THE CARNITINE AND CARNITINE ESTER CONTENT OF RAT BILE AND HUMAN DUODENAL FLUID

By

JENNIFER JANE HAMILTON

B.Sc., The University of Guelph, 1983

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Division of Human Nutrition

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 1986

ABSTRACT

Carnitine is necessary for the beta-oxidation of long-chain fatty acids. Carnitine esters are produced to transport fatty acids across the otherwise impermeable inner mitochondrial membrane. Although carnitine has been investigated in many tissues, the content and possible roles of carnitine in the gastrointestinal tract have not been completely investigated. The kinetics of carnitine absorption have been determined but the treatment and sources of carnitine in the intestinal tract are unknown.

This study investigated the possible contribution of bile to the carnitine content of the intestinal lumen. First, the amounts of carnitine and carnitine esters in rat bile were measured. Then, the origin of carnitine in the bile was studied. Finally, the carnitine content of human bile-rich duodenal fluid was investigated.

Bile was collected from eleven anaesthetized (pentobarbital) adult male rats. Bile flow was measured gravimetrically and carnitine and its esters were determined using a radiochemical carnitine assay. Specific types of carnitine esters were quantitated after first extracting the samples with chloroform and methanol. The origin of carnitine in the bile was investigated indirectly by measuring the carnitine content of bile from rats who were fasted for 72 hours or orally administered with tetradecylglycidic acid, an inhibitor of carnitine palmitoyltransferase I. These treatments differently affect the amounts and types of carnitine esters found in the liver

and extrahepatically. The bile carnitine was compared to the serum and liver carnitine after these treatments to see if similarities existed. The origin of carnitine in the bile was further studied by administering [^{14}C]carnitine intravenously and determining its recovery in the bile. Human bile-rich duodenal fluid was collected from ten patients with suspected cholelithiasis by duodenal aspiration using nasogastric intubation after pancreozymin-cholecystokinin injection.

Large quantities of total carnitine and long-chain carnitine esters were found in rat bile relative to other tissues. It appears that carnitine enters the bile both directly from the newly synthesized or stored hepatic carnitine pool and also following hepatic uptake of carnitine from serum. The latter is first esterified in the liver before entering the bile. The types and amounts of carnitine esters in the bile, thus, appear to reflect the metabolic state at the time of bile formation. Carnitine was also found in human bile-rich duodenal fluid. The percentage of long-chain carnitine esters was similar to that found in rat bile.

The discovery of carnitine in rat bile challenges the common assumption that only dietary carnitine is present in the intestinal tract. It also disputes the theory that urine is the sole route for carnitine excretion. In addition, it suggests that carnitine depletion could result if carnitine in the bile is not reabsorbed in the intestine. This might occur with general malabsorption syndromes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

The author would like to thank the following people who helped with this project:

-Dr. Peter Hahn for his supervision and great friendship.

-committee members, Dr. Sheila Innis and Dr Joseph Leichter for their time and guidance.

-Leighton James and Dr. David Seccombe for their kind assistance.

-Marta Taller, Livia Srubiski, Wendy Cannon, Vicky Louie and Nada Hahn for their technical advice, and ever stimulating conversation.

-the Cystic Fibrosis Foundation of Canada for financial support.

-both her husband, Drew Meikle, and her parents for moral support.

LIST OF ABBREVIATIONS

BW	body weight
°C	degrees Celsius
c-	centi-
Ci	Curie
CAT	carnitine acetyltransferase
CoA	coenzyme A
CPT	carnitine palmitoyltransferase
CV	coefficient of variation
DPM	disintegrations per minute
EDTA	ethylenediaminetetraacetate
g	gram
GOT	glutamate-oxaloacetate transaminase
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IP	intraperitoneally
IV	intravenously
l	liter
m-	milli-
M	molar
min	minute
mol	mole
n-	nano-
NEM	N-ethylmaleimide
SE	standard error
TDGA	tetradecylglycidic acid

TLC	thin layer chromatography
rpm	rotations per minute
u-	micro-
UBC	University of British Columbia
v	volume

CHAPTER 1

Purpose and Organization

The metabolism of carnitine has been widely studied in many organs. The presence and role of carnitine in the intestine, however, has been largely ignored. The objective of this work was to investigate the content and origin of carnitine in rat bile and measure its presence in human duodenal fluid. This might reveal an endogenous source of carnitine in the intestine and help to elucidate a potential metabolic role for carnitine in this organ.

A review of the literature relevant to this study is presented in Chapter Two. In Chapter Three, the experimental design and rationale for each experiment are described. The methods used in this work are described in Chapter Four. The experimental results are reported and discussed in Chapters Five, Six and Seven. Chapter Eight includes a discussion of the results of other related investigations. In Chapter Nine the experimental results are summarized and conclusions are made. Suggestions for further studies and the possible implications of this work are also presented.

CHAPTER 2

Literature Review

2.1 Discovery of Carnitine

In 1905 carnitine was independently discovered by two laboratories as a component of Liebig's meat extract (Gulewitsch and Krimberg; Kutscher). The structure of carnitine was determined in 1927 (Tomita and Sendju) and in 1952 it was identified as a growth factor for the mealworm, Tenebrio molitor (Carter et al., 1952). By 1955 the role of carnitine in lipid metabolism was known (Friedman and Fraenkel; Fritz).

2.2 Chemistry of Carnitine

The structure of carnitine, gamma-trimethylamino-3-hydroxybutyrate, is shown in Figure 1. L-carnitine is the biologically active isomer. Fatty acids are bound enzymically to the hydroxyl group of carnitine to form short-chain (2-6 carbons), medium-chain (8-12 carbons) and long-chain (14 or more carbons) carnitine esters. Free carnitine and short and medium-chain carnitine esters are uniformly distributed in aqueous solutions but long-chain carnitine esters migrate to the air/water interphase (Pande, 1981).

Yalkowsky and Zografi (1970) showed that carnitine esters with between 8 and 16 carbons are amphiphiles and form micelles. The critical micellar concentration decreases with increasing molecular weight. Pande (1981) showed that an uneven distribution of palmitoylcarnitine also occurs at concentrations below the critical micellar

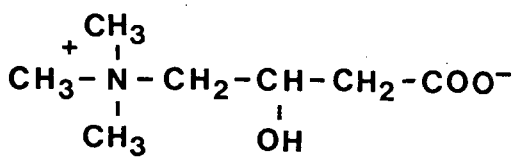


FIGURE 1

The structure of carnitine.

concentration. Long-chain carnitine esters are evenly distributed in solutions of methanol and chloroform (Pande, 1981).

2.3 Function of Carnitine

The principal function of carnitine is to transport long-chain fatty acids across the inner mitochondrial membrane to the mitochondrial matrix where beta-oxidation occurs (Fritz, 1968). It is necessary because fatty acyl-CoA formed in the cytosol cannot cross the inner mitochondrial membrane which is impermeable to CoA. The formation of fatty acylcarnitine is catalyzed by carnitine palmitoyltransferase I (CPT I, E.C.2.3.1.21) which is located on the outer side of the inner mitochondrial membrane. The transport of the carnitine ester across the membrane is facilitated by acylcarnitine translocase (Pande and Parvin, 1976) and the dissociation of the carnitine ester is catalyzed by CPT II which is located on the inside of the membrane. Carnitine can then return to the outer side of the membrane and repeat this process.

Carnitine is also involved in the beta-oxidation of short and medium-chain fatty acids. Usually these are activated to acyl-CoA within the mitochondria and carnitine is not needed for membrane transport (Aas and Bremer, 1968). Shorter-chain fatty acids may arise as a result of oxidation of very long-chain fatty acids. Peroxisomes partially shorten these fatty acids independently of carnitine

(Osmundsen and Neat, 1979). The shortened esters are then bound to carnitine and transferred to the mitochondria as carnitine esters for complete oxidation (Bieber et al., 1981). Peroxisomal and mitochondrial carnitine acetyl- and octanoyl- transferases are required for this process (Bremer, 1983).

Shorter-chain carnitine esters may also be involved in the maintenance of the inner mitochondrial acyl-CoA/CoA ratio (Pearson and Tubbs, 1967). Acetylcarnitine is formed from carnitine and acetyl-CoA freeing CoA within the mitochondrial matrix for further fatty acid oxidation (Bremer, 1983).

Carnitine may also play a role in certain disease or toxicity states. As a result of some inborn errors of metabolism, organic acids that undergo intramitochondrial chain shortening accumulate at the point of the metabolic defect causing an abnormally high acyl-CoA/CoA ratio (Engel and Rebouche, 1984). An apparent carnitine deficiency results as the carnitine is used to accept the organic acid from the CoA. The carnitine esters then leave the cell and are excreted in the urine. The same situation occurs during valproic acid therapy (Ohtani et al., 1982). Thurston and colleagues (1984) have hypothesized that high levels of valproyl-CoA are formed within the mitochondria and that carnitine is again used to buffer the acyl-CoA/CoA ratio. In both of these conditions, carnitine administration allows

the excretion of the accumulated acid from the mitochondria and alleviates the symptoms of carnitine deficiency.

2.4 Synthesis of Carnitine

In the synthesis of carnitine, protein-bound lysine and free methionine first form trimethyllysine (Paik and Kim, 1975). There are several additional steps in the process before the eventual hydroxylation of gamma-butyrobetaine by gamma butyrobetaine hydroxylase (E.C.1.14.11.1) to produce carnitine (Lindstedt and Lindstedt, 1965). In humans, this final step in carnitine synthesis occurs in the liver, kidney and brain (Englard, 1979), but in rats, is limited to the liver (Bohmer, 1974).

2.5 Presence and Distribution of Carnitine

Muscle contains 95-98% of the total body carnitine in man and dogs (Rebouche and Engel, 1983; Rebouche and Engel, 1984) and 98% in rats (Cederblad and Lindstedt, 1976). The remaining carnitine is in the serum, liver and kidneys. Both double (Cederblad and Lindstedt, 1976) and triple compartment models (Rebouche and Engel, 1983) have been hypothesized to explain the distribution of carnitine in mammals. The two compartment model includes a small serum pool of rapidly turning over carnitine and a large stable muscle pool. The three compartment model defines the liver and kidney carnitine as a separate pool having a very high turnover rate (Rebouche and Engel, 1983). Both models have

been mathematically tested using data obtained from radiolabelled tracer experiments but in both cases, the calculated carnitine excretion was greater than the amount of carnitine found in the urine. Similar results were also found by Yue and Fritz (1962) and Brooks and McIntosh (1975). No other means of carnitine breakdown or excretion, other than urinary, is known.

Methods for the assessment of carnitine status are controversial. There is no correlation between muscle and serum carnitine concentrations (Cederblad and Linstedt, 1976). Both muscle and serum carnitine levels are low only in the case of severe muscle depletion (Mitchell, 1978b). Carnitine deficiency is very rare and although not always recognized, the symptoms are distinct. The importance of subclinical carnitine deficiencies is not known.

The degree of esterification of serum carnitine is a useful index of metabolic state. For instance, highly esterified serum carnitine is indicative of fasting (Brass and Hoppel, 1978), a high dietary fat content (Seccombe et al., 1978) or some types of metabolic aciduria (Engel and Rebouche, 1984). The specific carnitine esters present give additional information about the metabolic state of the individual.

2.6 Carnitine in the Intestine

2.6.1 Carnitine in Food

The carnitine content of food has been studied by many laboratories; the results have been reviewed (Mitchell, 1978a). Carnitine is most abundant in meats but is also present in plants.

2.6.2 Oral Administration of Carnitine

The absorption of carnitine was first demonstrated by Karpati and colleagues (1975). Upon administering oral carnitine to a carnitine deficient patient, the serum carnitine concentration increased. Since then, similar observations have been made by many other authors. Generally, after treatment with oral carnitine, the symptoms associated with carnitine deficiency disappeared, and serum and urine carnitine levels increased (Gilbert, 1985; Engel and Rebouche, 1984). In 1983, Bach and colleagues studied carnitine absorption using twelve apparently healthy students. Two grams of L-carnitine were administered orally to six of the students; the remainder received a placebo. Free and total serum carnitine were monitored during the subsequent 24 hours. Carnitine levels peaked after 3.5 hours and had not returned to baseline levels at the end of the 24 hour period. Other investigators have reported that serum carnitine levels are still elevated in humans 4 hours after an oral dose (Seccombe et al., 1984; Frohlich et al., 1978). Similar observations have been made in rats.

Gudjonsson and colleagues (1984) observed that after an intraluminal dose of labelled carnitine, the amount of label in the serum had not yet peaked 8 hours later. Similarly, Gross and Henderson (1984) found 35% of an intraluminal dose of labelled carnitine in the serum 7 hours after administration.

2.6.3 Carnitine in the Contents of the Gastrointestinal Tract

Sachan and Ruark (1985) studied fed and fasted adult rats to determine the content of endogenous carnitine in the intestinal lumen and tissue. Although the diets contained only free carnitine and long-chain carnitine esters, short and medium-chain carnitine esters were found in the intestinal lumen. With fasting, this fraction decreased. The authors hypothesized that these short and medium-chain carnitine esters may have been released from intestinal tissue, formed due to intraluminal esterification or produced by intestinal microorganisms.

Investigations of the intestinal contents of suckling rats by Hahn and coworkers (1985) found that the degree of carnitine esterification increased throughout the length of the gastrointestinal tract. It was hypothesized that this was due to preferential absorption of free carnitine.

2.6.4 Kinetics of Carnitine Uptake into Intestinal Mucosal Cells

The kinetics of carnitine uptake and release from intestinal mucosal cells have been studied by groups led by Gross of the University of Minnesota (Gross et al., 1983; 1984; 1985) and Olsen of the University of Wisconsin (Gudjonsson et al., 1984; 1985a; 1985b; Hamilton et al., 1983; Li et al., 1983; 1985; Shaw et al, 1982; 1983). Both *in vitro* and *in vivo* experiments were done using humans, rats, guinea pigs and rabbits. The techniques used included intestinal lumenal and vascular perfusion, intralumenal bolus dose injection, everted intestinal rings and sacs, enterocyte tissue culture, mucosal incubation, isolated brush border membrane vesicles and the Ussing chamber mounted with intestinal epithelium.

The results of both the Minnesota and Wisconsin groups concur: carnitine and acetylcarnitine are taken up more rapidly into jejunal mucosal cells than ileal cells while the duodenum does not absorb carnitine or carnitine esters. Carnitine uptake into enterocytes is active, sodium dependent and potentially carrier mediated. This is similar to carnitine uptake reported in other tissues including the liver (Christiansen and Bremer, 1976), the skeletal muscle (Willner et al., 1978; Rebouche, 1977), the kidney cortex (Huth and Shug, 1980), the epididymal cells (James et al., 1981), the heart (Sartorelli et al., 1982; Bahl et al., 1981; Bohmer et al., 1977) and the cerebral cortex (Huth et

al., 1981). Both groups also showed that at very high carnitine concentrations passive diffusion occurs.

The Gross and Olsen groups also demonstrated that once inside the mucosal cell, as much as 50% of the carnitine is esterified to acetylcarnitine. Other authors have also shown a high degree of acetylcarnitine production within the enterocyte (Hahn et al., 1985; Sachan and Ruark, 1985). All investigators agree that the release of carnitine from the mucosal cells is very slow.

2.6.5 Carnitine Acyltransferases in the Intestine

In 1981, Hanson and Carrington found that the activity of CPT in adult rat intestinal mucosa (umol/min/g wet weight) was only one third the activity found in the liver. Carnitine acetyltransferase (CAT, nmol/min/mg protein), however, was approximately 40% more active in the mucosa than the liver (Gudjonsson et al., 1985a). As the energy metabolism of intestinal cells is based on glutamine metabolism (Windmueller and Spaeth, 1980), the lesser activity of CPT probably reflects the fact that fat is not a major energy source for the mucosal cell. The high activity of CAT in the mucosa is probably responsible for the high degree of esterification of carnitine to acetylcarnitine within the mucosal cell. Hahn and colleagues (1985) found that the activities of carnitine acyltransferases (nmol/min/mg protein) in the intestinal mucosa of developing rats were elevated after birth and decreased to adult levels

at weaning. They also found that mucosal cell CPT I activity was much greater than that of CPT II. This implies that palmitoylcarnitine is formed in the mucosal cell but that the palmitate is not oxidized there.

2.6.6 Carnitine Degradation in the Intestine

There is no evidence that carnitine is metabolized to other compounds in rat tissues (Brooks and McIntosh, 1975). Bacterial degradation of carnitine in the intestinal tract was suspected for several years before Rebouche and colleagues (1984) used normal and germ-free rats to demonstrate that endogenous intestinal flora is responsible for degradation of carnitine in the lower gastrointestinal tract.

In Rebouche's experiments, labelled carnitine and metabolites were found in the feces of rats who had received either oral or intravenous carnitine. Hahn and colleagues (1985) showed a similar phenomenon in suckling rats who had been injected subcutaneously with labelled carnitine. Twenty-four hours after injection, labelled carnitine had accumulated in the intestinal mucosa.

2.6.7 Carnitine Malabsorption

Three situations of possible carnitine malabsorption have been studied. Frohlich and coworkers (1980) reported that serum carnitine levels were reduced after jejunoileal bypass surgery and continued to decrease post operatively.

Whether decreased carnitine or carnitine precursor ingestion contributed to this is unknown. Seccombe and coworkers (1984) measured carnitine absorption in similar patients. Serum carnitine was measured upon administration of an oral dose of carnitine. After surgery, the increase in serum carnitine was 38% less than before surgery. Thus, it appears likely that jejunoileal bypass surgery results in carnitine malabsorption.

Mikhail and Mansour (1976) found decreased serum carnitine levels in patients suffering from intestinal schistosomiasis infections. The patients with the most severe cases had the lowest serum carnitine levels. With nutritional repletion and drug treatment, serum carnitine levels were restored. Serum carnitine levels were also decreased in patients with Crohn's disease and some patients with a history of villous atrophy and malabsorption (Bohmer et al., 1974). Although the reduced serum carnitine levels in these cases could be due to reduced absorption of the precursors for carnitine synthesis, carnitine malabsorption itself might have contributed.

2.7 Carnitine Metabolism During Fasting

Adipocyte lipolysis is elevated after fasting and fatty acid availability and utilization is increased. As carnitine is intimately involved in fatty acid oxidation, the types of carnitine esters in the rat liver are altered

and the percentage of carnitine esterification in the serum is changed (Brass and Hoppel, 1978).

The results of studies on the effects of fasting on the carnitine content of rat liver are shown in Table 1. All data is given as a percent of total liver carnitine. The increase in the oxidation of fatty acids during fasting gives rise to increased amounts of longer-chain carnitine esters. The accumulation of long-chain carnitine esters is also due to the higher activity of hepatic activity of CPT I than CPT II (Bremer, 1981; Aas and Daae, 1971; Norum, 1965). The decrease in the amount of free carnitine in the liver is a result of the increased levels of long-chain carnitine esters.

Serum carnitine esterification in rats increased from 41% to 69% after a 72 hour fasting period (Brass and Hoppel, 1978). This increase was mainly in the form of acetylcarnitine and was likely the result of carnitine being used to buffer the intramitochondrial acyl-CoA/CoA ratio. In humans, serum carnitine esterification also increased upon fasting (Frohlich et al., 1978; Hoppel and Genuth, 1976).

2.8 The Effect of Tetradecylglycidic Acid on Carnitine Metabolism

Tetradecylglycidic acid (TDGA, McN-3802) is a fatty acid structural analogue (Figure 2). It was first reported as a CPT I inhibitor by McNeil Laboratories (Tutweiler et al,

TABLE 1

Effects of Fasting on the Relative Amounts of Carnitine and Carnitine Esters in Rat Liver

Length of Fast (hours)	Carnitine and Carnitine Esters(% of Total)								Reference
	Free		Acetyl		Short and Medium-chain		Long-chain		
	fed	fast	fed	fast	fed	fast	fed	fast	
24	-	-	26	51	-	-	2	20	Bohmer and Bremer, 1968
24	73	45	-	-	25	42	2	13	Bohmer, 1967
48	60	49	-	-	38	38	3	16	Bohmer et al., 1966
24	48	30	17	26	51	50	1	19	Brass and Hoppel, 1981
24	53	41	-	-	43	44	3	18	Brass and Hoppel, 1978
48	53	43	-	-	43	43	3	14	Brass and Hoppel, 1978
72	53	47	-	-	43	45	3	11	Brass and Hoppel, 1978
48	-	29	-	-	-	58	-	14	French et al., 1985
36-48	56	31	13	23	27	28	3	18	Pearson and Tubbs, 1967

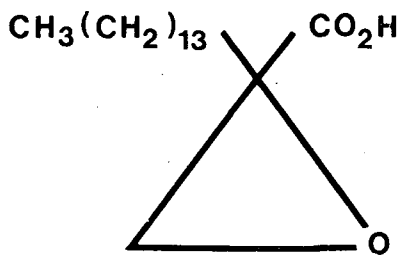


FIGURE 2

The structure of tetradecylglycidic acid.

1978; Tutweiler and Dellevigne, 1979). TDGA very potently and specifically inhibits CPT I *in vivo* and *in vitro* in rats by irreversibly inactivating the active site (Tutweiler and Dellevigne, 1979). It, therefore, inhibits the oxidation of long-chain but not short or medium-chain fatty acids. Long-chain acyl-CoA accumulates in the cytosol of the hepatocyte as it cannot cross the inner mitochondrial membrane. Concentrations of long-chain carnitine esters decrease and the concentration of free carnitine is increased (Tutweiler et al., 1978). As a result of the decreased fatty acid oxidation, gluconeogenesis is inhibited when TDGA is administered in the fasting state resulting in hypoglycemia in both man and rats (Tutweiler and Dellevigne, 1979; Mandarino et al., 1984).

French and colleagues demonstrated the effects of oral TDGA administration on the livers of rats previously fasted for 48 hours (1985). Free carnitine increased significantly, short and medium-chain acylcarnitine levels remained constant and long-chain acylcarnitine decreased. An increase in the total carnitine content of the liver was also noted. Frost and Wells (1982), studying neonatal rats, showed that TDGA produced no change in liver weight but increased the size of the hepatic carnitine pool. This was primarily due to an increase in free carnitine and was hypothesized to be a compensatory mechanism to overcome the inhibition of the TDGA.

CHAPTER 3

Experimental Design and Rationale

The study of carnitine in the intestine is important for several reasons. For example, although oral carnitine is often used therapeutically, the efficiency of carnitine absorption under various conditions is not known. Normal carnitine absorption should be defined in order to investigate the claim that defective carnitine absorption might be the cause of some cases of systemic carnitine deficiency (Engel and Rebouche, 1984). Finally, carnitine might play a role in fat absorption; both are absorbed in the jejunum. Carnitine esters, because of their amphiphilic nature, are possibly involved in the mixed micelles of the intestinal lumen. Alternatively, carnitine might shuttle fatty acids across some of the membrane barriers encountered in the transport of fat across the enterocyte and into the lymph. This is similar to a role that has been proposed for carnitine in the epididymis, where it has been hypothesized to transport fatty acids between the blood stream, epididymal epithelium, epididymal lumen and sperm mitochondria (Brooks, 1980).

The endogenous carnitine content of the intestine must be studied before the intestinal absorption of dietary carnitine can be investigated. The objective of the present study was to determine whether or not bile contributes to the carnitine found in the intestinal lumen. This was done in a series of experiments which were designed to answer the following questions: 1) Are carnitine and carnitine esters present in rat bile? 2) What is the origin of the carnitine

in bile? 3) Does carnitine exist in human bile-rich duodenal fluid?. The rationale and protocol for each experiment is described below. The specific methods and materials used are described in Chapter 4.

3.1 The Carnitine and Carnitine Ester Content of Rat Bile

The results of several studies indicate that carnitine of the intestinal lumen might be derived from other than dietary sources. Sachan and Ruark found some types of carnitine esters in rat intestinal contents that were not present in the diet. Hahn and colleagues (1985) and Rebouche and associates (1984) found labelled carnitine in the intestinal tract after it was injected subcutaneously or intravenously. Data from body pool studies suggest that there may be another route for carnitine excretion besides the urine (Rebouche and Engel, 1984; Cederblad and Lindstedt, 1976; Yue and Fritz, 1962). Possibly, carnitine is excreted into the intestine, via the bile, where it is either reabsorbed or degraded by bacteria of the large intestine (Rebouche et al., 1984).

To clarify the presence of carnitine in rat bile, bile was collected from eleven fed adult rats. Bile flow, bile and serum carnitine and carnitine ester concentrations were measured. Long-chain carnitine ester and acetylcarnitine concentrations were determined in randomly selected samples.

3.2 The Origin of Carnitine in Rat Bile

Carnitine found in the bile could be of either hepatic or extrahepatic origin. Large amounts of long-chain carnitine esters are present in bile. Because these long-chain carnitine esters are present in the serum to only a very limited degree, this suggests that carnitine is derived from the liver. The origin of the carnitine in bile was investigated by imposing conditions on rats which affect carnitine esterification in the liver and serum differently. The conditions studied were fasting and TDGA treatment. The amount and type of carnitine esters in the bile were examined to see if they reflected the changes occurring in the liver or serum. A third group of rats was injected intravenously with labelled carnitine to further investigate the source of carnitine in the bile.

3.2.1 The Effects of a 72 Hour Fast on the Carnitine Content of Rat Bile

Fasting increased the proportion of long-chain carnitine esters in the liver, whilst the amount of shorter-chain carnitine esters decreased. In the serum, the overall carnitine esterification increased (Brass and Hoppel, 1978). Five rats were fasted for 72 hours prior to bile collection. The bile flow, carnitine and carnitine ester concentrations were measured in all samples. Long-chain carnitine esters were determined in randomly selected samples. Serum was also collected and the

carnitine and carnitine ester concentrations measured. The results of this fasting experiment were compared with those from rats in the fed state.

3.2.2 The Effects of Tetradecylglycidic Acid Treatment on the Carnitine Content of Rat Bile

As TDGA inhibits CPT I, hepatic long-chain carnitine ester production is decreased and the concentration of free carnitine is increased (French et al., 1985). In the serum, both esterified carnitine and total carnitine concentrations are decreased.

Six rats were fasted for 72 hours and intubated with TDGA, according to the protocol of French and colleagues (1985). Bile was collected and the bile flow, carnitine and carnitine ester concentrations were measured in all of the samples. Long-chain carnitine esters were determined in randomly selected samples. The serum carnitine concentration was also measured. The results from these studies were compared to those in the previous study in which the rats were fasted for 72 hours.

3.2.3 The Recovery of Intravenously Injected [^{14}C]Carnitine in Rat Bile

Bile was collected from six rats following intravenous injection of labelled carnitine. The types of carnitine esters in the bile and the specific activity of both the serum and the bile carnitine, were determined. Possible

associations between the bile and the hepatic and extrahepatic sources of carnitine were examined.

3.3 The Carnitine Content of Human Bile-Rich Duodenal Fluid

The results of four studies have indicated that carnitine malabsorption might exist in humans (Seccombe et al., 1984; Frohlich et al., 1980; Bohmer et al.,1974; Mikhail and Mansour,1976). If an enterohepatic circulation of carnitine is present, carnitine depletion might result during malabsorptive states or excessive bile losses. Before investigating this further, the carnitine content of human bile should be determined.

Seventeen bile-rich duodenal fluid samples were collected from ten adult patients with suspected cholelithiasis. These samples were then analysed for carnitine and its esters.

CHAPTER 4

Methods and Materials

4.1 Chemicals and Isotopes

All chemicals and isotopes were purchased from the Sigma Chemical Company, St. Louis, MO., 63178, except where otherwise noted.

[1-^{14}C]Acetyl-Coenzyme A, 57.6 mCi/mmol, Amersham Ltd., Arlington Heights, Illinois.

L-[U-^{14}C]Aspartate, 227 Ci/mol, Amersham Ltd., Arlington Heights, Illinois.

Carnitine Acetyltransferase, from pigeon breast muscle, 85 units/mg protein.

L-Carnitine Hydrochloride, Sigma-Tau Company, Rome, Italy.

L-[methyl-^{14}C]Carnitine Hydrochloride, 58 mCi/mmol, Amersham Limited, Arlington Heights, Illinois.

Citrate Synthase, Boehringer Mannheim Biochemica, Dorval, Quebec.

EDTA (ethylenedinitrilotetraacetic acid), disodium salt.

Glutamate-oxaloacetate transaminase, Boehringer Mannheim Biochemica, Dorval, Quebec.

HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid, BDH Chemicals Canada Ltd., Vancouver, B.C.

N-ethylmaleimide (NEM).

Somnotol, sodium pentobarbital (65 mg/ml) in an aqueous propylene glycol base, M.T.C. Pharmaceuticals, Mississauga, Ont.

TDGA (tetradecylglycidic acid), donated by Dr. John McNeill, Dean and Professor of Pharmaceutical Science, University of British Columbia.

Tragacanth Gum, grade III powder.

4.2 Housing and Care of Animals

Adult male Wistar rats (380.3 ± 4.4 g, mean ± SE) were used. These were bred and raised either at the Centre for Developmental Medicine or in the Animal Care Centre of the University of British Columbia (UBC). The rats were housed in groups of two or three in plastic cages with wood shavings for bedding. They had free access to Purina Rat Chow and water at all times. Lighting was on a reversed 12 hour cycle with the lights turned on at 4 PM and turned off at 4 AM. All surgical procedures were conducted between 8 and 10 AM to control for possible diurnal variations in serum carnitine levels (Bohmer and Bremer, 1968).

4.3 Rat Bile Collection

Rats were anaesthetized with an intraperitoneal (IP) injection of Somnotol (6.5 mg/100 g body weight, BW) immediately before the surgery. Bile was collected according to Waynforth (1980) with some modifications. Each rat was placed on its back on a heating pad which maintained its body temperature, 37 °C, measured rectally. A 2 cm lateral incision was made at the level of the xiphoid cartilage and a small piece of duodenum, located directly beneath the liver, was pulled gently out of the peritoneal cavity until resistance was felt from the attachment of the bile duct. The bile duct was then carefully dissected from the surrounding tissue and a short length of doubled thread was inserted around the duct to form two loose ligatures.

The bile duct was then held with forceps while it was semi-transected with fine scissors. A polyethylene cannula (internal diameter 0.5 mm) was then drawn out, inserted in the opening and pushed forward beyond the pancreas (towards the liver for 3-4 mm). The point of entry of the cannula was then sealed with cyanoacrylate glue. Once bile flow was established, the intestine was replaced into the peritoneal cavity and the incision sutured. Bile was collected into preweighed glass test tubes held beside the heating pad at the same height as the rat.

Bile was collected from each rat for a total of two hours. The collection tube was replaced every 15 minutes, and flow determined gravimetrically assuming a density of 1 g/ml. Bile flow was calculated as ul/min/100 g BW. After each tube was weighed, bile samples were aliquoted into disposable conical polystyrene tubes for subsequent carnitine analysis and frozen at -20 °C. Freezing does not affect the serum carnitine concentration (Seccombe, 1981). The pH of the samples were monitored to confirm neutrality.

After two hours of bile collection, a blood sample was taken from the tail vein of each rat. Rats were then killed with excess Somnotol. The blood was centrifuged at 3,000 rpm for 5 minutes at 4 °C and the serum removed and frozen at -20 °C until carnitine analysis. In one case, the blood samples were collected at 15 minute intervals throughout the bile collection to determine the effect of fluid loss, due to bile drainage, on serum carnitine concentrations.

4.4 Human Bile-Rich Duodenal Fluid Collection

Bile-rich duodenal fluid was obtained from ten, male and female, adult patients with suspected cholelithiasis. The samples were collected by the Gastroenterology Unit of Shaughnessy Hospital for Dr. N. Urquhart (Department of Pathology, University of British Columbia). Patients were instructed to fast for at least 12 hours before the procedure which was either conducted at 10 AM or 1 PM. A nasogastric tube was placed into the first segment of the duodenum and its position was confirmed by fluoroscopy. In some cases, a basal fluid sample was collected. The patients were then injected (IV) with 80 units of concentrated pancreozymin-cholecystokinin (Boots Drugs Ltd., Toronto, Ont.). This stimulates the release of bile from the gallbladder (Ivy et al., 1928). One or two fluid samples were then collected at 15 minute intervals. All samples were neutralized to prevent ester hydrolysis and stored frozen at -20 °C for later carnitine analysis.

4.5 Determination of L-Carnitine in Serum and Bile

4.5.1 Principle of the Carnitine Assay

Originally, carnitine was measured by bioassay using the carnitine requiring organism, Tenebrio molitor (Fraenkel, 1951). In 1958, this was replaced by a colorimetric assay which was not very specific to carnitine (Friedman, 1958). The carnitine assay now used requires commercial CAT (Marquis and Fritz, 1964). Free CoA is measured

spectrophotometrically after incubation of each sample with acetyl-CoA. The reaction is as follows:

$$\text{L-Carnitine} + \text{Acetyl-CoA} \xleftrightarrow{\text{CAT}} \text{Acetylcarnitine} + \text{CoA}$$

In 1972, Cederblad and Lindstedt increased the sensitivity of the assay into the picomole range by introducing the use of [^{14}C]acetyl-CoA. The amount of labelled acetylcarnitine formed after incubation is proportional to the amount of carnitine in the sample. One problem with this method is the reversibility of the reaction: i.e. the dissociation of acetylcarnitine to free carnitine and acetyl-CoA. This can be overcome by the addition of tetrathionate (McGarry and Foster, 1976) or N-ethylmaleimide (Parvin and Pande, 1977), which binds to the free CoA, a product of the forward reaction. Parvin and Pande (1977) have suggested that N-ethylmaleimide (NEM) is superior to tetrathionate.

Another limitation of the Cederblad and Lindstedt method (1972) is the use of a DOWEX column to separate the unreacted [^{14}C]acetyl-CoA from the [^{14}C]acetylcarnitine. In place of this, McGarry and Foster (1976) added an ion exchange (DOWEX) resin slurry to each reaction vial; the supernatant is then free of any unreacted [^{14}C]acetyl-CoA. Instead, Parvin and Pande (1977) recommend the addition of a charcoal slurry containing acidified ethanol. Charcoal adsorbs nucleotide compounds and ethanol is needed to

prevent the retention of carnitine and acetylcarnitine as well as acetyl-CoA. Both methods are effective; the charcoal, however, is faster and least expensive.

4.5.2 Procedure used

All carnitine assays were performed in disposable polystyrene conical tip tubes (12 x 75 mm). Fifty microliters of each sample, standard or control were used for the assessment of each of free carnitine and total carnitine (free plus esterified carnitine). The assays were done in duplicate. A carnitine standard curve was prepared by serial dilution of carnitine and water. The control was human serum. All tubes were kept on ice during preparation for the assay in order to prevent the hydrolysis of carnitine esters.

All samples, standards and controls were deproteinized prior to assaying for carnitine. Although, McGarry and Foster (1976) reported that this was not necessary, Seccombe (1981) found that interference from serum proteins exaggerated the results. Even though bile contains very little protein (LaRusso, 1984), it was also deproteinized. The protein was precipitated by the addition of 200 ul zinc sulfate (0.087 M) to each sample for analysis of free carnitine. Subsequently, 200 ul of barium hydroxide (0.083 M) was added for neutralization. The assay of total carnitine requires the prior hydrolysis of carnitine esters. For these samples, the barium hydroxide was added first and

the samples were then incubated for 2 hours at 75 °C before addition of the zinc sulfate. The protein and barium sulfate precipitate were removed by centrifugation for 15 minutes at 3,500 rpm at 4 °C.

In preparation for the assay, 100 ul of the deproteinized supernatants were placed in new polystyrene tubes with 100 ul of a buffered substrate solution. The substrate solution contained HEPES buffer (20 mM), pH 7.6, EDTA (0.8 mM), NEM (2 mM) and [1-^{14}C]acetyl-CoA (2.5 uM, specific activity 0.02 uCi/nmol).

The reaction was initiated by the addition of 50 ul of CAT (containing 1 unit of enzyme). After incubation for 60 minutes at room temperature, the reaction was terminated with 600 ul of a stirred charcoal slurry. Each batch of slurry was prepared immediately before use and contained 4.0 g of neutralized activated charcoal, 54.5 ml of ethanol, 1.25 ml 85% phosphoric acid and 4.25 ml distilled water. After mixing with the slurry, the reaction tubes were placed on ice, and centrifuged at 3,500 rpm for 15 minutes at 4 °C. The supernatants were transferred to clean tubes and centrifugation repeated.

An aliquot of the final supernatant (500 ul) was then added to a disposable mini-counting vial containing 400 ul distilled water. As the supernatant contained ethanol, which is difficult to pipette, the pipettor tip was rinsed with the water in the vial to ensure quantitative transfer. Finally, 5 ml of scintillation fluid (ACS) was added before

the vial was mixed and counted. The samples were counted on a Beckman LS9000 Scintillation counter with an external standard quench correction.

The standard curves for both the free and total carnitine assays were then plotted and linear regression analysis was performed. If carnitine concentrations were beyond the range of the standard curve (0 to 100 uM), the assay was repeated with diluted samples. The concentration of acylcarnitine was derived by subtracting the concentration of free carnitine from the total carnitine concentration. Interassay variation was approximately 5%. Both the absolute quantity of carnitine in the bile per 100 g BW and the carnitine concentration were calculated for each 15 minute bile collection sample.

4.6 Acetylcarnitine Analysis

4.6.1 Principle of Method

Pande and Caramancion (1981) devised a radioisotopic assay for the determination of carnitine based on the following two reactions:

$$\text{1) Acetylcarnitine + CoA} \xrightarrow{\text{CAT}} \text{Acetyl-CoA + Carnitine}$$

$$\text{2) Acetyl CoA + [}^{14}\text{C]oxaloacetate} \xrightarrow{\text{Citrate Synthase}} \text{[}^{14}\text{C]citrate}$$

First, CAT causes the dissociation of carnitine and acetyl-CoA. Citrate synthase (E.C.4.1.3.7) then catalyzes the formation of [^{14}C]citrate from acetyl-CoA and

[^{14}C]oxaloacetate. The amount of [^{14}C]citrate formed is proportional to the amount of acetylcarnitine in the sample. Excess [^{14}C]oxaloacetate is removed by the subsequent addition of glutamate-oxaloacetate transaminase (GOT, E.C.2.6.1.1) which catalyses the formation of [^{14}C]aspartate. A cation exchange resin is used to remove [^{14}C]aspartate.

4.6.2 Procedure Used

The acetylcarnitine assays were performed by Leighton James (Department of Pathology, UBC) in Shaughnessy Hospital laboratories. Unless otherwise noted, all solutions were kept on ice to prevent hydrolysis of the carnitine esters. Aliquots of each sample (100 ul), standard or blank were deproteinized by addition of 400 ul of zinc sulfate (0.087 M) followed by the addition of the same volume of barium hydroxide (0.083 M). The precipitate was then removed by centrifugation for 10 minutes at 3,500 rpm at 4 °C. The standard curve ranged from 0.05 uM to 20 uM.

Pande and Caramancion (1981) found that the sensitivity of the assay was improved by the use of dialysed enzymes. All three enzymes were dialysed at 0-4 °C against 500 ml of 20 mM potassium phosphate (pH 7.4). The dialysing fluid was changed every 30 minutes for two hours.

Freshly reduced CoA reagent was prepared in advance and contained 0.2 mM CoA, 10 mM potassium diphosphate (pH 9) and 3.2 mM dithiothreitol. 400 ul of this was then left at

25 °C for 30 minutes before the addition of 800 ul of 0.5 M potassium phosphate (pH 7.4).

To prepare [^{14}C]oxaloacetate, 800 ul of a solution containing 200 nM EDTA, 5 mM alpha-ketoglutarate, 56 mM potassium phosphate (pH 7.4), 12 units/ml dialysed GOT and 50 nM L-[U-^{14}C]aspartate (227 Ci/mol) was incubated for 10 minutes at 25 °C. Then, 20 ul of 1 M perchloric acid was added, and left for 15 minutes. Immediately before the assay, this was neutralized with 40 ul 0.6 M chilled potassium hydroxide plus 60 ul of 67 mM EDTA (pH 7.4).

The assay was performed in conical borosilicate test tubes (12 mm x 75 mm). To each tube, the following was added in the order given: 155 ul sample, 10 ul 120-150 mM EDTA (pH 7.4) and 30 ul freshly reduced CoA reagent, 1.5 unit dialysed citrate synthase, 0.8 unit CAT and 5 ul [^{14}C]oxaloacetate. The reaction mixture was then incubated at 25 °C for 20 minutes. 30 ul of glutamate plus GOT were then added and the mixture was incubated for a further 20 minutes. 320 mg of the resin (AG 50W-X8, BioRad) and 0.6 ml water were then added to each tube which was allowed to stand for 10 minutes at room temperature. The resin was removed by centrifugation for 10 minutes at 3000 rpm. 0.3 ml of the supernatant, 0.6 ml of water plus 5 ml of scintillation fluid were then combined in mini-counting vials which were counted on a Beckman LS9000 Scintillation counter with an external standard quench correction. The standard curve was then plotted and the concentrations of

acetylcarnitine in each sample determined. Only bile samples from the initial and final bile collection periods were assessed.

4.7 Carnitine Ester Separation

4.7.1 Principle of Method

Thin-layer chromatography (TLC) has been used extensively to distinguish between short, medium and long-chain carnitine esters. Unfortunately, it is time consuming and may not be quantitative. To determine the relative amounts of long-chain carnitine esters in bile, another method has been devised based on the solubility of carnitine and its esters. Free carnitine and short and medium-chain carnitine esters are water-soluble but long-chain carnitine esters are only soluble in methanol and chloroform (Pande, 1981). Theoretically, the long-chain carnitine esters in bile can be quantitatively separated from the other forms of carnitine by extraction with chloroform:methanol.

4.7.2 Verification of Method

To verify the quantitative separation of long-chain carnitine esters by chloroform:methanol extraction, bile was collected from anaesthetized rats after injection of 0.5 uCi [1-^{14}C]carnitine (specific activity 2.5 mCi/nmol) into a jugular vein. The jugular vein was cannulated according to Waynforth (1980) with one exception. The hole into the vein

was made by inserting the tip of a bent (45 degrees) 21 gauge hypodermic needle into the blood vessel. The bile collected contained [^{14}C]carnitine and [^{14}C]carnitine esters.

To separate the aqueous and lipid fractions, 625 ul of 1:1 (v/v) chloroform:methanol was added to 50 ul of each sample, mixed and left to stand on ice for 30 minutes. Then, a further 440 ul of chloroform was added followed by 250 ul of water and the process was repeated twice. The resulting aqueous and lipid phases were separated, dried down under nitrogen, and redissolved in 50 ul of water and methanol, respectively. The samples were then applied to silica G plates (500 microns) along with standards. The plates were developed in the following solvent system: methanol, chloroform, water, concentrated ammonia, concentrated formic acid (55:50:10:7.5:2.5 v/v/v/v/v) as described by Gumpen and Norum (1973). After 30 minutes, the plates were removed from the solvent tank and the standards were visualized by exposure to iodine vapour. The distribution of the labelled carnitine was determined by scraping the plates and counting the silica in 1 cm bands. The results are shown in Figure 3. Only 3% of the label found in the aqueous phase migrated with the long-chain carnitine ester standard. The only label present in the lipid fraction migrated with the long-chain carnitine ester fraction. The recovery was 96.5%. Therefore, the extraction of bile with chloroform:methanol quantitatively separates

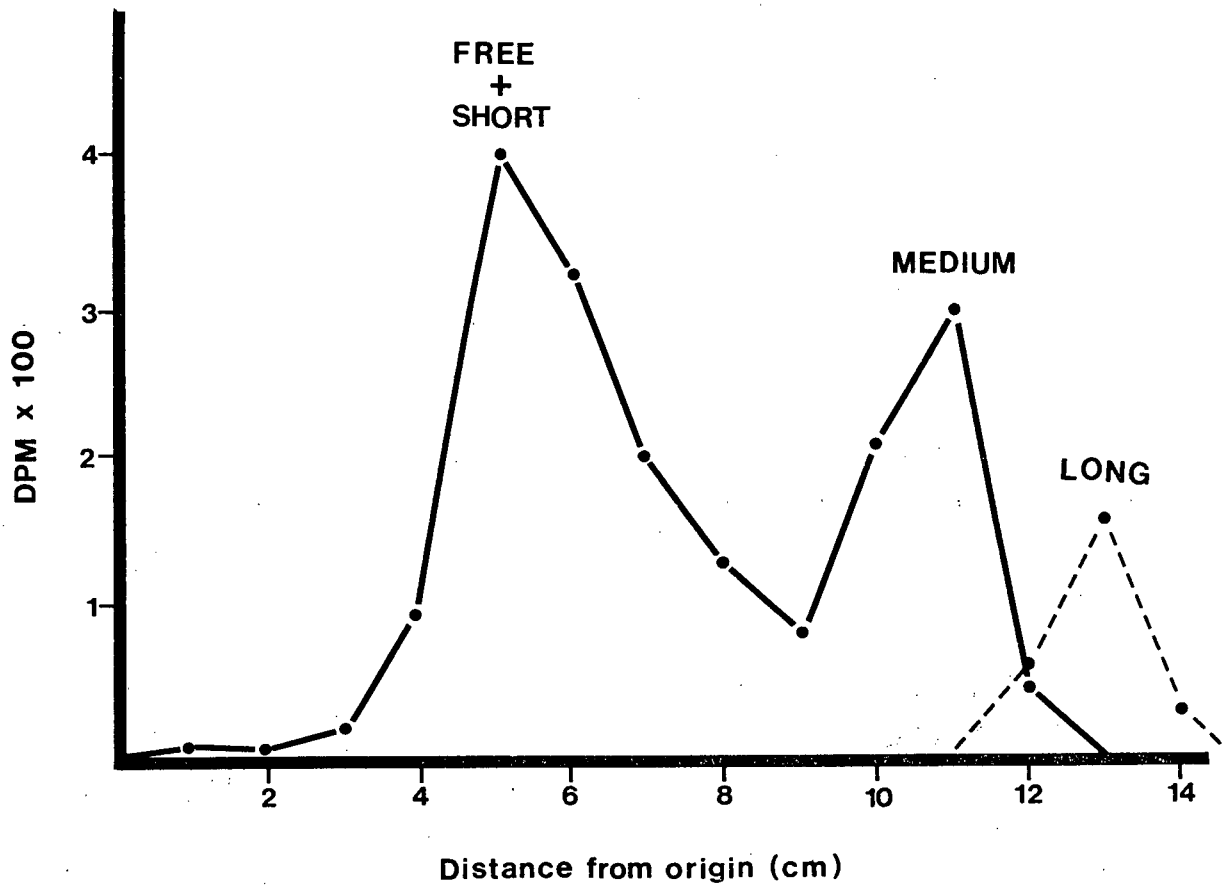


FIGURE 3

Verification of the separation of long-chain carnitine esters from other types of carnitine by lipid extraction using thin-layer chromatography (TLC). 200 ul of bile was collected from a rat intravenously injected with [^{14}C]carnitine. After extraction of the bile, silica TLC was performed on the resulting aqueous (solid line) and lipid (dashed line) phases. The silica was then scraped in 1 cm bands and counted in scintillation fluid.

long-chain carnitine esters from the other forms of carnitine in bile.

4.7.3 Procedure Used

Each sample, standard or control was separated into aqueous and lipid phases as described above. The phases were then dried under nitrogen, redissolved in 50 ul of water, hydrolyzed with 200 ul barium hydroxide, neutralized with zinc sulfate and assayed for carnitine.

The quantity of short and medium-chain carnitine esters were derived by subtracting the concentration of free carnitine determined in the carnitine assay from the concentration of carnitine found in the aqueous extract. Carnitine esters were analysed in randomly selected bile samples from the initial bile collection period of fed rats and both the initial and final collection periods of both fasted and rats treated with TDGA.

4.8 Fasting Procedure

The rats were weighed and placed individually into clean cages with no food but free access to fresh water 72 hours before the scheduled surgery. The rats were weighed again before bile collection. The amount of carnitine in the bile was reported on the basis of the initial body weight.

4.9 Tetradecylglycidic Acid Preparation and Administration

After the 72 hour fast, rats were intubated with TDGA suspended in 0.5 ml tragacanth (French et al.,1985). 2.5 mg of TDGA/100 g BW was administered to each rat. Each animal was restrained in a towel while a polyethylene tube attached to a syringe was placed into the esophagus. The TDGA solution was then injected. To ensure that the fluid was not regurgitated, the animals were observed closely after this procedure. Two hours later, the animals were anaesthetized and bile collection initiated. Two additional rats were fasted for 72 hours and intubated with the tragacanth vehicle alone. These animals served as controls for the administration vehicle used. Bile was then collected and the flow rate assessed gravimetrically.

4.10 Analysis of Data

The data obtained from these experiments are reported as the mean ± SE , unless otherwise noted. In some cases the coefficient of variation was also determined. The T-test was exclusively used to test for statistical differences. Where simultaneous comparisons were made, Bonferoni's correction was applied to attain overall significance of <0.05. Statistical differences are indicated on the Tables and Figures. The changes in the course of each experiment were determined by comparing the carnitine content of the bile from the first and last

collection periods. Duncan's multiple range test was also used to confirm these differences.

CHAPTER 5

The Carnitine and Carnitine Ester Content of Rat Bile

5.1 Results

5.1.1 Bile Flow

The bile flow during the course of the experiment is shown in Figure 4. The flow rate for the initial 15 minute collection period was significantly higher than for any of the subsequent collection periods. There were no further differences among the collection periods.

5.1.2 Carnitine Content of Bile

The concentrations of free, acyl and total carnitine in the bile are shown in Figure 5. Acyl and total carnitine concentrations increased during bile collection while the free carnitine concentration remained constant. The percentage of carnitine in the acyl form increased from 70 to 83% during the course of the experiment.

The amount of carnitine present in each 15 minute sample of bile is illustrated in Figure 6. The amounts of acyl and total carnitine again increased during bile collection. Although free carnitine decreased during the experiment, the percentage of acylcarnitine again increased.

5.1.3 Carnitine Esters in Bile

The distribution of the carnitine esters of rat bile is shown in Table 2. Long-chain carnitine esters made up 29% of the bile carnitine. Approximately 54% of the total carnitine was in the form of short and medium-chain carnitine esters. Only one quarter of this was as

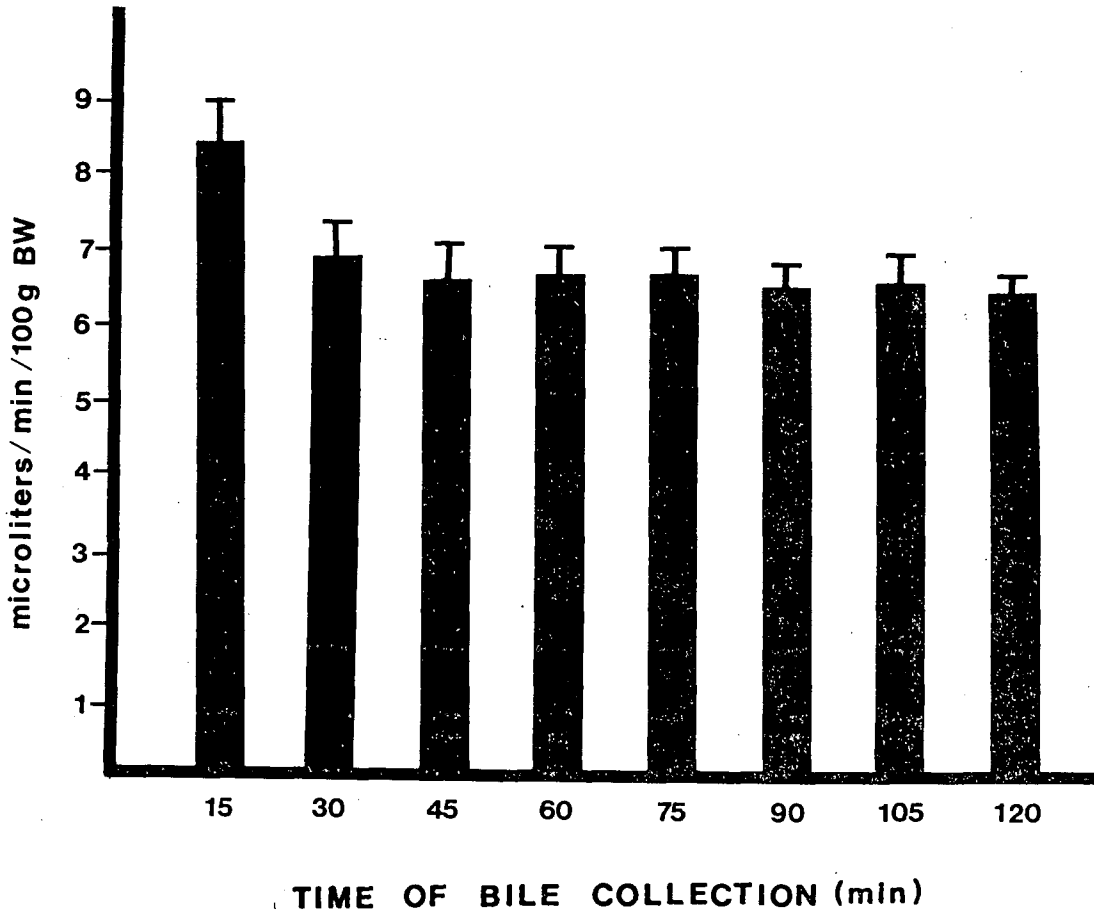


FIGURE 4

Bile flow (microliters/min/100 g BW) during bile collection from fed rats (mean ± SE). Bile was collected in eight consecutive 15 minute samples and the flow rate was assessed gravimetrically. Flow was greater in the first collection period than subsequent periods (at least $p<0.05$). There were no further differences between collection periods.

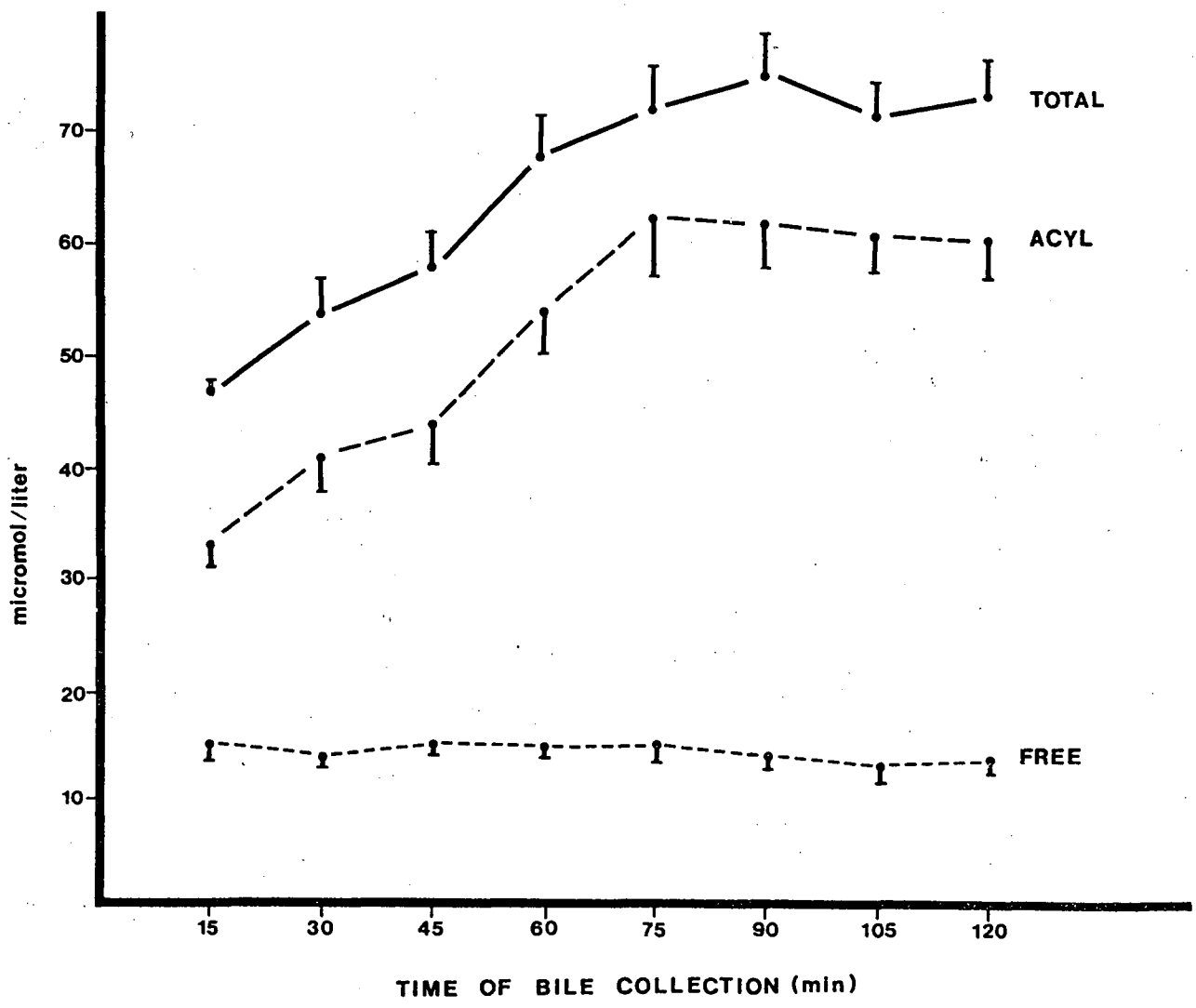


FIGURE 5

The concentrations of free, acyl and total carnitine in bile from fed rats collected at 15 minute intervals (mean ± SE). The concentrations of total and acyl carnitine were significantly greater after 120 minutes of bile collection than after 15 minutes ($p<0.001$). The concentration of free carnitine did not change during bile collection.

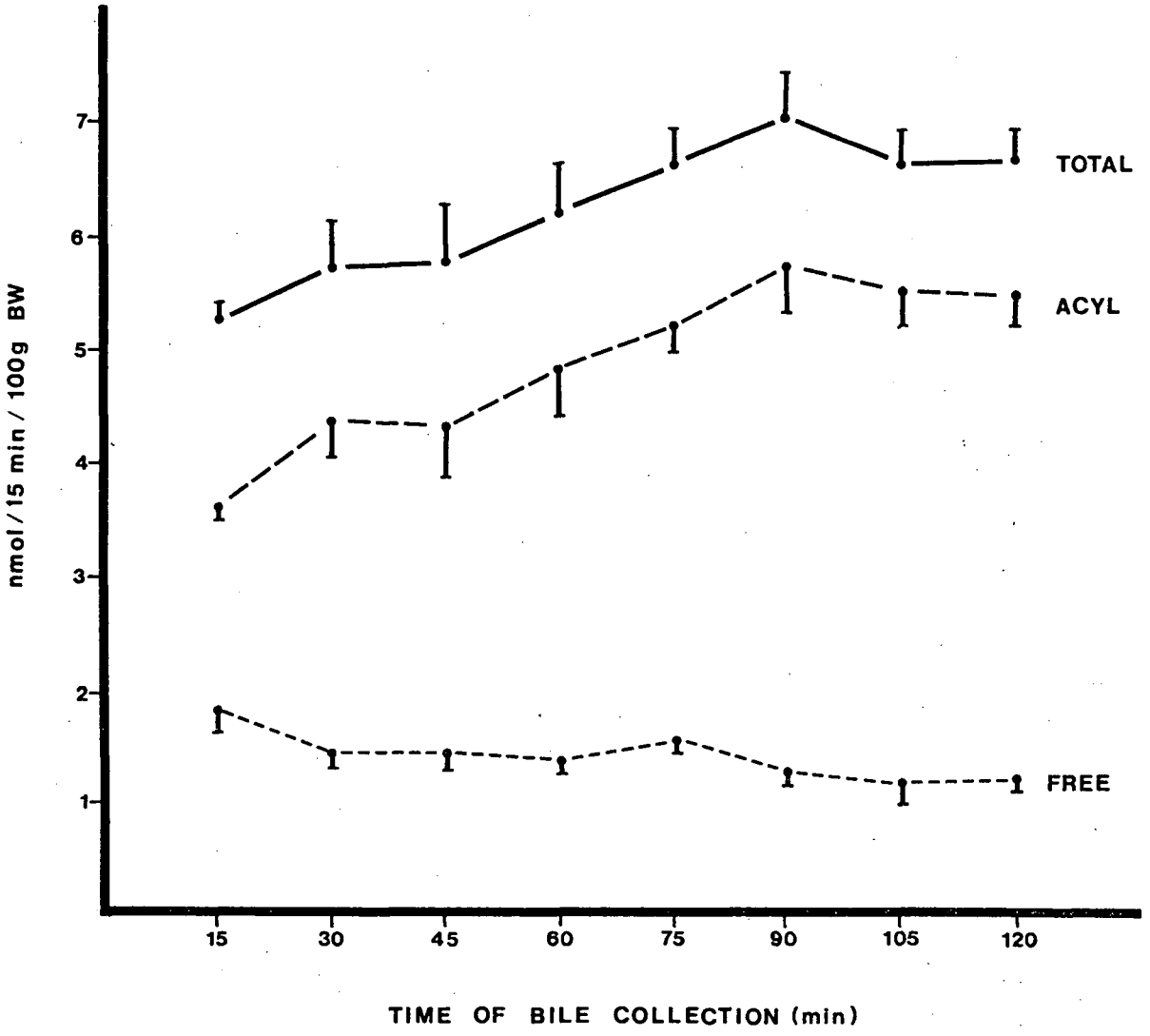


FIGURE 6

The amounts of free, acyl and total carnitine in bile collected from fed rats at 15 minute intervals (nmol/15 min/100 g BW, mean ± SE). The amounts of total and acylcarnitine were significantly greater after 120 minutes of bile collection than after 15 minutes ($p<0.001$). The amount of free carnitine decreased over this same time interval ($p<0.02$).

TABLE 2

The Amounts of Carnitine and Carnitine Esters in the Initial Bile Sample Collected from Fed Rats (n=4)

Type of Carnitine	nmol/15 min/ 100 g BW	%
Free	1.04 ± 0.22	17.2 ± 3.9
Short and Medium-chain	3.29 ± 0.21	53.6 ± 2.9
Long-chain	1.81 ± 0.26	29.2 ± 3.4
Total	6.13 ± 0.18	100 ± 2.9

acetylcarnitine (Table 3). The concentration of acetylcarnitine increased during the course of the bile collection. As a percentage of total carnitine, it remained constant, however.

5.1.4 Serum Carnitine

The concentration of carnitine in rat serum is shown in Table 4. Seventeen percent of the total carnitine was esterified. In the one additional rat studied, there was neither a change in the amount of carnitine nor the degree of carnitine esterification during the course of the experiment.

5.2 Discussion

Carnitine is present in similar concentrations in rat bile and serum. Results published by Gudjonsson and colleagues (1985b) concur. Gudjonsson found 27.3 and 34.3 uM carnitine in the bile of two rats. In this experiment, an average of 46.3 uM (n=11) was found in a comparable collection time period. In addition to the number of rats used, other differences exist between this study and those of Gudjonsson and colleagues: Gudjonsson used Sprague-Dawley rats weighing between 190 and 240 g which had been fasted for 18 hours. Larger, fed, Wistar rats were used in this experiment. These differences may account for the lower concentrations of carnitine found in

TABLE 3

The Concentration of Acetylcarnitine in Rat Bile from the Initial and Final Fifteen Minute Bile Collection Periods

	Initial Sample		Final Sample	
	umol/l	% of Total	umol/l	% of Total
MEAN	7.4	15.6	12.5*	17.7
SE	0.7	1.7	1.3	1.6
N	9	9	9	9

* $p<0.01$ versus initial sample

TABLE 4

The Concentration of Carnitine and Carnitine Esters in Rat Serum After Bile Collection (n=5)

	Free Carnitine umol/l	Total Carnitine umol/l	% Acyl Carnitine
MEAN	48.9	58.9	17.0
SE	4.4	5.0	2.0

the bile collected by Gudjonsson and colleagues. The method of collecting bile was the same in both studies.

Bile flow rate was measured to permit determination of the amount of carnitine in each 15 minute collection. Data published by Kuipers and colleagues (1985) indicate that bile flow decreases from approximately 7.5 to 4.0 ul/min/100 g BW in the 2 hours following initiation of bile collection from nonanaesthetised rats. They attributed this to a decrease in the bile acid dependent bile flow. Kuipers and coworkers also reported that sodium pentobarbital anaesthesia caused a decrease in bile flow in the hour following bile collection but increased flow in subsequent hours. Other investigators, however, have reported that pentobarbital does not affect bile flow (Bailey et al., 1975; Cooper et al., 1976).

The bile flow rate throughout this experiment was similar to that reported for nonanaesthetised rats at the initiation of bile collection (Kuipers et al., 1985). The flow rate was constant throughout the experiment except for the initial 15 minutes of collection at which it was higher. Although some investigators recommend discarding this initial bile sample, (Reuben et al., 1982; Minuk and Sarjeant, 1984), it was retained in this experiment.

The amount of carnitine found in bile in 24 hours may be estimated for a 350 g rat. Assuming a bile flow of 8 ul/min/100 g BW for 12 hours over 24 hours, the amount of carnitine found in the bile would be approximately 1% of the

rats total body pool every 24 hours (1.4 umol or 0.4 umol/24 h/100 g BW). The carnitine body pool size was obtained from the data of Cederblad and Lindstedt (1976). Gudjonsson and colleagues (1985b) estimated that in the rat, there is between 0.4 and 1.4 umol carnitine/24 h/100 g BW in the bile (1-3.5% of the total carnitine body pool).

The concentration and the amount of carnitine in the bile increased throughout the course of the experiment. This was due to increasing amounts of acylcarnitine; the amount of free carnitine remained constant throughout. This phenomenon is discussed in Chapter 8, but the cause is unknown. It is also not known whether the initial sample or the final sample is more representative of true bile, so both are reported.

The analysis of carnitine in the rat bile indicated that between 69% and 80% was esterified. This is comparable to the 75% carnitine esterification reported by Gudjonsson and colleagues (1985b). No other body tissue or fluid contains carnitine that is esterified to such a high degree. The serum from the rats in this study demonstrated 16% esterification. This concurs with the 23% reported by Pace and colleagues (1978). Skeletal muscle carnitine is approximately 51% esterified (Pace et al., 1978) whilst hepatic carnitine esterification ranges from 50 to 62% (Brass and Hoppel, 1981; Brass and Hoppel, 1978; Pace et al., 1978; Pearson and Tubbs, 1967; Bohmer et al., 1966).

The carnitine in the outflow of vascularly perfused rat liver, however, is 73% esterified (Sandor et al., 1985).

Only one quarter of the short and medium-chain carnitine esters in the bile are in the form of acetylcarnitine. In other tissues and fluids, acetylcarnitine is the predominant carnitine ester. Large concentrations of other short-chain or medium-chain carnitine esters are rarely observed.

The percentage of long-chain carnitine esters in rat bile, 30%, was also greater than reported for other fluids or tissues of the rat. In the liver, long-chain carnitine esters make up 1.5-4% of the total carnitine (Brass and Hoppel, 1981; 1978; Bohmer, 1968; 1967; 1966; Pearson and Tubbs, 1967). In the serum, long-chain carnitine esters have been reported as 1% (Pace et al., 1978) and 10% (Brass and Hoppel, 1981) of the total carnitine. Long-chain carnitine esters accounted for 15% of the carnitine exported from the liver during vascular perfusion (Sandor et al., 1985).

The concentration of long-chain carnitine esters in rat bile, is above the critical micellar concentration for palmitoylcarnitine of 15 uM (Seim and Dargel, 1978; Yalkowsky and Zografi, 1970). Therefore, long-chain carnitine esters, which are not water-soluble, are probably in the form of micelles in bile.

In summary, this experiment has demonstrated the presence of relatively large amounts of carnitine and

carnitine esters in rat bile. This suggests that carnitine in the intestinal lumen is not solely of dietary origin. Further, bile may represent a possible route for carnitine excretion. Bile is unique in that it contains more long-chain carnitine esters and a greater percentage of esterified carnitine than other body tissues or fluids.

The establishment of bile as an endogenous source of carnitine in the intestine may help explain the presence of short and medium-chain carnitine esters in the lumen of the rat small intestine (Sachan and Ruark, 1985). It may also explain the recovery of labelled carnitine from the intestine reported after intravenous or subcutaneous injection (Hahn et al., 1985; Rebouche et al., 1984). Lastly, it is possible that bile might serve as a route for carnitine excretion. This suggestion is supported by body pool studies in which carnitine losses could not be accounted for by urinary losses alone (Rebouche and Engel, 1984; Cederblad and Lindstedt, 1976; Yue and Fritz, 1962).

CHAPTER 6

The Origin of Carnitine in Rat Bile

6.1 The Effects of a 72 hour Fast on the Carnitine Content of Rat Bile

6.1.1 Results

6.1.1.1 Bile Flow and Weight Loss

Bile flow decreased in rats after fasting 72 hours (Figure 7). The rate of bile flow did not change during the course of the experiment. The fasted rats lost 14.1 $\pm$ 1.6% of their body weight.

6.1.1.2 Carnitine Content of Bile

Table 5 shows the concentrations of free, acyl and total carnitine in bile of fasted and normally fed rats. At all times, with one exception, the acyl and total carnitine concentrations were greater in the fasted than in the fed state. There was no difference, however, between the concentrations of free carnitine in the fasted and fed states. The concentrations of acyl and total carnitine increased over the course of the experiment while the free carnitine concentration did not change.

The amounts of free, acyl and total carnitine found in each 15 minute bile sample from fasted and normally fed rats are illustrated in Table 6. There were no differences between fed and fasted rats, except in the first collection period where there was less free carnitine upon fasting. The carnitine content of the bile did not change during the experiment.

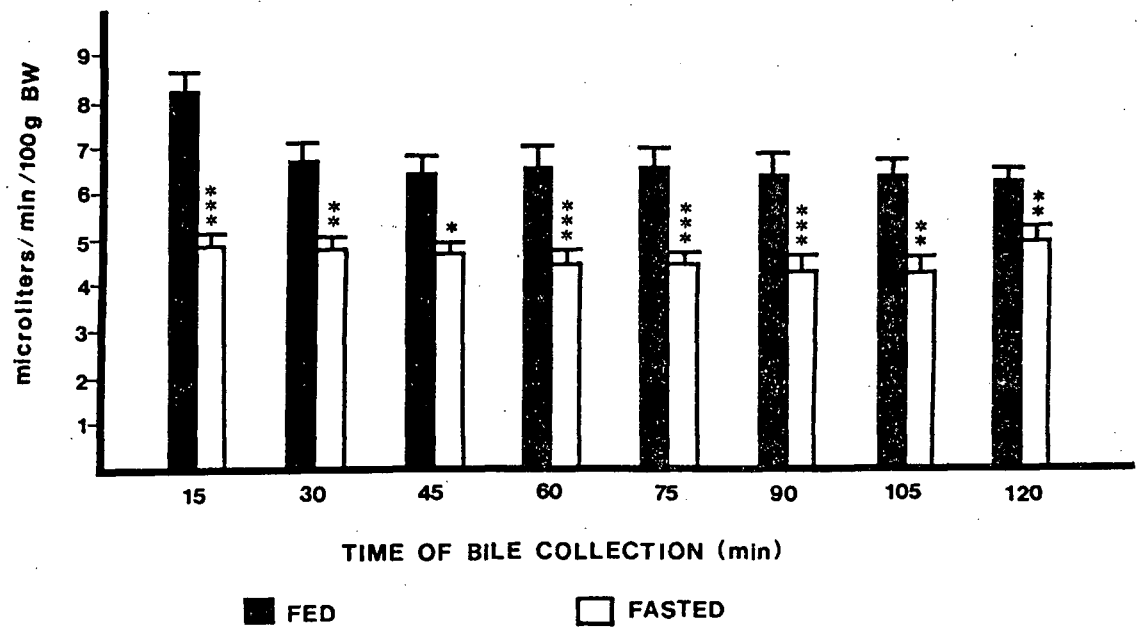


FIGURE 7

Bile flow (microliters/min/100 g BW) during bile collection from fed and 72 hour fasted rats (mean ± SE). Bile was collected in eight consecutive 15 minute samples and the flow rate was assessed gravimetrically. At all time periods, the bile flow decreased upon fasting. The levels of significance are as follows: * = $p<0.02$, ** = $p<0.01$, *** = $p<0.001$.

TABLE 5

The Concentrations of Free, Acyl and Total Carnitine in the Bile of Fed and Fasted Rats

			Consecutive bile collection periods (15 minutes each)							
			1	2	3	4	5	6	7	8
FREE										
	FED	MEAN	14.2	13.2	14.3	13.8	14.2	13.2	12.4	12.4
		SE	1.2	0.9	1.0	1.0	1.0	1.0	1.5	1.5
		N	9	11	11	11	11	11	11	10
	FASTED	MEAN	11.51	11.4	10.9	11.0	11.2	12.7	12.9	12.9
		SE	0.7	1.1	0.6	0.5	0.7	0.6	0.4	0.4
		N	5	5	5	5	5	5	5	5
		p	0.1378	0.2604	0.0470	0.0916	0.0780	0.7536	0.8298	0.8224
ACYL										
	FED	MEAN	32.6	40.2	43.0	53.0	61.5	60.9	60.0	59.4
		SE	1.5	3.3	3.6	3.8	5.3	4.1	3.7	3.6
		N	9	10	11	11	11	11	11	10
	FASTED	MEAN	70.4	73.7	81.2	85.4	88.5	90.2	92.1	89.3
		SE	4.9	1.2	2.0	2.0	1.6	1.3	1.1	3.5
		N	5	5	5	5	5	5	5	5
		p	0.0000 *	0.0000 *	0.0000 *	0.0000 *	0.0048	0.0004 *	0.0000 *	0.0002 *
TOTAL										
	FED	MEAN	46.3	53.2	57.0	67.0	71.0	74.2	70.5	72.3
		SE	1.1	3.3	3.3	3.7	3.9	3.8	3.3	3.0
		N	9	10	10	11	11	11	11	10
	FASTED	MEAN	81.9	85.1	92.0	96.4	99.7	102.9	105.0	102.2
		SE	4.5	1.9	2.3	1.6	1.6	1.5	1.1	3.6
		N	5	5	5	5	5	5	5	5
		p	0.0000 *	0.0000 *	0.0000 *	0.0002 *	0.0002 *	0.0002 *	0.0000 *	0.0000 *

* significant at overall the 0.05 level using Bonferoni's correction based on 48 tests

TABLE 6

The Amounts of Free, Acyl and Total Carnitine in the Bile of Fed and Fasted Rats

			Consecutive bile collection periods (15 minutes each)							
			1	2	3	4	5	6	7	8
FREE										
	FED	MEAN	1.81	1.38	1.41	1.37	1.45	1.27	1.20	1.20
		SE	0.16	0.14	0.13	0.37	0.13	0.11	0.16	0.16
		N	10	11	11	11	11	11	10	9
	FASTED	MEAN	0.84	0.81	0.76	0.75	0.75	0.84	0.82	0.95
		SE	0.05	0.05	0.26	0.03	0.04	0.04	0.06	0.09
		N	5	5	5	5	5	5	5	5
		p	0.0012 *	0.0186	0.0246	0.2866	0.0034	0.0228	0.1432	0.2946
ACYL										
	FED	MEAN	3.59	4.35	4.32	4.79	5.20	5.71	5.48	5.45
		SE	0.13	0.34	0.46	0.37	0.26	0.43	0.30	0.31
		N	8	12	11	11	10	11	10	9
	FASTED	MEAN	5.34	5.30	5.79	5.91	6.01	6.03	5.93	6.49
		SE	0.58	0.25	0.32	0.46	0.44	0.44	0.59	0.46
		N	5	5	5	5	5	5	5	5
		p	0.0036	0.1096	0.0622	0.0986	0.1148	0.9600	0.4590	0.0770
TOTAL										
	FED	MEAN	5.27	5.72	5.73	6.17	6.66	6.98	6.62	6.65
		SE	0.16	0.44	0.50	0.37	0.26	0.42	0.30	0.30
		N	8	11	11	11	10	11	10	9
	FASTED	MEAN	6.19	6.11	6.55	6.67	6.76	6.87	6.75	7.44
		SE	0.60	0.24	0.34	0.50	0.47	0.47	0.65	0.54
		N	5	5	5	5	5	5	5	5
		p	0.0958	0.5770	0.3150	0.4518	0.8422	0.9866	0.8370	0.1874

* significant at overall the 0.05 level using Bonferoni's correction based on 48 tests

6.1.1.3 Carnitine Esters in Bile

The amounts of short and medium-chain carnitine esters decreased from 53 to 33% although the total amount of carnitine in bile was not affected by fasting. At the same time the amount of long-chain carnitine esters increased from 29 to 54% of the total carnitine in the bile (Figure 8).

6.1.1.4 Serum Carnitine

Upon fasting the concentration of serum total carnitine did not differ from the fed state (Table 7). The concentration of free carnitine, however, was lower and the overall carnitine esterification was higher.

6.1.2 Discussion

The changes in the carnitine content of rat bile after a 72 hour fast were similar to those reported by Brass and Hoppel (1978) in rat liver (Table 1). In both, the amounts of free carnitine decreased slightly (from 17% to 13% in the bile and from 53% to 47% in the liver), the amounts of long-chain carnitine esters increased considerably (from 29% to 54% in the bile and from 3% to 11% in the liver) and there were no changes in the amounts of total carnitine. This reflects increased fatty acid oxidation in the fasting state. More carnitine is involved in the transfer of fatty acids across the inner mitochondrial membrane and thus is in the form of long-chain carnitine esters. Therefore, less

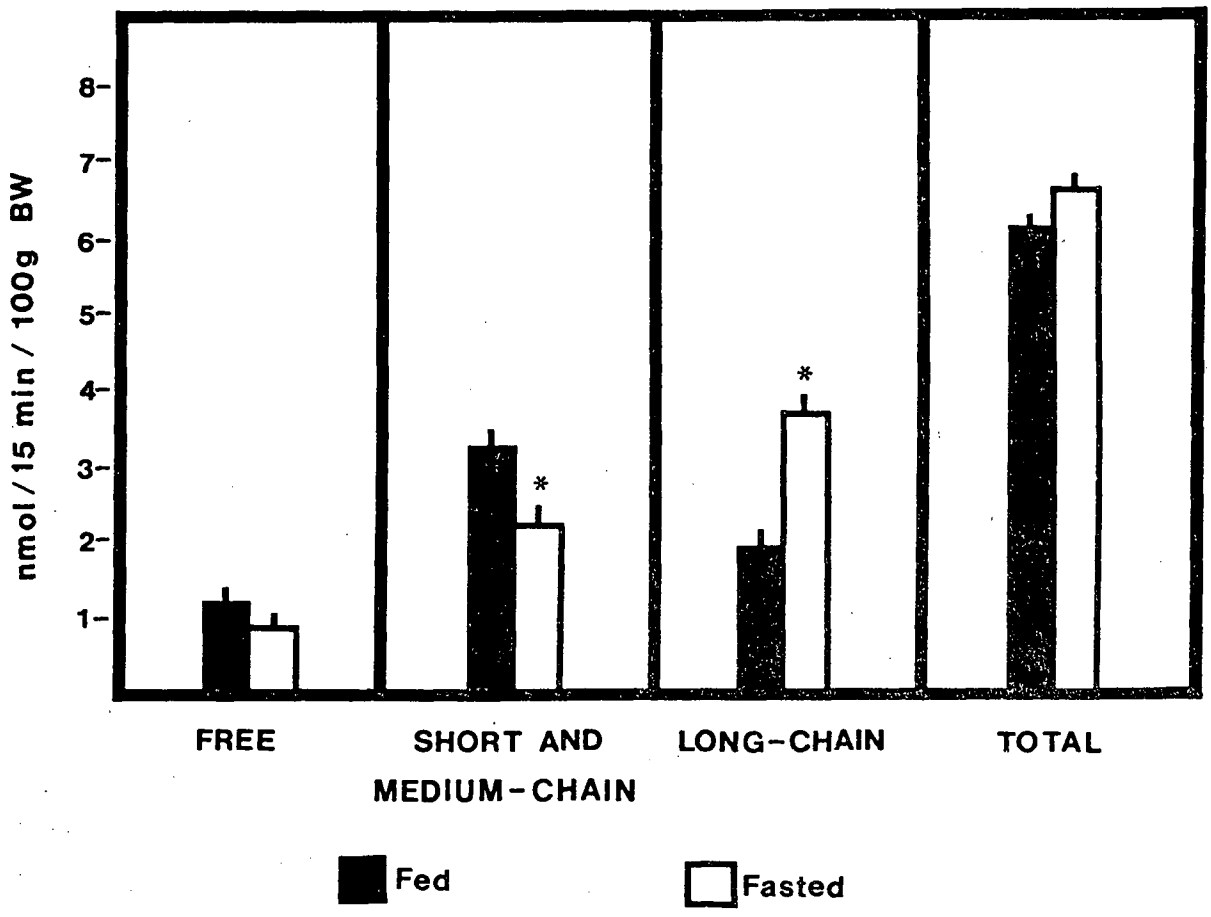


FIGURE 8

The amounts of carnitine and carnitine esters in the bile of fed and fasted rats immediately after the iitiation of bile collection (mean ± SE). Short and medium-chain acylcarnitine levels were decreased after fasting ($p<0.001$) while long-chain carnitine ester levels increased ($p<0.001$). The free carnitine content of the bile did not change after fasting.

carnitine is in the free state. One difference between the liver and bile is that the amount of short and medium-chain carnitine esters decreased in the bile (from 54% to 32%) but not in the liver (43%).

The changes in serum carnitine upon fasting for 72 hours do not parallel those in the bile. Serum carnitine esterification increased from 16 to 50% and the total carnitine did not change. This is comparable to the 51% serum carnitine esterification reported by Gudjonsson and colleagues in rats fasted for 16-18 hours (1985b). In other rats fasted for 72 hours, carnitine esterification increased from 41% to 69% (Brass and Hoppel, 1978). These initially higher carnitine levels might reflect a higher dietary fat content (Seccombe et al., 1978). In humans, serum carnitine esterification also increased upon fasting with no change in total carnitine (Frohlich et al., 1978; Hoppel and Genuth, 1976). Carnitine esterification in the bile did not increase upon fasting. Instead, the amount of long-chain carnitine esters increased while short and medium-chain carnitine esters decreased. After a 72 hour fast, there was no change in the amount of long-chain carnitine esters in the serum (Brass and Hoppel, 1978).

Although the concentration of carnitine in the bile increased upon fasting the amount of carnitine found in the bile did not change per unit time. This is a result of reduced bile flow during fasting which was also observed by Minuk and Sarjeant (1984). Similarly, the hepatic carnitine

content also increased during fasting (Brass and Hoppel, 1981; Bohmer, 1968) but Brass and Hoppel found that this was only a result of decreased liver weight.

Carnitine was also found by Gudjonsson and colleagues (1985b) in the bile of two rats who were fasted 16-18 hours. They found, 30.8 uM total carnitine, much less than the 81.9 uM found in this study after a 72 hour fast. The shorter fasting period might be responsible for this difference. Short and medium-chain carnitine esters made up 33% of the total carnitine compared to 32% in this study and long-chain carnitine esters made up 43% compared to the 54% in this study.

In conclusion, the changes that occur in the composition of carnitine in the bile, more closely parallel those occurring in the liver than the serum as illustrated below.

CARNITINE	SERUM	LIVER	BILE
-amount	no change	no change	no change
-% esters	increased	no change	no change
-types of esters			
-free	decreased	no change	no change
-short and medium	increased	no change	decreased
-long	no change	increased	increased

These results also support the theory that bile carnitine contributes to the carnitine content of the intestinal

lumen. Sachan and Ruark (1985) found that a 24 hour fast lowered the amount of short and medium-chain carnitine esters in the rat intestinal lumen by 54%. There was a 41% decrease in these esters in the bile of rats who were fasted for 72 hours. Lumen free carnitine decreased by 10% and long-chain carnitine esters increased by 12%. The decrease in free carnitine probably reflects the lack of dietary carnitine and the relative increase in long-chain carnitine esters is likely due to the carnitine in the bile which is rich in long-chain carnitine esters. This suggests an association between the bile and the intestinal carnitine contents.

6.2 The Effects of Tetradecylglycidic Acid Treatment on the Carnitine Content of Rat Bile

6.2.1 Results

6.2.1.1 Bile Flow and Weight Loss

The bile flow of fasted rats with and without TDGA treatment is shown in Figure 9. TDGA caused bile flow to increase above the fasting levels except in the second bile collection period. In two additional rats, bile flow was stimulated by the tragacanth vehicle alone. TDGA treated rats lost 14% of their body weights upon fasting for 72 hours. This is not different from the weight loss of the rats who were fasted only.

6.2.1.2 Carnitine Content of the Bile

TDGA treatment caused an increase in the concentration of free carnitine in the bile, a decrease in acyl and no change in total carnitine concentrations (Table 8). Free, acyl and total carnitine concentrations all increased during the experiment.

TDGA caused no changes in the amounts of acyl and total carnitine in each 15 minute bile collection (Table 9). There was, however, more free carnitine in the bile of TDGA treated rats in the third through eigth collection periods. Both the amounts of acyl and total carnitine increased throughout the experiment but free carnitine did not change.

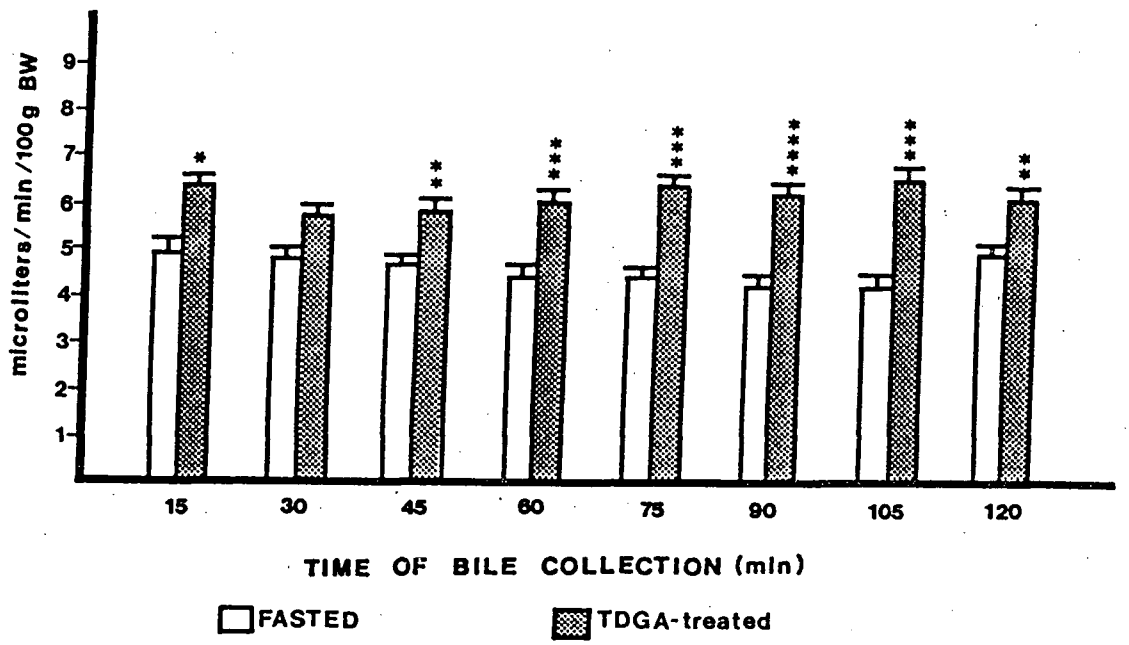


FIGURE 9

Bile flow (microliters/min/100 g BW) during bile collection from 72 hour fasted and TDGA treated rats (mean ± SE). Bile was collected in eight consecutive 15 minute samples and the flow ratewas assessed gravimetrically. At most time intervals the bile flow was increased after TDGA treatment. The levels of significance are as follows: * = $p<0.05$, ** = $p<0.02$, *** = $p<0.01$, **** = $p<0.001$.

TABLE 7

The Concentrations of Carnitine and its Esters in the Serum of Fed, Fasted and TDGA Treated Rats

Treatment	N	Free Carnitine umol/l	Total Carnitine umol/l	% Acyl Carnitine
Fed				
MEAN	5	48.9	58.9	17.0
SE		4.4	5.0	2.0
Fasted				
MEAN	5	35.4 *	71.8	50.4 **
SE		2.6	5.4	2.6
TDGA treated				
MEAN	5	35.0	48.1 @	27.6 @@
SE		3.3	3.0	3.6

* $p<0.05$ versus fed
** $p<0.001$ versus fed
@ $p<0.01$ versus fasted
@@ $P<0.001$ versus fasted

TABLE 8
The Concentrations of Free, Acyl and Total Carnitine in the Bile of Fasted and TDGA-Treated Rats

			Consecutive bile collection periods (15 minutes each)							
			1	2	3	4	5	6	7	8
FREE										
	FASTED	MEAN	11.5	11.4	10.9	11.0	11.2	12.7	12.9	12.9
		SE	0.7	1.1	0.6	0.5	0.7	0.6	0.4	0.4
		N	5	5	5	5	5	5	5	5
	TDGA	MEAN	21.2	23.3	22.9	24.2	25.0	25.4	26.4	26.4
		SE	0.2	1.4	1.0	0.7	1.1	1.0	1.0	1.4
		N	6	6	6	6	6	6	6	5
		p	0.0000 *	0.0004 *	0.0000 *	0.0000 *	0.0000 *	0.0000 *	0.0000 *	0.0000 *
ACYL										
	FASTED	MEAN	70.4	73.7	81.2	85.4	88.5	90.2	92.1	89.3
		SE	4.9	1.2	2.0	2.0	1.6	1.3	1.1	3.5
		N	5	5	5	5	5	5	5	5
	TDGA	MEAN	42.2	49.5	56.0	56.6	58.5	59.9	66.5	62.9
		SE	3.4	2.6	2.9	2.8	3.6	3.4	2.7	3.9
		N	6	6	6	6	6	6	5	5
		p	0.0008 *	0.0000 *	0.0000 *	0.0000 *	0.0000 *	0.0000 *	0.0000 *	0.0010 *
TOTAL										
	FASTED	MEAN	81.9	85.1	92.0	96.4	99.7	102.9	105.0	102.2
		SE	4.5	1.9	2.3	1.6	1.6	1.5	1.1	3.6
		N	5	5	5	5	5	5	5	5
	TDGA	MEAN	63.2	71.8	79.3	79.5	82.7	84.9	91.9	89.3
		SE	2.3	2.8	3.0	3.1	4.3	3.8	2.9	4.4
		N	6	6	6	6	6	6	5	5
		p	0.0036	0.0044	0.0100	0.0014 *	0.0072	0.0028	0.0028	0.0530

* significant at overall the 0.05 level using Bonferoni's correction based on 48 tests

TABLE 9
The Amounts of Free, Acyl and Total Carnitine in the Bile of Fasted and TDGA-Treated Rats

			Consecutive bile collection periods (15 minutes each)							
			1	2	3	4	5	6	7	8
FREE										
	FASTED	MEAN	0.84	0.81	0.76	0.75	0.75	0.84	0.82	0.95
		SE	0.05	0.05	0.26	0.03	0.04	0.04	0.06	0.09
		N	5	5	5	5	5	5	5	5
	TDGA	MEAN	2.00	1.86	2.03	2.02	2.25	2.26	2.42	2.40
		SE	0.26	0.23	0.12	0.13	0.13	0.13	0.11	0.18
		N	6	6	6	6	6	6	5	5
		p	0.0032	0.0028	0.0012 *	0.0000 *	0.0000 *	0.0000 *	0.0000 *	0.0000 *
ACYL										
	FASTED	MEAN	5.34	5.30	5.79	5.91	6.01	6.03	5.93	6.49
		SE	0.58	0.25	0.32	0.46	0.44	0.44	0.59	0.46
		N	5	5	5	5	5	5	5	5
	TDGA	MEAN	3.92	4.28	4.84	4.98	5.47	5.48	6.33	5.73
		SE	0.34	0.54	0.25	0.26	0.48	0.44	0.28	0.49
		N	6	6	6	6	6	6	5	5
		p	0.0550	0.1446	0.0414	0.0986	0.3692	0.4038	0.5576	0.2908
TOTAL										
	FASTED	MEAN	6.19	6.11	6.55	6.67	6.76	6.87	6.75	7.44
		SE	0.60	0.24	0.34	0.50	0.47	0.47	0.65	0.54
		N	5	5	5	5	5	5	5	5
	TDGA	MEAN	5.93	6.13	6.87	6.99	7.73	7.75	8.76	8.13
		SE	0.40	0.71	0.31	0.35	0.58	0.53	0.29	0.62
		N	6	6	6	6	6	6	5	5
		p	0.6928	0.9806	0.5040	0.6036	0.2386	0.2986	0.0224	0.4258

* significant at overall the 0.05 level using Bonferoni's correction based on 48 tests

6.2.1.3 Carnitine Esters in the Bile

In the first bile collection period, increased amounts of free carnitine and short and medium-chain carnitine esters were found in the bile of the TDGA treated rats (Figure 10). Long-chain carnitine ester levels were decreased. By the end of the 2 hour bile collection, the amounts of free carnitine and short and medium-chain carnitine esters had increased further but there was no additional change in the amount of long-chain carnitine esters in the bile.

6.2.1.4 Serum Carnitine

TDGA treatment caused a decrease in the concentration of total carnitine in rat serum (Table 7). Carnitine esterification also was lower upon TDGA treatment, decreasing from 50 to 28%.

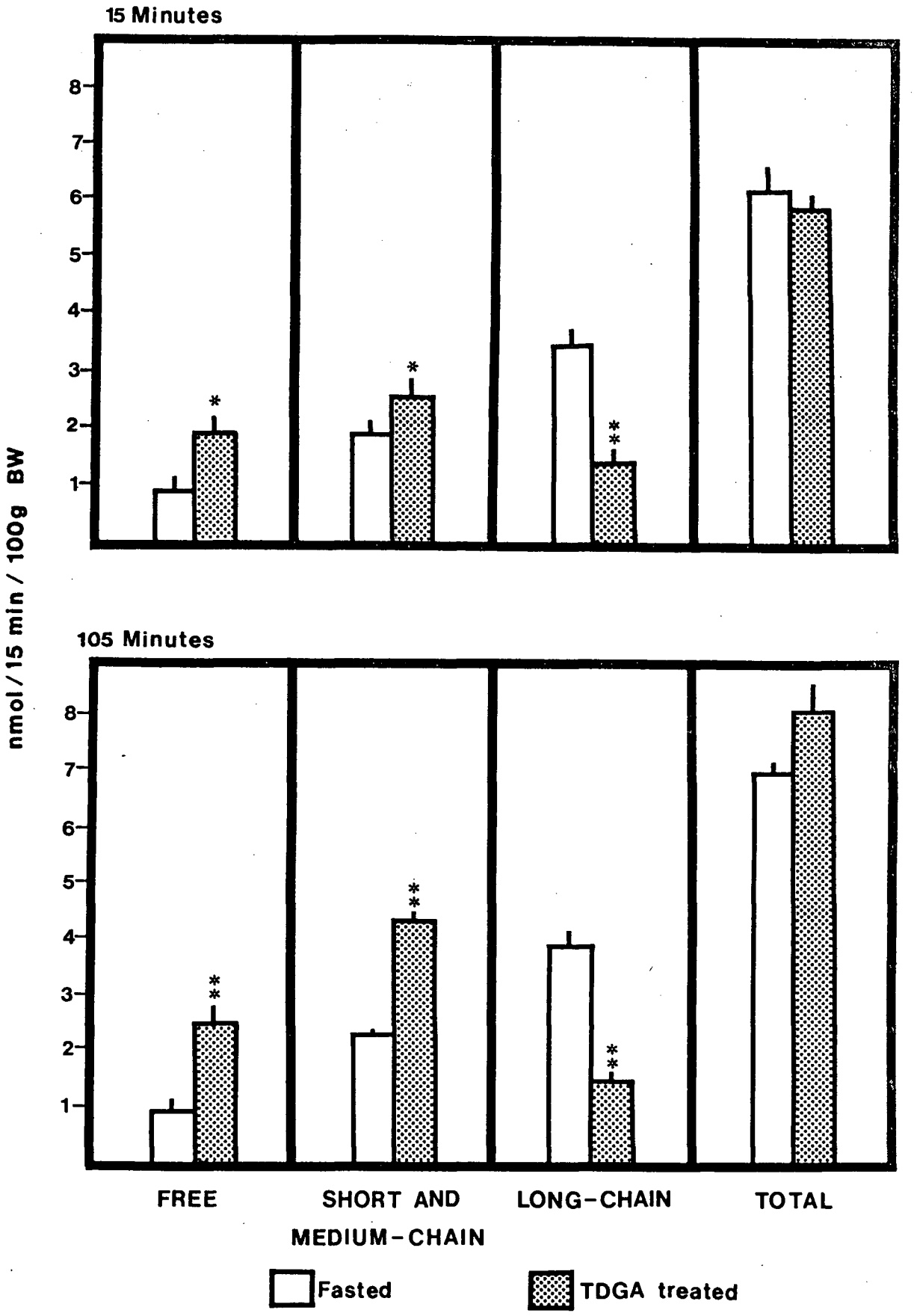
6.2.2 Discussion

The changes in the carnitine content of bile after administration of TDGA to fasted rats were similar to those reported by French and colleagues (1985) for rat liver after the same treatment. The amount of long-chain carnitine esters decreased below fasting levels 2 hours after TDGA intubation and free carnitine levels increased.

In the bile, the percentage of esterified carnitine remained at approximately 70% after TDGA treatment. In the liver, however, TDGA caused a decrease in carnitine

FIGURE 10

The amounts of carnitine and carnitine esters in the bile of fasted and TDGA treated rats 15 and 105 minutes after the initiation of bile collection (mean ± SE). After 15 minutes, free and short and medium-chain acylcarnitine levels were increased in the rats treated with TDGA. Long-chain carnitine ester levels were decreased below fasting levels. After 105 minutes, the free and short and medium-chain carnitine ester levels had increased further but the long-chain acyl carnitine content of bile remained at the 15 minute level. The levels of significance are as follows: * = $p<0.01$ and ** = $p<0.001$.


15 Minutes
105 Minutes
nmol / 15 min / 100g BW
1
2
3
4
5
6
7
8
*
**
FREE
SHORT AND MEDIUM-CHAIN
LONG-CHAIN
TOTAL
Fasted
TDGA treated

esterification from 69% to 55% (French et al., 1985). The fact that esterification in the bile was not affected by TDGA might be due to increased amounts of the short and medium-chain carnitine esters found in the bile upon TDGA administration. These were not changed in the liver after TDGA administration (French et al., 1985).

In rat serum after TDGA intubation, the total and acylcarnitine concentrations were both decreased. This is different from the situation in the bile where there was no change in the overall amount of acyl carnitine. The serum acylcarnitine content may be decreased due to increased uptake of carnitine esters by the tissues for use as energy.

French and colleagues (1985) found that the hepatic carnitine content increased above fasting levels with TDGA treatment. Frost and Wells (1982) also observed this increase in neonatal rats and hypothesized that it is a compensatory mechanism to overcome the inhibition of the TDGA. The amount of carnitine in the bile, however, did not differ with TDGA administration.

Overall, after a 72 hour fast and TDGA intubation, the changes that occur in bile carnitine more closely parallel those occurring in the liver than the serum as shown below.

CARNITINE	SERUM	LIVER	BILE
-amount	decreased	increased	no change
-% esters	decreased	decreased	no change
-types of esters			
-free	no change	increased	increased
-short and medium	decreased	no change	increased
-long	?	decreased	decreased

6.3 The Recovery of Intravenously Injected [^{14}C]Carnitine in Rat Bile

Labelled carnitine, injected intravenously, was almost immediately found in rat bile. The methods and results of this experiment are reported in Appendix 1. This finding supports the hypothesis that the labelled carnitine found in the intestine and feces after subcutaneous and intravenous injections (Hahn et al., 1985; Rebouche et al., 1984) arrives there via the bile.

Within 2 hours of injection, 0.5% of labelled carnitine was recovered in the rat bile. Gudjonsson and colleagues (1985b) found 2.4% of an injected label in the bile after 4 hours. The higher recovery in Gudjonsson's experiment is probably due to the large amount of nonlabelled carnitine injected with the label (8.7 versus 0.26 nmol carnitine/100 g BW) as he found larger amounts of carnitine in the bile after injection of massive doses.

Although the specific activity of the carnitine in the bile and the serum after 20 minutes was similar, the ester distribution was different. There were no long-chain carnitine esters in the serum while 50% of the label in bile was in this form. Of the non-labelled carnitine, only 30% was esterified to long-chain esters. Therefore, the distribution of labelled carnitine is not representative of the actual carnitine distribution in the bile. Therefore, there appears to be more than one pool of carnitine in the liver that enters the bile; one that enters from the serum, but is first esterified to long-chain carnitine esters in the hepatocyte and the other that originates from either stored or newly synthesized carnitine in the liver.

Brooks and McIntosh (1975) also reported the presence of two pools of carnitine in liver; a large (89%), rapidly turning over pool and a smaller, more stable pool. The labelled long-chain carnitine esters presumably come from the larger pool because of their rapid turnover. Alternatively, the injected labelled carnitine could be esterified in the skeletal muscle tissue, and transported to the liver via the serum. This is unlikely as long-chain carnitine esters in muscle cells are probably immediately oxidized. Skeletal muscle has a large, slowly turning over carnitine pool (Brooks and McIntosh, 1975).

Yue and Fritz (1962), when studying the uptake of labelled carnitine injected intravenously into dogs, found that less than 1% of the labelled carnitine taken up by the

skeletal muscle in the 7 hours after injection was chloroform extractable (long-chain carnitine). 6% of the labelled carnitine in the liver, however, could be extracted with chloroform. These results suggest that the liver is more active at producing long-chain carnitine esters from injected carnitine than is the muscle. This is another reason to suspect that labelled long-chain carnitine esters found in the bile originate in the liver.

Gudjonsson and coworkers (1985b) provide further evidence to support the concept that serum carnitine is first esterified in the liver before entering the bile. After administration of large amounts of free carnitine intravenously, the carnitine ester content of the bile increased dramatically while free carnitine levels remained constant.

CHAPTER 7

The Carnitine Content of Human Bile-Rich Duodenal Fluid

7.1 Results

The concentration of carnitine in each duodenal fluid sample is shown in Table 10. The percentage of acylcarnitine was consistent (81%), although free and total carnitine levels varied widely. The two samples collected before the stimulation of bile flow, contained very little carnitine most of which was in the free state. The types of carnitine esters in the duodenal fluid are shown in Table 11. Short and medium-chain carnitine esters made up 35% and long-chain carnitine esters made up 37% of the total carnitine.

7.2 Discussion

Rudman and colleagues (1980) reported no significant differences between the serum carnitine concentrations of cholelithiasis patients and normal controls. On this basis, the population of patients studied in this experiment was assumed to be homogeneous with respect to carnitine metabolism.

The concentrations of both free and total carnitine in bile-rich human duodenal fluid varied widely. The coefficients of variation for these two sets of data were 119% and 140% respectively. In rats, the total concentration of carnitine in the bile had a coefficient of variation of only 7%. When the amount of esterified carnitine in the duodenal fluid was expressed as a

TABLE 10

The Concentrations of Carnitine and Carnitine Esters in Human Duodenal Fluid After Stimulation of Bile Flow

Subject	Carnitine Concentration (uM)		% Acyl Carnitine
	Free	Total	
1 *	19.1	94.6	80
	5.1	27.3	81
2 *	28.3	89.5	68
	1.8	8.8	80
3 *	4.6	38.4	88
	26.0	113.4	77
4	6.1	35.7	83
5	8.0	42.6	81
6	0.6	4.3	86
7 *	4.0	25.4	84
	6.3	49.6	87
8 *	60.9	412.3	85
	58.9	388.8	85
9	2.4 **	2.5 **	4 **
	7.6	30.0	75
10	3.6 **	3.8 **	4 **
	7.8	23.7	67
MEAN ***	16.3	92.3	81
SE	5.0	33.3	2

* Samples, from the same patient, collected 15 minutes apart.
** Samples obtained before bile flow stimulation.
*** Excluding samples obtained before bile flow stimulation.

TABLE 11

The Relative Quantities of Carnitine and Carnitine Esters in Bile-Rich Human Duodenal Fluid

	Free Carnitine (%)	Short and Medium-Chain Carnitine Esters (%)	Long-chain Carnitine Esters (%)
MEAN	26.0	35.3	36.5
SE	3.8	1.5	8.7
N	4	4	4

percentage of the total, however, the coefficient of variation was only 8%.

The degree of carnitine esterification in the duodenal fluid (81%) was not significantly different from that of rat bile in the final bile collection period. These results also correspond with those obtained from one sample of pig bile also analysed for carnitine. This sample was collected directly from the gallbladder of a fasted, male Yucatan pig immediately post-mortem. 87% of the total carnitine (206.9 uM) was esterified.

There are two plausible explanations for the wide range of carnitine concentrations in bile-rich human duodenal fluid. First, humans have gallbladders, which are absent in rats. Bile is concentrated to varying degrees depending on the length of time that it remains in the gallbladder (Wheeler, 1971). Concentration of bile and, hence, carnitine might have occurred to different degrees in the patients studied as they were instructed to fast for 12-18 hours before the procedure. Secondly, the samples studied were duodenal fluid, not pure bile. Each sample could have been diluted to a different extent depending on the amounts of gastrointestinal fluid aspirated along with the bile. This was shown by collecting two samples from each of five patients at intervals of 15 minutes after bile flow was stimulated. In patients #7 and #8, there were no major differences between the carnitine concentrations measured in the two samples. In patients, #1, #2, and #3, however,

there were considerable differences. For example, patient #1 had 94.6 uM carnitine in the first sample but only 27.3 uM carnitine in the latter. The degree of carnitine esterification did not change however between the two samples (80% for the first and 81% for the second).

To ensure that the carnitine measured was from the bile and not other gastrointestinal secretions, the carnitine content of duodenal fluid before bile flow stimulation was measured in two subjects. In both cases, there was less than 4 uM carnitine present. It can be concluded, therefore, that gastrointestinal secretions do not contribute to the carnitine content of the samples. Also, the carnitine ester profile of the pre-stimulation fluid is different from the post-stimulation fluid. Before bile flow stimulation, carnitine was only 4% esterified while after, it was 81% esterified. The carnitine content of pancreatic fluid, the release of which is also stimulated by pancreozymin-cholecystokinin injection, is unknown. The carnitine content of pancreatic tissue, however, is very low (Brooks and McIntosh, 1975). The rat bile was not contaminated by pancreatic secretions because the cannula was placed beyond the pancreatic tissue.

Carnitine in bile-rich human duodenal fluid is distributed evenly between free carnitine, short and medium-chain carnitine esters and long-chain carnitine esters. The percentage of long-chain carnitine esters is not different from that of the bile collected from fed rats

and the percentage of short and medium carnitine esters is not different from that of fasted rat bile. This ester profile might reflect the short fast imposed upon the patients before bile collection.

The existence of an enterohepatic circulation for carnitine has been demonstrated in the rat (Gudjonsson et al., 1985b) and the presence of carnitine in the human bile has now been established. There is also some evidence that carnitine malabsorption in man is related to decreased serum carnitine levels (Seccombe et al., 1984; Frohlich et al., 1980; Mikhail and Mansour, 1976; Bohmer et al., 1975). Therefore, malabsorption in humans might lead to decreased body carnitine not only due to decreased absorption of dietary carnitine but also to decreased intestinal reabsorption of carnitine from the bile.

CHAPTER 8

Changes in the Carnitine Content of Rat Bile During Collection

Throughout this study, the concentration of acylcarnitine in bile increased during rat bile collection. The amount of carnitine found in each 15 minute collection period also increased with time.

Three hypotheses were proposed to attempt to explain this phenomenon. Each was investigated and the results are reported below. First, the effect of the fluid lost via the bile during the experiment was assessed. Secondly, the effect of interrupting the bile flow to collect the samples was investigated. Finally, the effects of the anaesthetic on bile flow were determined.

8.1 The Effect of Saline Infusion on the Carnitine Content of Bile

Over the course of two hours of bile collection, almost 3 ml of bile was collected from each 350 g rat. The effect of this loss on the carnitine composition of the bile was investigated by replacing the fluid lost with saline infused into a jugular vein. The results of this experiment are reported in Appendix 2.

Despite the infusion of saline, the carnitine ester content still increased during bile collection. Therefore, the loss of total body fluid from the rats in the form of bile had no effect on the carnitine content of the bile.

8.2 The Effect of Interruption of the Enterohepatic Circulation on the Carnitine Content of the Bile

Strasberg and colleagues, in a review of biliary sampling (1984), stressed the importance of investigating the effects of the interruption of the enterohepatic circulation on substances measured in bile. To determine whether or not this affects the acylcarnitine content of the bile, bile was recirculated in a rat as described in Appendix 3.

Again the amounts of carnitine esters increased during the course of the experiment. It cannot be concluded, however, that the interruption of the enterohepatic circulation does not alter the carnitine content of the bile, because the enterohepatic circulation was still reduced by 33% in this experiment. It would be advisable, therefore, to repeat this experiment and collect less bile.

8.3 The Effect of Pentobarbital on the Carnitine Content of Bile

In most cases, the carnitine content of bile usually increased during the first 75 minutes of bile collection and then plateaued. Bile collection was usually initiated 5-10 minutes after the rat was anaesthetized. If the anaesthetic was responsible for the increased bile carnitine during the bile collection, one would expect that the levels would have already plateaued after a rat had been anaesthetized for

some time. This was tested by an experiment described in Appendix 4.

The results suggest, however, that the anaesthetic does have an effect on the carnitine esterification in the bile but is not responsible for the increase in the carnitine content during the course of the experiment.

8.4 Summary

With the exception of the saline infusion experiment, no conclusions can be drawn from this work, because of the small number of animals used. One can, however, use the results to form new hypotheses and direct further studies. For instance, it seems apparent that the effect of the anaesthetic should be more closely investigated. Future studies should compare the effects of different anaesthetics on the carnitine composition of bile. The effect of interrupting the enterohepatic circulation should also be examined more thoroughly to determine whether acylcarnitine still increases during the experiment with removal of less than 33% of the bile.

If the increased carnitine in the bile cannot be accounted for by the effects of the anaesthetic or the interruption of the enterohepatic circulation, the effect of surgery itself should be investigated. Possibly, physical manipulation of the bile duct is responsible. Strasberg and colleagues (1984) report that with bile duct cannulation, the pressure-flow relationships of the bile are distorted.

Such effects might account for the change in the carnitine content of bile during bile collection.

CHAPTER 9

Conclusions

The conclusions of this study are summarized below. Their implications and suggestions for further research are also presented.

9.1 Summary

9.1.1 Carnitine Content of the Bile

The concentration of carnitine in rat bile was similar to that of rat serum. Bile contained more acylcarnitine and long-chain carnitine esters than other rat tissues or fluids. It was estimated that 1% of the total body pool of carnitine passes through the bile every 24 hours. Gudjonsson and colleagues (1985b) reported similar quantities of carnitine in rat bile.

Carnitine was also found in human bile-rich duodenal fluid. Although the quantities varied considerably, the degree of carnitine esterification was consistent and similar to that of rat bile. The long-chain carnitine ester content was also similar to that of rat bile.

9.1.2 Origin of Carnitine in the Bile

The origin of the carnitine in bile was not determined conclusively. Three experiments were performed to attempt to distinguish between hepatic and extrahepatic carnitine sources. In the first two, the rats were either fasted or intubated with tetradecylglycidic acid; treatments which affect the amounts of carnitine and types of carnitine esters in the liver and serum. The changes in the bile

carnitine more closely parallelled those occurring in the liver than the serum.

In the final experiment, [^{14}C]carnitine was intravenously injected into rats during bile collection. Results suggest that carnitine in the bile is derived from both newly synthesized or stored hepatic carnitine and serum carnitine which enters the liver and becomes esterified.

9.1.3 The Transport of Carnitine into Bile

Some information was obtained concerning the transportation of carnitine into the bile (see Appendix 5). Results suggested that the carnitine content of bile is both dependent on bile formation and the carnitine content of the serum. The increased concentrations of carnitine in the bile of fasted rats might be the result of water reabsorption after bile production.

9.1.4 Possible Functions of Carnitine in the Bile

The function of carnitine in the bile is not known. Although Gudjonsson and colleagues have determined that there is an enterohepatic circulation of carnitine, the efficiency of this is unknown. It is possible, therefore, that the bile could be an excretory route for carnitine. Data presented by Rebouche and colleagues (1984), who found labelled carnitine in the feces after intravenous administration, support this. As the excretion of energy-rich long-chain carnitine esters would be

inefficient, hydrolysis of the carnitine ester might occur in the intestinal lumen.

Another possible function of carnitine in the bile is to rid hepatocytes of excess organic acids resulting from organic acidurias or treatment with drugs such as valproic acid.

The involvement of carnitine in fatty acid absorption is another possible role. Long-chain carnitine esters in the high concentrations found in bile might aid in the emulsification of dietary fatty acids or be involved in the intracellular transport of fatty acids within the enterocytes. The long delay between uptake of carnitine into the mucosal cells and its release into the portal blood indicates that the carnitine is involved in some process within the mucosal cells.

9.2 Suggestions for Additional Work

9.2.1 The Absorption of Carnitine and its Esters

Only the absorption of free and acetylcarnitine have been studied. There is no knowledge of the intestinal handling of long-chain acylcarnitine which are present in large quantities in bile and the diet. A simple, and physiological way to study this in anaesthetized rats would be by mixing labelled long-chain carnitine esters with bile to measure its uptake from the rat intestine while interrupting further bile flow. This would avoid the problem of the water-insolubility of long-chain

acylcarnitines and prevent possible complications caused by the recirculation of the absorbed label in the bile.

The enterohepatic circulation of bile, reported by Gudjonsson and colleages who studied a single rat, should be further quantitated. The efficiency of reabsorption of all types of carnitine esters should be measured and the effects of dietary components determined.

9.2.2 The Involvement of Carnitine in Fat Absorption

The effects of carnitine on fat absorption were not studied in these experiments. Future experiments could investigate this in anaesthetized rats by injecting labelled fatty acids into tied off intestinal loops. The effect of carnitine on fatty acid absorption could then be determined by monitoring the disappearance of the label from the intestinal lumen of rats in the presence and absence of carnitine.

Another reason for the interest in fat absorption and carnitine is based on the observation that oral carnitine may have useful short term effects in acutely ill patients with cystic fibrosis (Benke et al., 1984). Because fat absorption is usually impaired in cystic fibrosis, a possible role for carnitine was suspected. Carnitine was measured in the serum of several children with cystic fibrosis. The results are shown in Appendix 6. Although these patients had serum carnitine levels in the normal range (Mitchell, 1978b), additional carnitine might be

beneficial at the level of the intestine. This should be investigated further.

9.2.3 The Malabsorption of Biliary Carnitine

In this experiment, it was calculated that 1% of the total body carnitine of the rat passed through the bile each 24 hours. Gudjonsson and colleagues (1985) reported similar results. As carnitine was also found in bile-rich human duodenal fluid, the possibility exists that general malabsorption could result in carnitine depletion in humans. Although some studies have reported reduced serum carnitine levels in patients with various malabsorption syndromes, only one experiment reported decreased carnitine absorption (Seccombe et al., 1984). It seems suitable that further studies should be made of patients with various malabsorptive disorders. First, it should be determined whether or not these patients have reduced serum or tissue carnitine levels. Then, the possibilities of reduced carnitine absorption and reduced lysine and methionine absorption should be distinguished. This could be done by determining whether serum and tissue carnitine depletion persists after parenteral feeding with formulas lacking in carnitine.

9.2.4 The Effects of Hepatic Dysfunction on Biliary Carnitine

The effect of liver disease on the carnitine content of

the bile should also be studied. The serum carnitine concentrations of patients with alcoholic cirrhosis were significantly lower than those of normal controls (Rudman et al., 1980). This could be due to a reduced hepatic synthesis of carnitine or to general malabsorption. Also, the possibility of uncontrolled release of carnitine into the bile should also be investigated.

9.2.5 Biliary Carnitine in Body Pool Studies

To understand completely the metabolism of carnitine in the rat, body pool studies have been attempted using labelled carnitine. None, however, has considered the possible excretion of carnitine through the bile. These studies should be repeated taking into account the bile.

9.2.6 Carnitine Absorption in Systemic Carnitine Deficiency Syndromes

In cases of systemic carnitine deficiency, the site of the disorder is often not known and oral carnitine therapy is not always successful at alleviating the symptoms. It has been hypothesized that intestinal absorption is a possible site of defect (Gilbert, 1985; Engel and Rebouche, 1984). In the future, this should be investigated further by monitoring the effect of a bolus oral dose of carnitine on serum carnitine levels in patients and controls.

9.3 Implications

The discovery of carnitine in rat bile challenges the common assumption that only dietary carnitine is present in the intestinal tract. It also disputes the theory that urine is the single route for carnitine excretion. Carnitine researchers must now consider the carnitine content of the bile in intestinal and excretion studies.

Another implication of this work is for patients with malabsorption syndromes. It is possible that these patients have compromised carnitine stores not only due to lack of absorption of dietary carnitine and its amino acid precursors but also from lack of reabsorption of carnitine from the bile. Until it is conclusively shown whether or not this bile loss is significant to the well being of the patient, serum and tissue carnitine levels should be measured in patients with chronic malabsorptive syndromes. If reduced carnitine concentrations do exist and symptoms of carnitine depletion result, it might be advisable to supplement these individuals with intravenous or large doses of oral carnitine.

References

Aas, M. and Bremer, J. (1968) Short-chain fatty acid activation in rat liver. A new assay procedure for the enzymes and studies on their intracellular localization. Biochim. Biophys. Acta 164, 157-166.

Aas, M. and Daae, L.N.W. (1971) Fatty acid activation and acyl transfer in organs from rats in different nutritional states. Biochim. Biophys. Acta 239, 208-216.

Bach, A.C., Schirardin, H., Sihr, M.-O. and Storck, D. (1983) Free and total carnitine in human serum after oral ingestion of L-carnitine. Diabete Metab. 9, 121-124.

Bahl, J., Navin, T., Manion, A. and Bressler, R. (1981) Carnitine transport in isolated rat heart myocytes and the effects of 7,8-diOH chloropromazine. Circ. Res. 48, 378-385.

Bailey, D.G., Paul, H. and Johnson, G.E. (1975) Pentobarbital inhibits the biliary excretion of organic acids: a study of succinylsulfathiazole in the rat. Can. J. Physiol. Pharm. 53, 470-474.

Benke, P.J., McKay, B. and Foradada, J. (1984) L-Carnitine for cystic fibrosis. Ped. Res. 18, 219A.

Bieber, L.L., Krahling, J.B., Clarke, P.R.H., Valkner, K.J. and Tolbert, N.E. (1981) Carnitine acyltransferases in rat liver peroxixomes. Arch. Biochem. Biophys. 211, 599-604.

Bohmer, T., Norum, K.R. and Bremer, J. (1966) The relative amounts of long-chain acylcarnitine, acetylcarnitine and free carnitine in organs of rats in different nutritional states and with alloxan diabetes. Biochim. Biophys. Acta 125, 244-251.

Bohmer, T. (1967) Tissue levels of activated fatty acids(acylcarnitines) and the regulation of fatty acid metabolism. Biochim. Biophys. Acta 144, 259-270.

Bohmer, T. and Bremer, J. (1968) Propionylcarnitine physiological variations in vivo. Biochem. Biophys. Acta 152, 559-567.

Bohmer, T. (1974) Conversion of butyrobetaine to carnitine in the rat in vivo. Biochim. Biophys. Acta 343, 551-554.

Bohmer, T., Rydning, A. and Solberg, H.E. (1974) Carnitine levels in human serum in health and disease. Clin. Chim. Acta 57, 55-61.

Bohmer, T., Eiklid, K. and Jonsen, J. (1977) Carnitine uptake into human heart cells in culture. Biochim. Biophys. Acta 465, 627-633.

Brass, E.P. and Hoppel, C.L. (1978) Carnitine metabolism in the fasting rat. J. Biol. Chem. 253, 2688-2693.

Brass, E.P. and Hoppel, C.L. (1981) In vivo studies of carnitine and fatty acid metabolism; problems with the use of anaesthetics. Anal. Biochem. 110, 77-81.

Bremer, J. (1981) The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. Biochim. Biophys. Acta 665, 628-631.

Bremer, J. (1983) Carnitine - Metabolism and functions. Physiol. Rev. 63, 1420-1480.

Brooks, D.E and McIntosh, J.E.A. (1975) Turnover of carnitine by rat tissues. Biochem. J. 148, 439-445.

Brooks, D.E. (1980) Carnitine and the male reproductive tract and its relation to the metabolism of the epididymis and spermatozoa. In: Carnitine Biosynthesis, Metabolism and Function. Edited by R.A. Frenkel and J.D. McGarry. Academic Press, London, New York, San Francisco, pp. 219-236.

Carter, H.E., Bhattacharya, P.K., Weidman, K.R. and Fraenkel, G. (1952) Chemical studies on vitamin Bt isolation and characterization of carnitine. Arch. Biochem. Biophys. 38, 405-416.

Cederblad, G. and Lindstedt, S. (1972) A method for the determination of carnitine in the picomole range. Clin. Chim. Acta 37, 235-243.

Cederblad, G. and Lindstedt, S. (1976) Metabolism of labelled carnitine in the rat. Arch. Biochem. Biophys. 175, 173-180.

Christiansen, R.Z. and Bremer, J. (1976) Active transport of butyrobetaine and carnitine into isolated liver cells. Biochim. Biophys. Acta 448, 562-577.

Cooper, B., Eakins, M.N. and Slater, T.F. (1976) The effect of various anaesthetic techniques on the flow rate, constituents and enzymic composition of the rat bile. Biochem. Pharm. 25, 1711-1718.

Engel, A.G. and Rebouche, C.J. (1984) Carnitine metabolism and inborn errors. J. Inherited Metab. Dis. 7, S38-S43.

Englard, S. (1979) Hydroxylation of gamma-butyrobetaine to carnitine in human and monkey tissues. FEBS Lett. 1102, 297-300.

Fraenkel, G. (1951) Effect and distribution of vitamin Bt. Arch. Biochem. Biophys. 34, 457-463.

French, T.J., Goode, A.W., Holness, M.J. and Sugden, M.C. (1985) Carnitine metabolism in livers and hearts of 48H-starved rats: the contribution of fat and carbohydrate to acylcarnitine formation. Biochem. Int. 11, 265-272.

Friedman, S. and Fraenkel, G. (1955) Reversible enzymic acetylation of carnitine. Arch. Biochem. Biophys. 59, 491-501.

Friedman, S. (1958) Determination of carnitine in biological materials. Arch. Biochem. Biophys. 75, 24.

Fritz, I.B. (1955) The effects of muscle extracts on the oxidation of palmitic acid by liver slices and homogenates. Acta Physiol. Scand. 34, 367-385.

Fritz, I.B. (1968) The metabolic consequences of the effects of carnitine on long-chain fatty acid oxidation. In: Cellular Compartmentalization and Control of Fatty Acid Metabolism. Edited by F.C. Gran. Academic Press, London, pp. 39-63.

Frohlich, J., Seccombe, D.W., Hahn, P., Dodek, P. and Hynie, I. (1978) Effect of fasting on free and esterified carnitine levels in human serum and urine: correlation of serum levels of free fatty acids and B-hydroxybutyrate. Metabolism 27, 555-561.

Frohlich, J., Hahn, P. and Cleator, I. (1980) Changes in plasma carnitine levels after jejunoileal bypass. Lancet 1, 1085.

Frost, S.C. and Wells, M.A. (1982) Effect of inhibition of fatty acid oxidation on neonatal liver carnitine content. Biochem. J. 204, 861-863.

Gilbert, E.F. (1985) Carnitine deficiency. Pathology 17, 161-169.

Gross, C.J. and Henderson, L.M. (1983) Carnitine absorption by the rat small intestine. Fed. Proc. 42, 1320.

Gross, C.J. and Henderson, L.M. (1984) Absorption of D- and L-carnitine by the intestine and kidney tubule in the rat. Biochim. Biophys. Acta 772, 209-219.

Gross, C.J., Henderson, L.M. and Savaiano, D.A. (1985) Absorption of L and D carnitine by isolated guinea pigs enterocytes. Fed. Proc. 44, 763.

Gudjonsson, H., Li, B U.K., Stancher, J., Shug, A.L. and Olsen, W.A. (1984) In vivo studies of small intestinal carnintine absorption. Gastroenterology 86, 1101.

Gudjonsson, H., Li, B U.K., Shug, A. and Olsen, W.A. (1985a) In vivo studies of intestinal carnitine absorpton in rats. Gastroenterology 88, 1880-1887.

Gudjonsson, H., Li, B U.K., Shug, A.L. and Olsen, W.A. (1985b) Studies of carnitine metabolism in relation to intestinal absorption. Am. J. Physiol. 248, G313-G319.

Gulewitsch, W. and Krimberg, R. (1905) Zur kenntnis der extraktivstoffe der muskeln, 2 mitteilung. Ueber das Carnitin. Ztschr f. Physiol. Chem., Strassb. 45, 326-330.

Gumpen, S.A. and Norum, K.R. (1973) The relative amount of long-chain acylcarnitines, short-chain tissue from rats fed on rapeseed oil. Biochim. Biophys. Acta 316, 48-55.

Hahn, P., Chanez, C. and Hamilton, J. (1985) Carnitine and carnitine transferases in the intestinal mucosa of suckling rats. Biol. Neonate 48, 77-84.

Hamilton, J.W., Li, B U.K., Shug, A. and Olsen, W.A. (1983) Studies of L-carnitine absorption in man. Gastroenterology 84, 1180.

Hanson, P.J. and Carrington, J.M. (1981) Activity of 3-oxo acid CoA-transferase, D-3-hydroxybutyrate dehydrogenase, hexokinase and carnitine palmitoyltransferase in the stomach and small and large intestine of the rat. Biochem. J. 20, 349-355.

Hardison, W.G.M. and Apter, J.T. (1972) Micellar theory of biliary cholesterol excretion. Am. J. Physiol. 222, 61-67.

Hoppel, C.L. and Genuth, S. (1976) Fasting alters carnitine metabolism. Clin. Res. 24, 580A.

Huth, P. and Shug, A. (1980) Properties of carnitine transport in rat kidney cortex slices. Biochim. Biophys. Acta 602, 621-634.

Huth, P., Schmidt, M., Hall, P., Fariello, R. and Shug, A. (1981) The uptake of carnitine by slices of rat cerebral cortex. J. Neurochem. 36, 715-723.

Innis, S.M. (1985) Hepatic transport of bile salt and bile composition following total parenteral nutrition with and without lipid emulsion in the rat. Am. J. Clin. Nutr. 4, 1283-1288.

Ivy, A.C., Oldberg, E., Kloster, G. and Lueth, H.C. (1928) A hormone mechanism for gallbladder contraction and evacuation. Am. J. Physiol. 85, 381-383.

James, M., Brooks, D. and Snoswell, A. (1981) Kinetics of carnitine uptake by rat epididymal cells; androgen dependence and lack of stereospecificity. FEBS Lett. 126, 53-56.

Karpati, G., Carpenter, S., Engel, A.G., Watters, G., Allen, J., Rothman, S., Klassen, G. and Mamer, O.A. (1975) The syndrome of systemic carnitine deficiency. Neurology 25, 16-24.

Kuipers, F., Dijkstra, T., Havinga, R., Van Asselt, W., and Vonk, R.J. (1985) Acute effects of pentobarbital-anaesthesia on bile secretion. Biochem. Pharmacol. 34, 1731-1736.

Kutscher, F. (1905) Ueber Liebig's fleischextrakt. Ztschr, f. Untersuch. d.Nahrungs-u. Genussmittel, Berlin. 10, 528-537.

LaRusso, N.F. (1984) Proteins in bile: how they get there and what they do. Am. J. Physiol. 247, G199-G205.

Li, B U.K., Hamilton, J.W., Shaw, R.A., Olsen, W.A., Shug, A.L. (1983) Carnitine transport in rat jejunal microvillous membrane vesicles. Gastroenterology 84, 1230.

Li, B U.K., Graves, L.L. and Olsen, W.A. (1985) Unidirectional carnitine fluxes in isolated rat jejunum: evidence of diffusion. Fed. Proc. 44, 763.

Lindstedt, G. and Lindstedt, S. (1965) Studies on the biosynthesis of carnitine. J. Biol. Chem. 240, 316-321.

Mandarino, L., Tsalikian, E., Bartold, S., Marsh, H., Carney, A., Buerkin, E., Tutweiler, G. Haymond, M., Handwerger, B. and Rizza, R. (1984) Mechanism of hypoglycemia and response to treatment with an inhibitor of fatty acid oxidation on a patient with insulin resistance to antiinsulin receptor antibodies. J. Clin. Endocrinol. Metab. 59, 658-664.

Marquis, N.R. and Fritz, I.B. (1964) Enzymological determination of free carnitine concentrations in rat tissues. J. Lipid Res. 5, 184-187.

McGarry, J.D. and Foster, D.W. (1976) An improved and simplified radioisotopic assay for the determination of free and esterified carnitine. J. Lipid Res. 17, 277-281.

Mikhail, M.M. and Mansour, M.M. (1976) The relationship between serum carnitine levels and the nutritional status of patients with schistosomiasis. Clin. Chim. Acta 71, 207-214.

Minuk, G.Y. and Sarjeant, E.J. (1984) Gammaaminobutyric acid (GABA) and the hepatic bile flow in the rat. Clin. Invest. Med. 7, 193-197.

Mitchell, M.E. (1978a) Carnitine metabolism in human subjects I. Normal metabolism. Am. J. Clin. Nutr. 31, 293-306.

Mitchell, M.E. (1978b) Carnitine metabolism in human subjects II. Values of carnitine in biological fluids and tissues of "normal" subjects. Am. J. Clin. Nutr. 31, 481-491.

Norum, K.R. (1965) Activation of palmityl-CoA:carnitine palmityltransferase in the livers from fasted, fat-fed, or diabetic rats. Biochim. Biophys. Acta 98, 652-654.

Ohtani, Y., Endo, F. and Matsuda, I. (1982) Carnitine deficiency and hyperammonemia associated with valproic acid therapy. J. Ped. 101, 782-785.

Osmudsen, H. and Neat, C.E. (1979) Regulation of peroxisomal fatty acid oxidation. FEBS Lett. 107, 81-84.

Pace, J.A., Wannemacher, R.W. and Neufeld, H.A. (1978) Improved radiochemical assay for carnitine and its derivatives in plasma and tissue extracts. Clin. Chem. 24, 32-35.

Paik, W.K. and Kim, S. (1975) Protein methylation: chemical enzymoloogical and biological significance. Adv. Enzymol. 42, 227-286.

Pande, S.V. and Parvin, R. (1976) Characterization of carnitine acylcarnitine translocase system of heart mitochondria. J. Biol. Chem. 251, 6683-6691.

Pande, S.V. (1981) Uneven distribution of palmitoyl carnitine in solutions because of migration to air/water interphase. Biochim. Biophys. Acta 663, 669-673.

Pande, S.V. and Caramancion, M.N. (1981) A simple radioisotopic assay of acetylcarnitine and acetyl-CoA at picomolar levels. Anal. Biochem. 112, 30-38.

Parvin, R. and Pande, S.V. (1977) Microdetermination of (-)carnitine and carnitine acetyltransferase activity. Anal. Biochem. 79, 190-210.

Pearson, D.J. and Tubbs, P.K. (1967) Carnitine and derivatives in rat tissues. Biochem. J. 105, 953-963.

Rebouche, C. (1977) Carnitine movement across muscle cell membranes studied in isolated rat muscle. Biochim. Biophys. Acta 471, 145-155.

Rebouche, C.J. and Engel, A.G. (1983) Kinetic compartmental analysis of carnitine metabolism in the dog. Arch. Biochem. Biophys. 220, 60-70.

Rebouche, C.J. and Edmonson, P.F. (1984) Metabolism of oral carnitine in the rat. Fed. Proc. 43, 617.

Rebouche, C.J. and Engel, A.G. (1984) Kinetic compartmental analysis of carnitine metabolism in the human carnitine deficiency syndromes; evidence for alterations in tissue carnitine transport. J. Clin. Invest. 73, 857-867.

Rebouche, C.J., Mack, D.L. and Edmonson, P.F. (1984) L-carnitine dissimilation in the gastrointestinal tract of the rat. Biochemistry 23, 6422-6426.

Rudman, D., Ansley, J.D. and Sewell, C.W. (1980) Carnitine deficiency in cirrhosis. In: Carnitine Biosynthesis, Metabolism and Function. Edited by R.A. Frenkel and J.D. McGarry. Academic Press, London, New York, San Francisco, pp. 307-320.

Sachan, D.S. and Ruark, R.A. (1985) Distribution of carnitine and acylcarnitine in small intestine of carnitine-supplemented and fasted rats. J. Nutr. 115, 865-871.

Sandor, A., Kispal, G., Melegh, B. and Alkonyi, I. (1985) Release of carnitine from the perfused rat liver. Biochim. Biophys. Acta 835, 83-91.

Sartorelli, L.M., Ciman, M., Rizzoli, V. and Siliprandi, N. (1982) On transport mechanisms of carnitine and its derivatives in rat heart slices. Ital. J. Biochem. 31, 261-268.

Schanker, L.S. and Solomon, H.M. (1963) Active transport of quaternary ammonium compounds into bile. Am. J. Physiol. 204, 829-832.

Scharschmidt, B.F. and Schmid, R. (1978) The micellar sink. J. Clin. Invest. 62, 1122-1131.

Seccombe, D.W., Hahn, P. and Novak, M. (1978) The effect of diet and development on blood levels of free and esterified carnitine in the rat. Biochim. Biophys. Acta 528, 483-489.

Seccombe, D.W. (1981) Carnitine and carnitine acetyltransferase: their role in development. Ph.D. Dissertation, University of British Columbia, Vancouver, Canada.

Seccombe, D., Burget, D., Frohlich, J., Hahn, P., Cleator, I. and Gourlay, R.H. (1984) Oral l-carnitine administration after jejunoileal by-pass surgery. Int. J. Obes. 8, 427-433.

Seim, H. and Dargel, R. (1978) Induktion mitochondrialer volumen anderungen dursh homologe o-acylcarnitin-isomere. Acta Biol. Med. Ger. 37, 73-82.

Shaw, R.D., Shug, A.L. and Olsen, W.A. (1982) Studies of carnitine transport by rat small intestine. Gastroenterology 82, 1177.

Shaw, R.D., Li B U.K., Hamilton, J.W., Shug, A.L. and Olsen, W.A. (1983) Carnitine transport in the rat small intestine. Am. J. Physiol. 245, G376-G381.

Strasberg, S.M., Harvey, P.R.C. and Gallinger, S. (1984) Anatomy, visualization and sampling of the biliary tree in animals and man. Hepatology 4, 1S-3S.

Thurston, J.H., Carroll, J.E., Hauhart, R.E. and Schiro, J.A. (1984) Reply to letter: Valproate treatment and carnitine deficiency. Neurology 34, 1128-1129.

Tomita, M. and Sendju, Y. (1927) Uber die oxyaminoverbindungen, welche die Biuretreaktion zeigen. III. Spaltung der gamma-amino-beta-oxybuttersaure in die optisch-ak-tiven komponenten. Hoppe-Seyer's Z. Physiol. Chem. 169, 263-277.

Tutweiler, G.F., Dellevigne, P., Mohrbacher, R. and Ho, W. (1978) Methyl 2-tetradecylglycidate; an orally effective hypoglycemic agent which inhibits long-chain fatty acid oxidation. Fed. Proc. 37, 1308.

Tutweiler, G.F. and Dellevigne, P. (1979) Action of the oral hypoglycemic agent 2-tetradecylglycidic acid on hepatic fatty acid oxidation and gluconeogenesis. J. Biol. Chem. 254, 2935-2941.

Waynforth, H.B. (1980) Experimental and Surgical Technique in the Rat. Academic Press, London.

Wheeler, H.O. (1971) Concentration function of the gallbladder. Am. J. Med. 51, 588-595.

Willner, J., Ginsburg, S. and DiMauro, S. (1978) Active transport of carnitine into skeletal muscle. Neurology 28, 721-724.

Windmueller, H.G. and Spaeth, A.E. (1980) Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. J. Biol. Chem. 255, 107-112.

Yalkowsky, S.H. and Zografi, G. (1970) Potentiometric titration of monomeric and micellar acylcarnitines. J. Pharm. Sci. 59, 798-802.

Yue, K.T.N. and Fritz, I.B. (1962) Fate of tritium-labelled carnitine administered to dogs and rats. Am. J. Physiol. 202, 122-128.

APPENDIX 1

The Recovery of Intravenously Injected [^{14}C]Carnitine in Rat Bile

The objective of this experiment was to determine the recovery of labelled carnitine injected into the jugular vein in rat bile.

A1.1 Methods

A1.1.1 Purity of the Isotope

The purity of the [^{14}C]carnitine was verified by using the cellulose TLC system recommended by Amersham. The mobile phase was composed of n-butanol:acetic acid:water(12:3:5, v/v/v).

A1.1.2 Surgical Procedure

The bile duct and a jugular vein were cannulated as described previously. One ml of saline containing 0.05 uCi of [1-^{14}C]carnitine (2.5 mCi/nmol) was slowly injected into the jugular vein. Immediately after the labelled carnitine was injected, bile was collected. The collection tube was changed every 5 minutes for 60 minutes so that the rate of recovery of the label in the bile could be measured. Subsequent bile samples were collected at 10 minute intervals until 2 hours had elapsed. At the termination of the experiment, a blood sample was taken from the tail vein and serum was prepared by centrifugation as described earlier.

A1.1.3 Analysis of Samples

Bile and serum samples (50 ul each) were placed in disposable mini-counting vials with 6 ml of aqueous scintillation fluid. The samples were then mixed and

counted on an LS9000 Scintillation counter. In order to quantitate the labelled long-chain carnitine esters in the bile, the lipid phase from three samples was mixed with scintillation fluid and counted. The specific activities in the bile and serum were determined by assaying the samples for carnitine.

A1.2 Results

The amounts of [^{14}C]carnitine recovered in the bile samples collected are plotted for each rat separately in Figure 11. Also shown are the amounts of label in the serum collected at the termination of the experiments. The labelled carnitine recovered in the bile increased to a maximum approximately 30 minutes after injection in all cases. 50%, 47% and 50% of the labelled carnitine was present in the long-chain form in the three samples analysed.

The specific activity of the carnitine in the bile and the serum is shown in Table 12. The specific activity in the bile was less than in the serum for three of the five rats.

FIGURE 11

The appearance of intravenously injected [^{14}C]carnitine in 50 ul of bile of six rats collected at various intervals up to 2 hours after injection. Approximately 0.05 uCi was injected into each rat. The amount of [^{14}C]carnitine found in the serum at the termination of the experiment is also indicated for each rat (square symbol).

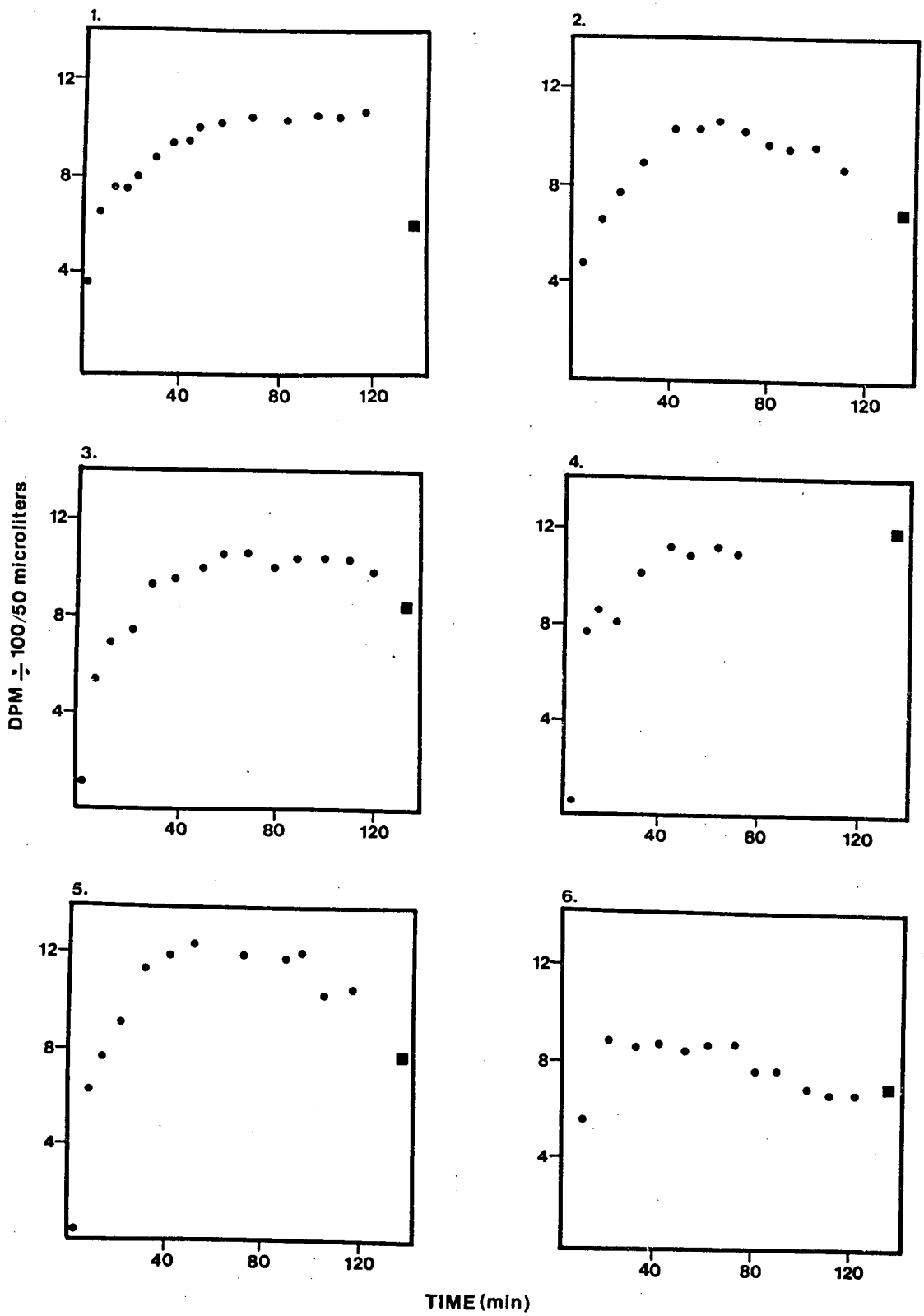

1.
2.
3.
4.
5.
6.
12
8
4
40
80
120
DPM ÷ 100/50 microliters
TIME (min)

TABLE 12

The Specific Activity of Carnitine in the Serum and Bile of Rats Two Hours After the Intravenous Injection of [^{14}C]carnitine

	SPECIFIC ACTIVITY (DPM/nmol Carnitine)	
RAT	SERUM	BILE
1	131	232
2	158	214
3	299	247
4	254	287
5	300	284

APPENDIX 2

The Effect of Saline Infusion on Bile Collection and the Carnitine Content of Rat Bile

The objective of this experiment was to determine whether or not the bile fluid lost during two hours of bile collection (approximately 3.2 ml) affects the bile flow rate and the carnitine content of rat bile. Rats were infused (IV) with saline to replace the fluid lost as bile. The bile flow rate was assessed and free and acyl carnitine determined. The results were compared to those in Chapter 5 where no fluid was replaced.

Unless specified, all procedures were performed as described Chapter 4. Seven adult male Wistar rats were anaesthetized. In each rat, a jugular vein was cannulated and a sterile saline solution was infused at a rate of 27.8 ul/min using a peristaltic pump (Scientific Industries, Model 603). The bile duct was then cannulated and bile was collected for two hours in 15 minute samples.

Bile flow was constant throughout the collection period (7.0 ± 0.1 ul/min/100 g BW). There was no significant difference between this and the bile flow in non-infused rats except in the final collection period where the bile flow was significantly greater in the infused rats ($p<0.01$).

The concentration of total carnitine in the bile of the infused rats only differed from the non-infused rat bile at the fourth time interval ($p<0.01$); there was a lower carnitine concentration in the bile of the infused rats. Saline infusion did not affect the free carnitine and acylcarnitine concentrations of rat bile at any of the time intervals. The amounts of carnitine in the rat bile in each

15 minute collection also did not change with saline infusion.

On the basis of these results, it was concluded that fluid replacement did not affect the carnitine content of rat bile collected continuously for up to two hours. Therefore, it was decided that for bile collection experiments, fluid replacement was not necessary.

APPENDIX 3

The Effect of Interruption of the Enterohepatic Circulation on the Carnitine Content of the Bile

The objective of the following procedure was to enable collection of rat bile from a cannulated bile duct while only partially interrupting the enterohepatic circulation.

A rat was anaesthetized and its bile duct cannulated as described in Chapter 4. The cannula was then inserted into another cannula of slightly greater diameter. The other end of the second tube was subsequently inserted into the intestine of the rat one inch below the pyloric sphincter and secured with cyanoacrylate glue. Bile was sampled at 15 minute intervals by disconnecting the two interlocking tubes and collecting 100 ul. Carnitine and carnitine ester analysis was performed as described in Chapter 4.

APPENDIX 4

The Effect of Pentobarbital on the Carnitine Content of the Bile

The following experiment was performed to assess the effect of pentobarbital on the changes in the acylcarnitine content of rat bile that occur after the initiation of collection.

Bile was collected from a rat who had been anaesthetized. The bile carnitine content was then compared to that of others where bile collection was established immediately after the anaesthetic took effect.

Despite the delayed surgery,the acylcarnitine content of the bile again increased during the course of the experiment. The free carnitine remained the same level as in the control bile while the total carnitine concentration was more than double that of the controls.

APPENDIX 5

Potential Mechanisms for the Transport of Carnitine Into Rat Bile

The mechanism for carnitine transport into bile has not been investigated. Possibly there are separate mechanisms for the entry of the various forms of carnitine into the bile. Of interest are the large quantities of long-chain carnitine esters. The "micellar pull theory" which has also been hypothesized for the entry of cholesterol into the bile (Hardison and Apter, 1972) may also be applicable to long-chain carnitine esters. Both cholesterol and the long-chain carnitine esters have the capacity to form micelles.

A5.1 Micellar Pull Theory

Assuming that the micellar pull theory accurately describes the transport of palmitoylcarnitine into the bile, the following would be true: monomeric palmitoylcarnitine would passively enter the bile from the hepatocyte achieving equal concentrations on both sides of the canalicular membrane. Once in the bile, the palmitoylcarnitine would associate with bile acid and phospholipid mixed micelles forming a "micellar sink" (Scharschmidt and Schmid, 1978). This would decrease the concentration of monomeric palmitoylcarnitine in the bile causing more carnitine esters to be "pulled" across the canalicular membrane from the hepatocyte.

Some properties of long-chain carnitine esters seem appropriate for the "micellar pull" theory. First, long-chain carnitine esters are amphiphiles and the

concentration of palmitoylcarnitine in bile is above its critical micellar concentration. It would therefore likely associate with bile acid and phospholipid micelles as well as forming micelles of its own. Secondly, this could explain how long-chain acylcarnitines are more concentrated in the bile than both the serum and the hepatocytes while free carnitine is not.

A5.2 The Effects of Bile Acid Infusion on the Carnitine Content of the Bile

To test the hypothesis, rats were infused (IV) with either taurocholate (1.2 umol/min/100 g BW), according to Innis (1985), or dehydrocholate (0.24 umol/min/100 g BW), according to Hardison and Apter (1972), during bile collection. Solutions of the bile acids and albumin (4%) were infused into a cannulated jugular vein of each rat. Taurocholic and dehydrocholic acids increase the rate of bile-acid dependent bile flow. Taurocholate is a micelle forming bile acid. If the micellar theory applies to carnitine, one would expect more acylcarnitine in the bile of the two rats infused with taurocholate. To distinguish between the effects of the micelle formation and the increased bile flow, dehydrocholate, a non-micelle forming bile acid was infused in two additional rats.

Despite an increased bile flow, the concentration of free and acylcarnitine in the bile of taurocholate infused rats remained the same as in the bile of saline infused

rats. Hence, the amount of carnitine found in the bile in each 15 minutes was three times higher than for the saline infused controls.

Increased bile flow and carnitine concentration also resulted when dehydrocholate was infused. This suggests that the carnitine content of the bile increased due to the increased bile volume rather than to micelle formation and disputes the micellar theory for the transport of long-chain carnitine esters into the bile.

A5.3 Other Observations

While investigating the carnitine content of rat bile, other observations were made. For instance, although the concentration of carnitine in bile was higher in fasting than in fed rats, the overall amount of carnitine found in each 15 minute collection did not differ. This might indicate that carnitine in the bile is independent of bile flow. Alternatively, the bile might have been concentrated in the fasted rats after its formation. The results of the bile acid infusion suggests that the latter is true. With both taurocholate and dehydrocholate infusion, the concentration of bile carnitine remained at the same levels as in saline infused controls but the amount of carnitine increased. Therfore, when bile is formed at the cannalicular membrane, there appears to be a constant concentration of carnitine. With increased bile flow, as in bile acid infusion, the concentration of the carnitine in

the bile, thus, remains constant. In fasting, however, the concentration of the initially formed bile might be the same as in fed rats but subsequent water reabsorption from the bile might occur.

In these studies, the overall ratio of free to total carnitine was not affected by the treatments imposed but carnitine esterification did increase throughout the course of each experiment. Gudjonsson and colleagues (1985b), however, showed a two fold increase in the amount of carnitine esters in the bile after injection with bolus doses of carnitine. Free carnitine levels did not change. This suggests that the transport of free and acylcarnitine into bile are not necessarily dependent on one another.

It is still unknown whether or not carnitine is actively or passively transported into the bile. Other quaternary ammonium compounds are actively transported (Schanker and Solomon, 1963), especially those with molecular weights greater than 300 g/mol. As short-chain carnitine esters have molecular weights less than 300 g/mol, and long-chain esters have greater molecular weights, this might explain why large amounts of long-chain carnitine esters are found in bile. The actively transported long-chain carnitine esters could then be available for mixed micelle formation.

APPENDIX 6

The Serum Carnitine Content of Cystic Fibrosis Patients

TABLE 13

The Concentration of Free and Total Carnitine in the Serum of Cystic Fibrosis Patients

Patient #	Sample #	Free Carnitine (uM)	Total Carnitine (uM)	Acyl Carnitine (%)
1	1	38.0	54.5	30
	2	45.0	53.2	15
2	1	52.1	57.2	9
3	1	71.8	84.9	15
4	1	33.2	42.2	21
5	1	35.4	47.5	25
6	1	31.0	42.0	26
7	1	32.2	50.1	36
	2	43.5	49.8	13
8	1	50.3	58.6	14
	2	51.6	64.4	20
9	1	8.0	10.1	21
10	1	18.9	24.5	23
11	1	49.9	45.2	45
12	1	40.2	54.3	46
	2	34.9	49.3	29
	3	7.5	11.5	35
13	1	47.3	52.6	10
14	1	61.9	68.3	9
15	1	48.7	69.5	18
	2	49.5	61.5	20
	3	49.5	61.5	20
16	1	28.4	50.1	43
17	1	54.6	60.6	9
18	1	33.2	42.2	21
19	1	49.5	56.0	19
20	1	39.7	44.9	12
21	1	56.2	66.1	15
22	1	49.7	57.5	14
	2	47.0	60.6	22
23	1	40.3	46.8	14
24	1	39.0	48.8	20
25	1	25.4	36.8	31
26	1	43.0	65.4	33
	2	38.1	55.4	31
	3	36.7	55.9	34
27	1	38.6	49.2	21
	2	37.7	49.3	24
	3	44.7	66.0	32
28	1	29.8	47.2	37
29	1	50.7	79.0	36
30	1	38.3	53.4	28
31	1	40.5	52.0	15
32	1	45.6	52.0	15
33	1	39.7	47.7	17