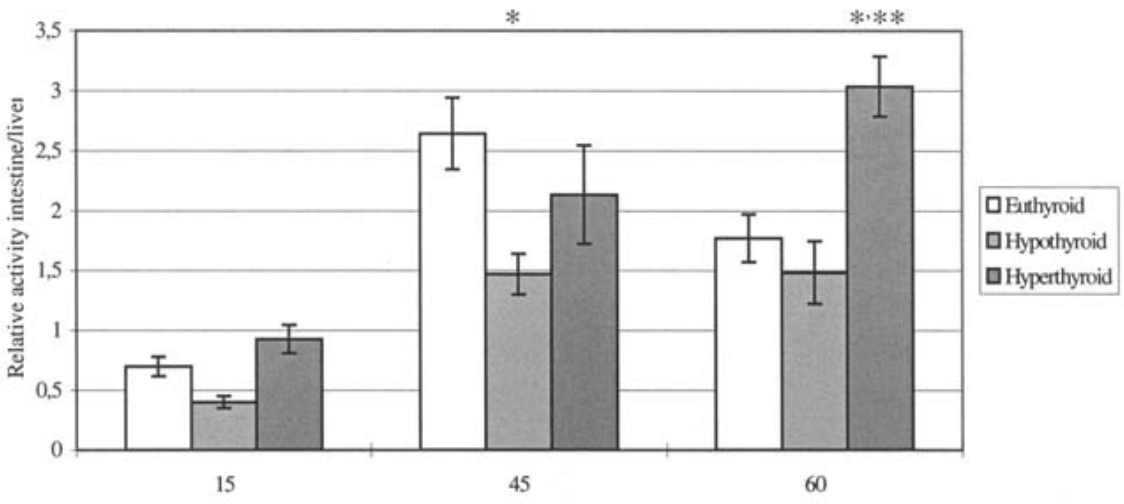

Figure 7. *Relative activity intestine v. liver in euthyroid, hypothyroid and hyperthyroid rats at 15, 45 and 60 minutes after Tc HIDA i.v. injection (mean ± SEM). Significance with regard to euthyroid rats in the time group: * $p < 0.005$. Significance with regard to hypothyroid rats in the time group: ** $p = 0.001$.*

Light microscopy of the liver showed a normal appearance in all rats. No morphological signs of cholestasis or cholangitis were seen in any of the groups.

In the hypothyroid (group 2) rats, S-ALP, S-T-Chol, S-HDL-C and S-LDL-C were significantly higher, and B-Hb significantly lower compared to the euthyroid (group 1) rats. In the hyperthyroid (group 3) rats S-ALP, S-T-Chol, S-HDL-C and S-TG were significantly higher, and B-Hb and S-Crea significantly lower compared to the euthyroid (group 1) rats. S-ALT and S-Bil did not differ between the study groups. (Study IV, Table I)

6 Study V. Is bile flow reduced in patients with hypothyroidism?

In the euthyroid stage all patients were clinically euthyreotic, although one had borderline values in S-FT_4 and S-TSH. In the hypothyroid stage the patients studied had low S-FT_4 levels (4.5 (3.8-6.4) v. 18.2 (14.5-26.9) pmol/l; $p<0.001$; median and range) and high S-TSH levels (75.6 (41.0-180.0) v. 0.6 (0.1-2.4) pmol/l; $p=0.002$; median and range) compared to the euthyroid stage. fS-T-Chol was significantly increased and above the normal range in the hypothyroid stage when compared to the euthyroid stage. FS-TG, fS-HDL-C, fS-ALT, fS-ALP, fS-Bil, fS-Ca and fS-P were within the normal range in the both stages of the study (Study V, Table I).

In quantitative ^{99m}Tc HIDA cholescintigraphy, the appearance of radioactivity in the large bile ducts at the hilum (8 (2-12) v. 9 (3-10) minutes from the beginning of the experiment; median and range) and the hepatic maximal uptake (21 (16-31) v. 21 (16-28) minutes; median and range) were similar in the hypothyroid and euthyroid stages. Hepatic clearance was decreased in the hypothyroid stage at 45 minutes (28 (11-38) v. 50 (33-54) %; $p=0.028$; median and range) and at 60 minutes (55 (28-80) v. 69 (61-79) %; $p=0.028$; median and range) compared to the euthyroid stage (Fig. 8). The HDT tended to increase from the median 13 (4-22) minutes measured in the euthyroid stage by 31 % in the hypothyroid stage (17 (5-27); median and range), although the difference did not reach statistical significance. In biliary ultrasonography, no changes were seen in the gallbladder or bile ducts in the hypothyroid compared to the euthyroid stage. The size of the gallbladder, the thickness of the gallbladder wall and the diameter of the CBD were normal in all patients in both the hypothyroid and euthyroid stages and without difference between the two stages. No stones or sludge were seen in the gallbladder or CBD in any of the patients during this study of a minimum of two months.

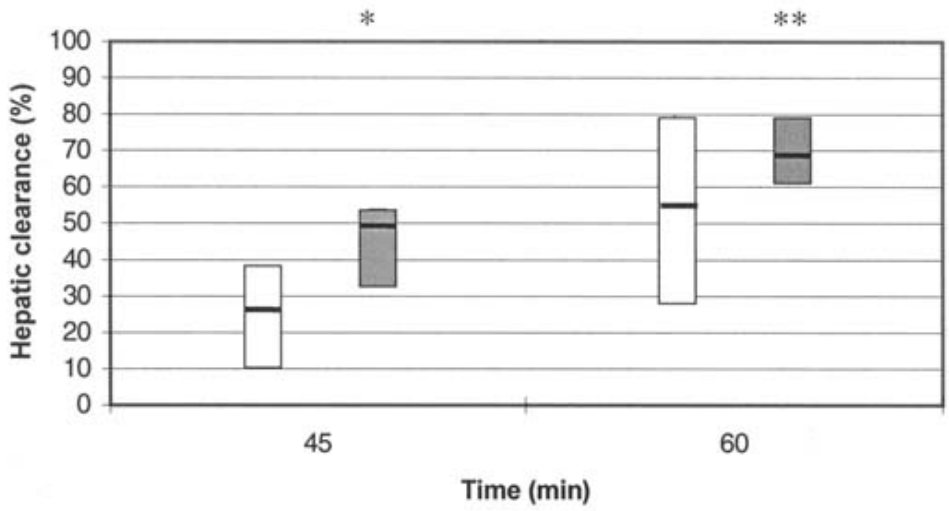

Figure 8. *Hepatic clearance (%) at 45 and 60 minutes in the study patients in hypothyroid* □ *and euthyroid* ■ *stages (median and range). Significance with regard to hypothyroid stage: * p=0.009, ** p=0.025.*

DISCUSSION

1 Methods for investigation of biliary motility

The SO has a major role in regulating the passage of about 1 litre of bile and 2 litres of pancreatic juice into the duodenum daily. SO stenosis or SO dyskinesia may lead to a mechanical obstruction of the biliary tract, causing biliary stasis and ultimately the formation of CBD stones (Osnes et al. 1981, Sandstad et al. 1994, Thistle 1998). In recent years knowledge of the normal and abnormal motility of the SO has increased by applications of electromyographic, radiographic, scintigraphic and endoscopic manometric techniques, and by investigations evaluating the interplay between hormonal and neural factors controlling SO motility (Toouli and Baker 1991, Coelho and Wiederkehr 1996).

Techniques for studying SO motility in vivo are complicated. Direct endoscopic biliary manometry is considered the gold standard in assessing possible SO dysfunction (Becker 1993). However, the disadvantages of SO manometry include the facts that the approach is invasive and difficult to perform, and has the same potential for complications as ERCP, the risk of acute pancreatitis being even higher than after diagnostic ERCP (Lehman 1991). Therefore, SO manometry should not be performed routinely in ERCP (Coelho and Wiederkehr 1996), and it cannot be used for studying SO motility in patients without biliary symptoms.

Monitoring of SO electrical activity in vivo by electromyography (EMG) has proved somewhat unreliable. In the pig the phasic contractions of the SO are difficult to detect during the resting period, since continuous monitoring is hampered by interference from respiratory movements (Coelho et al. 1985, Sand et al. 1997).

Laboratory tests in evaluating possible SO dysfunction are neither sensitive nor specific. Elevated serum values of hepatic and/or pancreatic enzymes may occur in patients with

SO dysfunction, as well as in those with other disorder, such as liver or pancreatic disease, but they may also be normal in patients with proved dysfunction of the SO (Coelho and Wiederkehr 1996).

Several imaging approaches have been used to study the SO. These tests in question are not invasive, but they lack specificity and sensitivity (Coelho and Wiederkehr 1996). Determination of the CBD diameter in ultrasonography after administration of a fatty meal or CCK-octapeptide has been proposed to evaluate SO function (Fein et al. 1984). This test is safe and inexpensive, but operator-dependent (Coelho and Wiederkehr 1996).

Dynamic cholescintigraphy is useful in identifying patients with partial CBD obstruction, including those with benign SO stenosis or dyskinesia (Persson et al. 1993). It is non-invasive and highly sensitive, and has been suggested as an early diagnostic procedure to evaluate patients with clinical suspicion of SO stenosis or dyskinesia (Coelho and Wiederkehr 1996). The hilum time-activity curve, the 45-minute clearance value and the HDT are the most useful tools to differentiate normal subjects from those with partial biliary obstruction (Persson et al. 1993, Kalloo and Pasricha 1996, Sand et al. 1999). ^{99m}Tc HIDA has been shown to possess high hepatobiliary specificity, low renal extraction and rapid hepatocellular transit in humans and in the rat (Rosenthal et al. 1980, Nunn et al. 1983). Distribution studies made in organs removed from rats at 30 minutes after ^{99m}Tc HIDA i.v. injection show that at this time the distribution of injected radioactivity is essentially complete in normal rats, that is, little or no distribution of radioactivity is to be detected elsewhere in the body except liver and gastrointestinal tract (Nunn et al. 1983). In humans the highest radioactivity is found in the liver around 15 minutes after injection (Mäkinen et al. 1998). Hepatic clearance is commonly studied at 45 or 60 minutes after ^{99m}Tc HIDA injection in cholecystectomized patients (Mäkinen et al. 1998, Sand et al. 1999) as well as in patients with gallbladder in situ (Mäkinen et al. 1997). In these last-mentioned the gallbladder contraction may be stimulated by an energy-rich drink taken p.o. 20 minutes after the ^{99m}Tc HIDA injection, to avoid the

retention of bile in the gallbladder, which might disturb measurements (Mäkinen et al. 1997).

Ex vivo the SO offers a useful model for studying the effect of different physiological and pharmacological agents on SO motility. However, the motility change may vary with the resistor-like SO (type II; human, pig and cat), and the pump-like SO (type I; guinea pig, opossum, Australian possum, rabbit and prairie dog) (Sand et al. 1997), and thus results from the two categories of SO must not be generalized. In many previous studies the drug doses used have been more pharmacological and not physiological, and results must be interpreted with caution. The physiological impact of several agents on the SO is not yet clear (Coelho and Wiederkehr 1996).

In the present study the effect of T_4 on the pig and human SO contractility was assessed. Because it is rather difficult to obtain suitable human SOs, the pig SO was chosen because of its similarities to the human counterpart (Sand et al. 1993a and 1993b, Mourelle et al. 1993, Sand et al. 1994, Slivka et al. 1994). The method used to analyse the effects of hormones and drugs on SO contractions, in which SO rings are placed between two stainless steel hooks and suspended in an organ bath chamber in oxygenated salt solution, has also previously been successfully employed in the smooth-muscle testing (Kähönen et al. 1994, Sand et al. 1997, Sand et al. 1998). To be able to study the relaxation of the SO in this in vitro preparation, the SO must be precontracted. KCl, ACh and Hist were used to induce the contraction in the smooth muscle of the SO. Each of these three substances represents a different kind of mechanism to induce the contraction, a high concentration of KCl being an unspecific smooth-muscle contractor causing direct cell membrane depolarization, while ACh causes contraction by binding to the muscarinic receptors and Hist by binding to the histamin-1 receptors (Sand et al. 1997, Sand et al. 1998, Sand et al. 2000).

Biliary dynamics in relation to altered thyroid gland function were investigated in humans and in rats. Unlike humans, the rat has a pump-like SO. However, when studying the SO dynamics in total the rat offers an excellent possibility since it does not have a gallbladder. A variety of methods have been used to investigate changes in the bile

composition and secretion, and in the rate of bile flow from the liver into the bile ducts in the rat. In previous studies cannulation of bile ducts has been used to collect bile, and bile flow has been measured either directly from the amount of bile collected during a certain time period (Field et al. 1986a, Adreini et al. 1994) or gravimetrically (van Steenbergen et al. 1989). Such methods were not suitable for the present purpose of investigating the bile flow from the bile ducts into the intestine, because cannulation of the duct blocks out the possible regulating effect of distal bile ducts, i.e. the SO. In this study ^{99m}Tc HIDA was therefore injected i.v., and study made of the radioactivity of liver and intestine removed at 15, 45 and 60 minutes after the injection. Bile flow was then measured by counting relative intestine v. liver activities. However, there are also certain disadvantages in this method. In the rat ^{99m}Tc HIDA is rapidly excreted into the intestine, and thus hepatic maximal uptake is not easy to determine. In addition, because the method for measuring radioactivity in the tissue specimen is not very accurate, very small changes in the bile flow cannot be detected. A sufficient number of animals must thus be used, as was done in the present study. Preparation of the intestine must be done very carefully so that no content and thus no radioactivity is wasted.

In humans biliary dynamics was studied in relation to altered thyroid gland function by cholescintigraphy. All patients involved had gallbladder in situ. To minimize and standardize the effect of the gallbladder on bile flow, the bladder was contracted by a standard drink at 20 minutes after ^{99m}Tc HIDA i.v. injection, as in a previous study in healthy volunteers (Mäkinen et al. 1997). The energy-rich drink may also to some extent cause the opening of the SO, which may interfere with the measurements, but this possible effect is presumably similar in the hypothyroid and euthyroid stages of the study, and thus hardly explains the differences found in bile flow. Even though the effect of the gallbladder was minimized by the standard stimulation of contraction, it may still have some effects on the results. To ensure that the investigations of biliary dynamics in hypothyroidism and euthyroidism were well comparable, measurements were made in the same patients in the two stages, each patient thus serving as her own control. Because diseases of the biliary tract could affect the results, such pathologies were ruled out by ultrasonography and liver chemistry. When analysing the results of cholescintigraphy by

computer, determination of the areas of liver, hilum and gallbladder must be made by hand. Because of the long study period, the movement of the patient may also affect the situation of the organs studied. In the present case these disadvantages were minimized by having experienced doctors perform the research and analyse the results.

2 Association between common bile duct stones and treated hypothyroidism

In clinical practice it was recognized that a number of patients with CBD stones had hypothyroidism in their medical history. In view of the lack of previous studies on this topic, the possible relation between CBD stones and diagnosed hypothyroidism was investigated in a case-controlled retrospective study. It was noted that the CBD stone patients had seven times more diagnosed hypothyroidism than the control patients. In patients over 60 years of age there was significantly less diagnosed hypothyroidism in the gallbladder stone group than in the CBD stone group but significantly more than in the control group.

In the gallbladder stone patient group (group III) only those patients with whom the CBD stones were judged very unlikely by liver chemistry plus ultrasound and/or magnet cholangiography were taken into the study, to create a group of patients with exclusively gallbladder stones. In the classic case of CBD stones, there is an elevation of the serum Bil and ALP, with minimal elevations of ALT (Jordan 1982, Way and Sleisenger 1989). However, in some CBD stone patients there may be no abnormal laboratory determinations (Jordan 1982). In ultrasonography the dilatation of bile ducts, signifying ductal obstruction, but rarely the CBD stones can be detected. The sensitivity of ultrasound in detecting obstruction is about 85% (Way and Sleisenger 1989). When positive, the reliability is extremely high (Jordan 1982). However, a negative result does not prove the absence of stones or obstruction. Magnet cholangiography produces a high degree of accuracy with pathology delineated in over 80% of patients in whom a positive test is recorded (Way and Sleisenger 1989). The gallbladder stone patients taken into the study had normal liver chemistry and no signs of CBD stones in ultrasound and/or

magnet cholangiography, but, despite this, it is possible that some of the CBD stones may have been overlooked.

The CBD stone patients with diagnosed hypothyroidism were receiving T_4 replacement therapy and were already clinically euthyreotic at the time of the diagnosis of CBD stones. Thus, CBD stones may have formed during the period of undiagnosed hypothyroidism, later T_4 replacement therapy having no effect on stones already formed, or T_4 replacement therapy might even play a role in the forming of CBD stones. Extremely high doses of T_4 have namely been reported to induce gallbladder stones in hamsters (Bergman and van der Linden 1966). However, there are no previous data to indicate that therapeutic doses of T_4 are associated with gallstone formation; in fact in one patient report gallstones have been shown to disappear after treatment with T_4 (Vassilakis and Nikolopoulos 1981). The 11% prevalence of previously diagnosed hypothyroidism in the CBD stone patients over 60 years of age would suggest that CBD stone patients in this age group should be screened for current thyroid dysfunction.

THs are known to have a number of effects on cholesterol metabolism (Andreini et al. 1994). When serum cholesterol values rise in hypothyroidism, bile may also become supersaturated with cholesterol, leading to gallbladder hypomotility (Donovan 1999), depressed contractility (Behar et al. 1989) and impaired filling (Jazrawi et al. 1995), giving rise to a prolonged residence of bile in the gallbladder. This may contribute to retention of cholesterol crystals in the organ, thereby allowing sufficient time for nucleation and continual growth into mature gallstones (Donovan 1999). In addition, the rate of bile secretion may be decreased (Field et al. 1986), physically impairing clearance of precipitates from the bile ducts and gallbladder. If the effect of T_4 or the absence of T_4 affected only the cholesterol metabolism and the hepatic bile secretion, the patients with gallbladder and CBD stones would presumably evince an equally increased prevalence of diagnosed hypothyroidism. In the current study it was, however, noted that the CBD stone patients had two times more diagnosed hypothyroidism than the gallbladder stone patients. This might be due to the effect of T_4 on the function of the SO. A group of

studies was therefore performed to further investigate the mechanisms behind this association between CBD stones and hypothyroidism.

3 The effect of thyroxine on sphincter of Oddi contractility

The effect of T_4 on the SO precontracted by KCl, ACh or Hist was investigated ex vivo. T_4 did not affect the unspecific high concentration KCl-induced SO contraction, but it did reduce the receptor-mediated ACh- and Hist-induced SO contraction, which suggests a direct effect on the control mechanisms of SO motility. Because the effect of T_4 on the SO is prorelaxing, a lack of T_4 may result in an increased tension of the SO.

To study the specificity of the effect of T_4, the effects of T_3, progesterone, cortisone, oestrogen and testosterone on the precontracted SO were studied. T_3 had a similar effect to T_4, whereas cortisone, oestrogen and testosterone had no effect. Thus the effect of THs is not an unspecific effect of any hormone. Progesterone reduced the KCl-, ACh- and Hist-induced SO contractions. Since progesterone also affected the high concentration KCl-induced contraction this effect seems to be fairly unspecific. This is not, however, surprising since progesterone is thought to be involved in the smooth-muscle relaxation seen in pregnancy (Fomin et al. 1999).

To study the mechanism of action of the T_4 on SO contraction, various concentrations and incubation times of T_4 were studied. The T_4 concentrations used (0.001 - 10 nM) were below, within and above the physiological concentrations measured in humans. The effect of the physiological T_4 concentration (0.01 nM) on SO contraction was remarkable, and could only be doubled when using 10, 100 and 1000 times over the physiological. A concentration 10 times the physiological (0.1 nM) was the lowest to induce the maximal effect on the precontracted SO, which was why this concentration was chosen for all the subsequent studies. As physiological concentrations of T_4 have prorelaxing effects on the SO ex vivo, T_4 may influence SO tone also in vivo. An incubation time of 30 minutes with T_4 was sufficient to create the maximal prorelaxing effect on the SO, while no effect was seen with shorter 2- or 15-minute incubation times.

Thus, the underlying cellular mechanisms involved do not act immediately but require a certain time lag. In the action of TH, TH enters the cell, passes through the cytoplasm, locates itself in the nucleus, and binds to a nuclear protein, TR. These are relatively fast events whereas the regulation via time-consuming transcription and translation probably explains the time lag required for the effect of T_4 on the SO.

T_4 could exert its effect either via the nerves or directly the smooth muscle of the SO. Therefore the prorelaxing adrenergic and NO-ergic mechanisms were studied by specific inhibitors as well as by a neurotoxin. α- and β-adrenoceptor antagonists, NO-synthesis inhibitor, and the elimination of the nerve function with tetrodotoxin did not affect the T_4-induced prorelaxation of SO. These results, together with the finding that a certain time lag is required for the prorelaxing effect of T_4 on the SO, strongly suggest that other than direct neural effects are involved.

To study whether T_4 might act via TR binding and TH responsive genes, we tested whether protein synthesis at transcriptional or translational level was required for the prorelaxing T_4 effect. Experiments demonstrated that at least some protein synthesis at the mRNA level is required for the effect of T_4. Thus, the effect of T_4 could be mediated by regulatory proteins partly synthesized as a result of T_4-induced gene expression.

Prostaglandins can relax smooth muscle (Beech 1997), and oestrogen is one hormone known to affect prostaglandin synthesis (Wakasugi et al. 1989). Thus, the hypothesis that T_4 could act indirectly via the modulation of prostaglandin synthesis was also tested. However, prostaglandin synthesis inhibition did not affect the T_4-induced prorelaxation of the SO, and it can be concluded that prostaglandin synthesis is not required in the mediation of the prorelaxing effect of T_4.

Besides directly Ca^{2+}-channels, also K^+-channels can be modulated by neurotransmitters and other messengers in smooth-muscle cells. These effects are often functionally important in the whole tissue (Shepard and Eberhardt 1993). In a previous study with rat skeletal muscle arteries, the ATP-sensitive K^+-channel blocker glibenclamide attenuated

T_3-induced vasodilatation, and thus vasodilatation may be mediated by ATP-sensitive K^+-channels (Park et al. 1997). Therefore, it was investigated whether the effect of T_4 was mediated via ATP-sensitive K^+-channels. Glibenclamide totally reversed the T_4-induced SO prorelaxation, but glibenclamide alone was without any effect on SO contractility. Thus, the effect of T_4 on the SO smooth muscle appears to be mediated via the opening of ATP-sensitive K^+-channels. This results in hyperpolarization, which closes all membrane Ca^{2+}-channels, and reduces Ca^{2+} influx, allowing only reduced contraction of the smooth-muscle (Allen and Walsh 1994).

When testing the effect of different antagonists and inhibitors, it is important to use adequate concentrations to induce a sufficiently high level of inhibition. Otherwise negative results cannot be considered reliable. Therefore, in the present study of the receptor antagonists and protein and prostaglandin synthesis inhibitors a high concentration of 10 μM was applied. Thus, the present negative results can hardly be due to insufficient doses of blockers.

Because the response of the SO to T_4 may vary between different animals, the effect of T_4 was studied also on human SO. A similar prorelaxing effect of T_4 shown in the pig SO was also observed in the human SO. The observation is thus not ”pig-specific”, but may also be of clinical significance, as suggested by the retrospective study I.

The time course and the transcription - translation antagonist studies suggested that the T_4 effect might be exerted via TRs. To study whether of TRs are expressed in the SO, an analysis was made by immunoblotting, and the antibody recognised 53 kDa and 58 kDa proteins in the SO tissue but not in the negative control HeLa cells. The molecular weights of the proteins corresponded to the β_1 and β_2 isoforms of TR, respectively. Both TR β_1 and β_2, but not α_1, would thus appear to be expressed in the human SO. Previously TR β_1 has been identified to be highly expressed in the liver and kidney e.g. in human, and TR β_2 in the pituitary (Chatterjee and Tata 1992).

The presence of TRs in the SO is a prerequisite, but does not serve as evidence, that T_4 exerts its prorelaxing effect via a hormone-receptor complex action. However, no specific TH receptor antagonist exists, excluding such inhibitor studies. The TR antibody used for immunoblotting cannot serve as such an antagonist in functional organ bath studies, since its large molecular weight prevents it from entering the smooth-muscle cell. Furthermore, the TR antibody does not inhibit binding of TH to TR, but recognizes both bound and unbound forms of TRs (Lazar 1993).

In summary of these findings, T_4 has a direct prorelaxing effect on the human SO, which expresses TR β_1 and β_2. This effect is mediated through a mechanism requiring new mRNA and protein synthesis and leading subsequently to the activation of K^+ channels.

More specifically, on the basis of what is generally known of hormone action and the regulation of smooth-muscle contraction, the hypothesis is the following: T_4 binds to the nuclear receptor demonstrated here to be expressed in the SO smooth muscle cell. Through interaction with this receptor, gene transcription is promoted. The mRNA formed turns into a protein, or a group of proteins, which regulate the opening of cell membrane K^+ channels. The opening of these channels is followed by hyperpolarization, which closes cell membrane Ca^{2+} channels and reduces Ca^{2+} influx, and results in reduced contraction of the SO smooth-muscle cell in response to any specific stimulus.

The studies discussed above have suggested that CBD stones are associated with previously diagnosed hypothyroidism and that one possible explanation for this is slow bile flow resulting from the reduced prorelaxing effect on the SO by diminished T_4. To establish whether hypothyroidism really affects the bile flow, rat and human ^{99m}Tc HIDA studies were performed.

4 Bile flow in relation to altered thyroid function in the rat

In the rat the highest ^{99m}Tc HIDA activity in the liver was measured at 15 minutes after administration in each group, and it did not differ between the study groups. Relative activity intestine v. liver was at 45 minutes decreased by 44 % in hypothyroid rats compared to euthyroid rats, and at 60 minutes increased by 73 % in hyperthyroid compared to euthyroid rats and by 107 % compared to hypothyroid rats. Although liver circulation was not measured, when taking into account that ^{99m}Tc HIDA is uptaken almost entirely by the liver, and the weight of the organs, blood pressure at injection, and the maximal uptake of radioactivity by the liver were similar between the groups, changes in the distribution of circulation are very unlikely explanations for the observations made. The findings strongly indicate that the bile flow to the duodenum is reduced in hypothyroid rats and enhanced in hyperthyroid rats. This may be explained by the possible changes in the regulation of bile duct emptying, e.g. the function of the SO, this conception being supported by the ex vivo organ bath studies (studies II and III), but also by the possible changes in the bile composition and in the bile excretion rate.

Since no signs of cholestasis or cholangitis were seen under light microscopy, intrahepatic cholestasis is an improbable explanation for the observations. There was no difference in ALT and Bil values between the study groups, which refers to normal hepatocytic function, but ALP was increased in the hypothyroid rats. ALP isoenzymes were not measured, which is why also ALP of bone origin may explain the change. In fact, a moderate increase of ALP values in hyperthyroidism could well be because of the possible increase in bone turn-over, but is unlikely the case in hypothyroidism. Therefore, the high ALP values measured in hypothyroid rats may rather be considered as a sign for partial or functional biliary obstruction. S-Ca did not differ between the study groups excluding hypocalcemia explaining the difference.

Serum cholesterol levels were significantly higher in hypothyroid animals, as has previously been shown (Field et al. 1986a), and can be assumed because of the known effects of T_4 on cholesterol metabolism (Andreini et al. 1994). S-TG was within normal

range in hypothyroid animals, but higher in hyperthyroid animals, a finding which was also reported in a previous study (Field et al. 1986b). Serum lipid determinations were not undertaken after fast, which must be taken into account when interpreting the results. The groups were, however, also comparable in this respect.

Bile composition was not measured in these animals. Any changes in bile composition associated with serum lipid alterations might also be a co-factor underlying changes in biliary dynamics. In hypothyroidism bile may become supersaturated with cholesterol and because of the viscous nature it may flow more slow to the duodenum. As supersaturated bile may cause hypomotility and depressed contractility of the gallbladder (which the rat does not have) (Donovan 1999, Behar et al. 1989), one could assume that it might have effects on SO contractility as well. The possible net effect of T_4 and supersaturated bile on SO motility is not known, but it is possible that the impaired relaxation of SO due to the lack of T_4 in hypothyroidism could be further impaired by supersaturated bile.

Hepatocytic bile secretion rate may be decreased in hypothyroidism (Gebhard and Prigge 1992, Gebhard et al. 1992). In the previous studies bile excretion rate measured by cannulating bile ducts proximal to the SO was found to be decreased by 24-50% in the hypothyroid compared to the euthyroid rats (Field et al. 1986, Van Steenbergen et al. 1989), but no effect on the bile excretion was seen in the hyperthyroid rats (Van Steenbergen at al. 1989). Cannulation blocks out the regulating effect of distal bile ducts, i.e. the SO. In the present study where the effects of T_4 on the SO are not excluded, both hypo- and hyperthyroidism altered the bile flow to duodenum. Therefore, the effect hardly is explained only by the altered hepatocytic excretion rate.

5 Bile flow in relation to altered thyroid function in humans

In humans hepatic clearance was significantly decreased and the HDT tended to increase in the hypothyroid compared to the euthyroid stage. Because the hepatic maximal uptake and the appearance of radioactivity in the large bile ducts were similar in the two stages of the study, the findings are not likely to be explained by different liver circulation and

^{99m}Tc HIDA accumulation, but strongly suggest that the bile flow into the duodenum is reduced in the hypothyroid stage. As in the rat, in addition to reduced bile flow regulated by SO, this may be explained by possible changes in the bile composition and in bile excretion rate, but also by the effects on the gallbladder, which is lacking in the rat but was in situ in all human subjects studied.

In biliary ultrasonography no changes were seen in gallbladder or bile ducts in the hypothyroid stage compared to the euthyroid stage. Thus, the 2-4 week period of hypothyroidism following thyroidectomy is not long enough to cause for example the formation of such gallbladder or CBD sludge or stones, or dilatation of bile ducts, which could be detected in transcutaneous ultrasonography.

In the hypothyroid stage the study patients had hypercholesterolaemia, as can be assumed from the known effects of T_4 on cholesterol metabolism (Andreini et al. 1994). The cholesterol levels in bile were not studied. In hypothyroidism serum hypercholesterolaemia may cause bile to become supersaturated with cholesterol and because of its viscous nature bile may also flow slower to the duodenum. Supersaturated bile may lead to gallbladder hypomotility (Donovan 1999), depressed contractility (Behar et al. 1989) and impaired filling (Jazrawi et al. 1995), giving rise to a prolonged retention of bile in the gallbladder. However, the biliary hypercholesterolaemia is hardly the only explanation for increased gallstone formation in hypothyroidism, because in the retrospective study I, CBDs were more strongly associated with hypothyroidism than were the gallbladder stones. As in the rat, it is also possible in humans that the impaired relaxation of SO due to the lack of T_4 in hypothyroidism could be further impaired by supersaturated bile.

The rate of bile secretion may be decreased in hypothyroidism (Field et al. 1986). However, in human subjects the appearance of radioactivity in the large bile ducts was found to be similar in the hypothyroid and euthyroid stages of the study, implying that reduced excretion of bile from the hepatocytes in hypothyroidism is not a likely explanation for the reduced bile flow. Thus, unlike in the study with rats, in this study

with human subjects the possible changes in the bile excretion rate seems not to be the explanation for the altered bile flow.

T_4 may also have effects on gallbladder contractility, but, to our knowledge, this has not so far been studied. When relaxing SO, T_4 could contract the gallbladder, and thus in hypothyroidism gallbladder contraction could be impaired, leading to reduced bile flow and gallstone formation, as is also the case because of the effect of supersaturated bile on the gallbladder (see above). However, this kind of effect of the lack of T_4 on the gallbladder should lead to increased prevalence of gallbladder stones over the CBD stones, which was not the case in the retrospective study I. Furthermore, the reduced bile flow in hypothyroidism was seen also in the rats without the gallbladder. In the study with human subjects the effect of gallbladder was standardised and minimised by an energy-rich drink. Even though the contraction of the gallbladder would be decreased in the hypothyroidism, it could not explain the finding that hepatic clearance was decreased in the hypothyroid stage of the study. Therefore, most likely, the diminished bile flow in hypothyroidism is not explained merely by any single mechanisms alone, but by the combination of the multiple mechanisms. The more frequent occurrence of CBD stones compared to gallbladder stones in hypothyroid patients is the strongest support for a high clinical impact of the reduced prorelaxing effect on SO in hypothyroidism.

In conclusion, it seems likely that the lack of T_4 in hypothyroidism gives rise to a reduction in bile flow in many ways. In addition to the increased cholesterol load in bile and the reduced bile excretion rate, which have been reported previously, the deficiency of the prorelaxing effect of T_4 on the SO appears to be a crucial factor leading to the reduced bile flow in hypothyroidism.

SUMMARY AND CONCLUSIONS

In the present study the association between CBD stones and hypothyroidism was established, which led to further studies on the effect of T_4 on the human and pig SO and the specificity and the mechanisms of this effect. The net impact of altered thyroid function on biliary motility was studied both in rats and in human subjects.

The major findings and conclusions were as follows:

1 In the retrospective study the CBD patients had significantly more diagnosed hypothyroidism compared to the gallbladder stone patients or to controls. The higher prevalence of hypothyroidism in the CBD stone patients compared to the gallbladder stone patients suggests that also factors other than merely changes in the cholesterol metabolism or bile excretion rate, particularly changes in the function of the SO, may be behind the association between CBD stones and hypothyroidism.

2 In the pig T_4 had no effect ex vivo on unspecific, high-concentration KCl-induced SO contraction but did reduce the receptor-mediated ACh- and Hist-induced SO contraction, which suggests a direct effect of T_4 on the control mechanisms of SO motility. Because the effect of T_4 on the SO was prorelaxing, the absence of T_4 might result in an increased tension in the SO.

3 A similar prorelaxing effect of T_4 on SO contractility observed in the pig was also found in human SO specimens. This observation is thus not ”pig-specific”, but may also be of clinical significance.

4 T_4 had a direct prorelaxing effect on pig SO contractility also in physiological concentrations, and T_4 might thus influence SO tone also in vivo. The human SO expressed TR β_1 and β_2. The prorelaxing effect of T_4 was mediated via a mechanism

which requires new mRNA and protein synthesis and subsequently leads to the activation of K^+ channels and reduced contraction.

5 In the rat the relative intestine v. liver ^{99m}Tc HIDA activity at 45 minutes decreased by 44 % in hypothyroid and at 60 minutes increased by 73 % in hyperthyroid rats compared to controls. Considering the ^{99m}Tc HIDA liver specificity, and that the weight of the organs, blood pressure at injection and the maximal uptake of radioactivity by the liver were similar between the groups, the results strongly suggest that the net bile flow to the duodenum is reduced in hypothyroidism and enhanced in hyperthyroidism in the rat.

6 In cholescintigraphy the hepatic clearance of ^{99m}Tc HIDA was decreased and the HDT tended to be increased in the hypothyroid stage in the study patients, the appearance of radioactivity in the large bile ducts at the hilum, and hepatic maximal uptake being the same in the hypothyroid and the euthyroid stage. These findings further support the view that hypothyroidism might decrease, in particular, bile flow into the duodenum.

In conclusion, the results of the present study suggest that the reduced prorelaxing effect of T_4 on the SO in hypothyroidism results in delayed emptying of the biliary tract, which may play an important role in the increased prevalence of CBD stones in hypothyroidism.

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ORIGINAL COMMUNICATIONS