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Betaine as a lipotropic agent and as an alleviator of osmotic stress

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Academic Dissertation

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Cover: Transmission electron microscope micrograph of a liposome in a hepatocyte of rat liver
to Aila and Kalervo
ABSTRACT

Betaine, discovered in the juice of sugar beets more than a hundred years ago, is a quaternary ammonium compound generally known to act as a lipotrope in the liver and as an intermediate metabolite in choline metabolism. Betaine has also been shown to function as an intracellular osmolyte in many different types of microbial, plant, and animal cells. In this work, both osmolytic and lipotropic effects of betaine were studied.

The osmoregulatory role of betaine in the physiological stress of salmonids during and after gradual or abrupt transfer from fresh water to seawater was examined (Study I) by using Atlantic salmon (*S. salar* L.) smolts. Atlantic salmon smolts were observed to better adapt to the physiological stress induced by transfer from fresh water to seawater when the commercial dry feed used was supplemented with a 1.5% commercial betaine/aminos acid additive (Finnstimp) (I). The main component in the additive used was betaine. When fish were fed with the diet containing the tested additive, their muscle water content decreased, desmoltification was delayed and the capacity to maintain ionic and osmotic balance improved.

The closely related osmoprotective role of betaine as a cryoprotective agent was studied in the deep-freezing process of stallion sperm (Study II). The effects of different concentrations of betaine in an extender while using different cooling rates on the freezing of stallion sperm were evaluated, and the post-thaw motility was measured using a laser Doppler technique. In the freezing of stallion sperm for artificial insemination, the post-thaw motility of spermatozoa was increased by the addition of 2.5% betaine to the extender. The measured post-thaw motility was 80-95% of the fresh sperm motility.

The lipotropic role of betaine in hepatic steatosis (fatty liver) was studied in the experimental models by using ethanol (III) and carbon tetrachloride (IV, V) to induce fatty liver.

In the ethanol experiment (Study III), research focused on the main metabolites of methionine biosynthesis, such as S-adenosylmethionine, and on liver triglycerides. The results showed that in Sprague-Dawley rats, 0.5% betaine in the diet prevented hepatic steatosis induced by chronic dietary ethanol feeding (36% ethanol of diet energy) and promoted generation of hepatic S-adenosylmethionine (SAM), which is the major methylating agent in the body.
When steatosis was induced by CCl₄, research (Study IV) was initially directed at the biochemical changes in the metabolites of methionine biosynthesis, and the histologic analysis of liver steatosis by light microscopy. The data revealed that dietary betaine (2% w/w) reduced centrilobular steatosis and increased biosynthesis of SAM in Sprague-Dawley rats, subsequent to induction of fatty liver by CCl₄.

In the next phase of the research on CCl₄-induced steatosis (Study V), quantitative morphometric histopathologic analyses were performed by means of transmission electron microscopy. Intracellular changes in the perinuclear cytoplasm of the hepatocytes of Han:Wistar rats after exposure to CCl₄ were studied. The results showed that oral administration of betaine (830 mg/kg/d) was related to higher volume densities of rough endoplasmic reticulum, Golgi complexes, and number of mitochondrial figures in the perinuclear cytoplasm of hepatocytes of rats exposed to CCl₄; a simultaneous, statistically significant reduction of liver steatosis was detected.

In conclusion, the results of the present work indicate that betaine can be used as an osmoregulatory agent in seawater transfer of Atlantic salmon smolts, and as a cryoprotectant in deep freezing of stallion sperm. Betaine can also be used as a lipotrope in hepatic steatosis induced by either ethanol or carbon tetrachloride. The effects of betaine in the liver are evidently related to improved transmethylation.
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This dissertation is based on the following original articles referred to in the text by Roman numerals I-V:


ABBREVIATIONS

AFOS  alkaline phosphatase
ALAT  alanine aminotransferase
ASAT  aspartate aminotransferase
ATP   adenosine triphosphate
ATPase adenosine triphosphatase
ATS   Atlantic salmon (Salmo salar, L.)
BHMT  betaine-homocysteine methyltransferase
BW    body weight
BWgain weight gain
CCl4  carbon tetrachloride
CF    condition factor
EC    enzyme coded
EDTA  ethylenediamine tetra acetic acid
ER    endoplasmic reticulum
GABA  gamma-aminobutyric acid
HPLC  high performance liquid chromatography
i.g.  intragastrically
i.m.  intramuscularly
i.p.  intraperitoneally
LM    light microscopy
MCHC  mean corpuscular hemoglobin concentration
mOsm  milliosmolar solution
MS    methionine synthetase
MTHF  methyltetrahydrofolate
NAD   nicotinamide adenine dinucleotide
ppt   part per thousand
PUFA  polyunsaturated fatty acid
RER   rough endoplasmic reticulum
rH    relative humidity
SAM   S-adenosylmethionine
s.c.  subcutaneously
SER   smooth endoplasmic reticulum
TCA   tricarboxylic acid
TEM   transmission electron microscopy
TG    triglyceride
TMA   trimethylamine
TMAO  trimethylamine oxide
VLDL  very low density lipoproteins
INTRODUCTION

Betaine is known as an intermediate metabolite in choline metabolism, and in the biochemical literature, it has been postulated to participate in transmethylation reactions as a methyl donor (Best and Huntsman 1932, Case et al. 1976, Kuksis and Mookerjea 1978, Harper et al. 1979, Mayes 1996). Furthermore, recent literature has found that betaine, as a molecule itself, plays an important role in the osmoregulation of different cells (Bowlus and Somero 1979, Daikoku 1980, Balaban and Knepper 1983, Le Rudulier et al. 1984, Robinson and Jones 1986, Haussinger and Lang 1991). Betaine has also been found to increase the stability of enzymes against heat and protect proteins against unfavorable consequences of dehydration-induced thermodynamic perturbation (Nash et al. 1982, Paleg et al. 1984, Plaza del Pino and Sanchez-Ruiz 1995).

When Atlantic salmon is raised and the fish are removed from fresh water to seawater as smolts, they face a marked physiological stress due to ionic and osmotic stress. In order to survive, smolts have to be able to restore their ionic and osmotic balance (Koch and Evans 1959). Similar stress takes place when living cells are deep frozen. In the freezing process, free water is removed from cells as ice crystals, and the cells become progressively dehydrated. Removal of water as ice leads to mechanical damage and a hypertonic media, which may be fatal for cell survival due to denaturation of structural proteins, membranes, and enzymes (Smith and Polge 1950, Arakawa and Timasheff 1985, Amann and Pickett 1987). To alleviate the perturbing effects of osmotic stress, organic osmolytes, such as betaine, can be used (Haussinger and Lang 1991).

Even though it is known that the metabolism of choline is closely linked to the metabolism of betaine and further to the transmethylation reactions in the liver, very little has been published about induced hepatic steatosis (fatty liver) and betaine in vivo. Hepatic steatosis can be induced by ethanol or carbon tetrachloride (Cameron and Karunarathe 1936, Stowell et al. 1951, Recknagel et al. 1960, Lieber et al. 1965, Hartroft and Porta 1968, Recknagel and Glende 1973, Krabenbuhl et al. 1991, Sherlock 1995); therefore, treatment with either of these compounds can be used when prevention of fatty liver is to be studied.
The purpose of this thesis was:

1) to study the osmotic role of betaine in the transfer of Atlantic salmon smolts from fresh water to seawater and in the deep-freezing process of stallion spermatozoa, and

2) to study the lipotropic effect of betaine on the metabolism of methionine and on hepatic steatosis (fatty liver) in experimental models when steatosis was induced either by ethanol or by CCl₄.
1. REVIEW OF THE LITERATURE

1.1. Betaine as a chemical compound

Betaine (s. N-trimethylglycine, glycine betaine, glycocoll betaine, oxyneurine, lycine), the designation for the compound 1-carboxy-\textit{N},\textit{N},\textit{N}\text{-trimethylmethanaminium} hydroxide was discovered by Schiebler as early as 1869 in the juice of sugar beets (\textit{Beta vulgaris}). The chemical formula of this quaternary ammonium compound is (CH$_3$)$_3$N$^+$CH$_2$COO$^-$, and its molecular weight is 117.15. It is highly soluble in water, slightly soluble in methanol and ethanol, and almost insoluble in organic solvents. It is present in 0.01-1.1\% of nitrogen found, for example, in green pasture grass, green lucerne, and maize grains. Betaine and other quaternary ammonium products are considered to represent the end-products of nitrogen metabolism in plants (Wood 1960, Bondi 1987, Budavari 1989). In addition to plants, high levels of betaine are found in many marine and freshwater invertebrates (Konosu and Hayashi 1975). In human plasma, betaine concentrations usually vary between 20 and 70 µmol/l; concentrations are well regulated even in serious metabolic diseases (Lever et al. 1994). Betaine is generally regarded as nontoxic and is used in the treatment of congenital homocysteinuria (Wilcken et al. 1983).

1.2. Metabolic interrelationships of betaine with choline and methionine

Biochemical literature reveals that the metabolism of betaine is closely linked to the metabolism of choline and methionine. All three are regarded as lipotropes, substances in the organism which prevent the formation of fatty liver and participate in transmethylation reactions (Harper et al. 1979, Mayes 1996). The main hepatic pathways of these compounds and their role in transmethylation reactions are shown in Fig. 1. As the basic metabolism of these three molecules is well documented, the main focus in this literature review will be the role of betaine in the liver. Choline and methionine are discussed only when necessary for better understanding of the topic under discussion.

The dietary requirement of choline and its lipotropic effects were first demonstrated by Best and Huntsman (1932). The metabolic relationship between betaine and methionine was reported by Du
Vigneaud et al. (1939). Numerous studies have shown (Forbes and Vaughan 1954, Sidransky and Farber 1958, Mookerjea 1965, Lombardi et al. 1966, Kuksis and Mookerjea 1978) that a deficiency in methionine or choline intake produces a fatty liver in rats and leads to a decrease in liver betaine content (Arvidson and Asp 1982).

Choline has three main functions in the body. First by participating in the biosynthesis of phosphatidylcholine and other complex choline containing phospholipids, choline has an important role in the synthesis of plasma phospholipids and in the structure of cell membranes (Lombardi et al. 1966, Haubrich et al. 1975, Kuksis and Mookerjea 1978, Tokmakjian et al. 1989). The second and third metabolic functions are the direct synthesis of acetylcholine, a neurotransmitter, and via betaine as a source of labile methyl groups (Du Vigneaud et al. 1950, Harper et al. 1979). It has been estimated (Mudd et al. 1980) that humans need approximately 0.3 mmol/kg/d of methyl groups. About 15% of the daily requirement is obtained from dietary choline, the rest coming from dietary methionine and from the recycling of homocysteine within the body (Frontiera et al. 1994).

Choline must be oxidized in the liver mitochondria to betaine in order to acts as a methyl donor (Fig. 1.) The oxidation of choline in rats has been shown to take place in the mitochondria of the liver and kidney (Mann et al. 1938, Wilken et al. 1970, Wong and Thompson 1972, Ridder and Dam 1975, Haubrich and Gerber 1981). Betaine is formed in mitochondria by a two-step process: the first reaction is catalyzed by choline dehydrogenase (EC 1.1.99.1), which converts choline to betaine aldehyde, and the second reaction is catalyzed by betaine aldehyde dehydrogenase (EC 1.2.1.8), which then oxidizes the aldehyde to betaine.

The oxidation of betaine aldehyde to betaine is a NAD$^+$ dependent reaction (Harper et al. 1979, Zhang et al. 1992). Salerno and Beeler (1973) have shown that the oxidation of choline to betaine in the rat liver is at least as great as its phosphorylation to phosphatidylcholine. Choline dehydrogenase may be involved in controlling the free choline level in cells by rapidly converting free choline by oxidative pathway to betaine. This enzyme has been found to exist in the liver of the rat, mouse, cat, dog,
- TCA-cycle, porphyrins, purines

Creatine →

Serine ← Glycine → SAM ↓

(phosphatidyl)ethanolamine ← SAM ↓

(phosphatidyl)methyllethanolamine ↓

SAM ↓

(phosphatidyl)dimethylethanolamine ↓

SAM ↓

(phosphatidyl)choline

Cell membranes ↓

Choline → Betaine

1. Choline → Mitochondrion → Betaine
2. Homocysteine ← Methionine → Protein
3. BHMT

*(CH₃)

Cystathionine ←

Cysteine ↓

SO₄²⁻ ← Glutathione

S-adenosylhomocysteine ← S-adenosyl-methionine (=SAM)

4. MS (N₅-THF)

5. →

6. ↓

7. →

Fig. 1. Main pathways in the metabolism of choline, betaine, and methionine. The numbers refer to the respective enzymes: 1 = choline dehydrogenase (EC 1.1.99.1); 2 = betaine aldehyde dehydrogenase (EC 1.2.1.8); 3 = betaine-homocysteine methyltransferase (BHMT)(EC 2.1.1.5); 4 = methionine synthase (MS)(EC 2.1.1.13); 5 = methionineadenosyltransferase (EC 2.5.1.6); 6 = adenosylhomocysteinase (EC 3.3.1.1); 7 = cystathionine-beta-synthase (EC 4.2.1.22) (Mudd and Poole 1975, Mudd et al. 1980, Finkelstein 1990).
monkey, sheep, man, rabbit, baboon, toad, and guinea pig (Haubrich and Gerber 1981). When choline oxidase activities from different species were compared, the activity within the rat liver was approximately 50 times higher than in the human liver, and clearly higher than in the baboon liver (Sidransky and Farber 1960, Hoffbauer and Zaki 1965). Conversely, it seems that the requirement of choline is smaller among primates than in the rat (Hoffbauer and Zaki 1965).

Betaine is released from the mitochondria into the cytosol (Ridder and Van Dam 1973, Kaplan et al. 1993, Porter et al. 1993), where it acts as a methyl donor (Mann et al. 1938). The labile methyl group donated by betaine can be used in transmethylation reactions in the liver to remethylate homocysteine to methionine (Fig. 1). Methionine is an essential amino acid, the homeostasis of which the body maintains even under conditions of extreme excess or deprivation (Harper 1979). The estimated nutritional requirement of methionine in humans is approximately 240 mmol/kg/d (Scott 1986). When methionine is given orally, about half of it is metabolized by the liver (Lieber 1999). In the case of excessive (3%) intake of methionine in rats methionine methyl group oxidation does not, for the most part, lead to an increased synthesis of choline (Case et al. 1976); instead, the addition of more than 2% L-methionine to the rat diet may have a negative effect on the growth of the animal due to its toxicity (Steele and Benevenga 1978).

Methionine intake is seldom limiting in the body, but homocysteine is a potentially toxic compound (Klavins and Johansen 1965, Dudman et al. 1993). Mammals are unable to synthesize homocysteine de novo (Banerjee and Matthews 1990). Mudd and Poole (1975) and Mudd et al. (1980) have estimated that about 50% of methionine, under normal dietary conditions in the human liver, is received from the recycling of homocysteine to methionine.

Two different enzymatic pathways can be used for remethylation of homocysteine to methionine in the liver; reactions by either a methylcobalamin and N5-methyltetrahydrofolate (N5-MTHF) dependent enzyme called methionine synthase (MS 2.1.1.13), or by betaine-homocysteine-methyltransferase (BHMT 2.1.1.5), which is independent of vitamin B-12 (Banerjee and Matthews 1990, Finkelstein 1990). Both enzymatic reactions are important for transmethylation in liver, and the reactions are closely linked to each other, as recently demonstrated by Varela-Moreiras et al. (1992); when rats were
kept on a choline-deficient diet for 2 weeks, folate concentration in the liver diminished markedly. Additionally, the concentration of methionine also affects on the concentration of methylfolate in rat liver (Lumb et al 1988). Methyl group transfer via the MS and N5-MTHF pathway occurs in all tissues (Case et al. 1976, Kuksis and Mookerjea 1978, Harper et al. 1979, Mayes 1996, Selhub and Rosenberg 1996). Certain compounds, e.g., ethanol, isovalerate and 3,3-dimethylbutyrate, are known to inhibit MS (Skiba et al. 1982, Barak et al. 1984a, 1985, 1987, Halsted 1996).

BHMT has been found to be present in rat, pig, and human liver, in pig and human kidney, and in the Rhesus monkey lens. The activity of this enzyme in rat liver is approximately 2.5 times higher than in man (McKeever et al. 1991, Rao et al. 1998). This enzyme may also exist in brain tissue (Mudd et al. 1969, Gaull et al. 1973), although results from neural tissue are somewhat contradictory (McKeever et al. 1991). Human BHMT, a zinc metalloenzyme (Millian and Garrow 1998), is a hexamer protein of identical subunits, which shows limited homology to bacterial vitamin B12-dependent methionine synthases (Garrow 1996, Millian and Garrow 1998).

Hepatic BHMT increases in the rat and chicken (Paul and Garrow 1999, Emmert et al. 1996) at low dietary methionine intake. Administration of betaine or choline to rats increased the level of liver BHMT, indicating that rats have a means of adaptation to excessive levels of dietary choline and betaine (Finkelstein et al. 1983, Xue and Snoswell 1985). In humans, BHMT did not seem to play a prominent role in the maintenance of methionine homeostasis after an oral administration of 0.7 mmol/kg betaine (Frontiera et al. 1994).

If homocysteine is not remethylated to methionine, it can be metabolized to cystathionine by cystathionine-beta-synthase (Fig. 1). In the case of (congenital) deficiency of this enzyme, homocystinuria results (Clarke et al. 1991). Pyridoxine and betaine have been used in the treatment of this disease (Wilcken et al. 1983, Anon. 1984, Garry and Vellas 1996). It has been estimated that in man, homocysteine is cycled to methionine 1.5-1.9 times before being converted to cystathionine. When labile methyl intake is curtailed, the number of cycles increases to 3-3.9 (Mudd and Poole 1975).

Storch et al. (1991) have shown that homocysteine remethylation and transmethylation rate tended to increase in man after oral betaine (3 g/d) supplementation. Plasma methionine concentrations became
elevated, as well. The close relationship between methionine homeostasis and recycling of homocysteine to methionine exists also in the chicken. In response to low dietary methionine, an enzymatic hepatic adaptation for recycling of homocysteine to methionine occurs (Saunderson and Mackinlay 1990, Emmert et al. 1996).

When methionine is formed from homocysteine, it is further metabolized to S-adenosylmethionine (SAM), an "active" methylating substance (Fig. 1.), unless otherwise utilized by the body (Cantoni 1953). SAM is regarded as, the major methyl donor for more than 100 reactions (phosphatidyl choline, DNA, neurotransmitters, creatinine etc.) (Harper et al. 1979, Hirata and Axelrod 1980, Tsukada et al. 1985, Frontiera et al. 1994, Selhub and Rosenberg 1996), and it contributes, as a source of labile methyl groups, to many vital biochemical reactions (synthesis, activation and metabolism) of e.g. nucleic acids, hormones, neurotransmitters, and cell membranes (Hirata and Axelrod 1980, Friedel et al. 1989, Finkelstein 1990). In liver, about 30% of produced SAM is taken back into mitochondria via a specific, carrier-mediated system (Horne et al. 1997).

In biosynthesis of cell membranes, SAM is considered to have an important role in the conversion of phosphatidylethanolamine to phosphatidylcholine. Hirata et al. (1978) and Hirata and Axelrod (1980) have demonstrated in adrenal medulla cells that SAM, by participating in sequential methylation within cell membranes, maintains proper membrane structure and sustains the essential function of cell membranes. Barak et al. (1994) have shown that orally administered betaine in rats stimulates endogenous methionine synthesis via the alternate BHMT pathway and further promotes generation of SAM in cases where the folate-dependent MS-pathway is inhibited. They conclude that by participating in the methylation of membrane phospholipids (Hirata et al. 1978, Hirata and Axelrod 1980), SAM may also play a role in maintaining the integrity of the liver (Duce et al. 1988, Barak et al. 1996a). The turnover time for SAM has been estimated to be 3.5-7 min in human liver (Mudd et al. 1980). However, methyl donor in cell culture did not prevent apoptotic death of rat hepatocytes induced by choline deficiency (Shin et al. 1997).

Thus, as shown, transmethylation reactions are important in the body’s metabolic functions and any disturbances to those functions may lead to diseases such as nutritional liver disease (Fischer and Kane

1.3. Betaine as an osmolyte

1.3.1. Osmoregulation

Considering osmoregulation at cell level, the cellular hydration state is a dynamic process, where swelling and shrinking take place depending on the osmotic pressure inside and outside cell membranes (Haussinger 1996, Luft 1996). In the intracellular fluid, mainly potassium, magnesium, phosphates, and proteins control cell turgor; in the extracellular fluid, sodium, chloride, and bicarbonate ions play the major roles (Reece 1993, Luft 1996). Cell volume homeostasis does not apparently focus on volume constancy, but the events of swelling and shrinkage allow cell hydration to play a physiological role as a regulator of cell function (Fig. 2; Haussinger 1996, Luft 1996). Fluctuations of cell hydration act as a signal for cellular metabolism and gene expression. The interaction between cellular hydration and cell function has been studied mostly in liver cells, but there is increasingly evidence that it also occurs in other cell types. Besides ionic mechanisms of cell volume control, certain organic osmolytes can play a role in cell shrinking or swelling (Haussinger 1996). For instance, protein and carbohydrate metabolism in the liver are sensitive to alterations in cell volume. In principle, swelling acts like an "anabolic signal", shrinkage as a "catabolic signal" (Haussinger and Lang 1991).

Cells in nature face osmotic stress under a variety of circumstances; organisms living in seawater, plants during dry season or winter, kidney medulla cells in high concentrations of urine, etc.. Only in the presence of functional mechanisms to counteract the existing stresses can cells survive. These mechanisms may be direct or indirect depending on the situation. In some cases, the stress may be man-made. For example, in the freezing of cells, the formation of extracellular and intracellular ice crystals reduce cell survival due to mechanical and physicochemical reasons. Removal of water as ice alters tonicity and leads to a hypertonic media which can be fatal for cells. In addition, damaging mechanisms during freezing involve mechanical fracture and disruption of proteins and cell membranes (Smith and Polge 1950, Pegg 1997).
1.3.2. Betaine in osmoregulation

In biochemical literature, betaine has been discussed primarily for its role as an intermediate product in choline metabolism. There is, however, increasing information to support that betaine itself acts as an active molecule, an intracellular osmolyte in different cells during osmotic stress. The results include microbes, marine invertebrates, plant cells, and different mammal cells (Sung and Johnstone 1969, Bowlus and Somero 1979, Daikoku 1980, Balaban and Knepper 1983, Le Rudulier et al. 1984, Arakawa and Timasheff 1985, Robinson and Jones 1986, Nakanishi et al. 1988, Haussinger and Lang 1991, Haussinger 1996, Wettstein and Haussinger 1997). Most recent studies have, however, concentrated on the role of betaine in the osmoregulation of different microbes.

Le Rudulier et al. (1984) were among the first to have convincingly shown the osmoregulatory role of betaine in *E.coli* in hyperosmotic media. The growth of bacteria was normal in media containing 0.8 M NaCl (almost double the osmotic strength of seawater) when 0.001 M betaine or choline was added. Based on the results of their work, the osmoregulatory role of betaine has become a focal point of research during recent years. A similar osmoregulatory effect has been found in *S.aureus* (Kunin and Rudy 1991). In *E. coli* and some other bacteria, a genetically controlled ProU transport system exists by which betaine is actively transported across the cytoplasmic membrane into the cell as an osmoprotectant under conditions of osmotic stress (Lucht and Bremer 1994). *S.aureus* cells grown in the presence of osmotic stress accumulated large amounts of betaine using a specific transport system, with choline being an ineffective competitor for betaine in the transport system (Stimeling et al. 1994). Kawahara et al. (1990) have demonstrated that the addition 10 mM of exogenous betaine stimulated the growth of *Brevibacterium lactofermentum* in media of inhibitory osmotic stress.

In marine invertebrates, betaine, trimethylamine, and certain other nitrogenous solutes prevent the perturbing effects of KCl, NaCl, and some organic molecules on enzymatic reactions in cells (Bowlus and Somero 1979, Daikoku 1980). Arakawa and Timasheff (1985) have shown that betaine, among other neutral osmolytes, stabilizes proteins against thermal denaturation and may be used as an osmoregulator in organisms under high osmotic pressure. In some fish (e.g. Atlantic salmon, rainbow
trout, chinook salmon) betaine seems to have a direct role as an intracellular osmolyte (Clarke et al. 1994, Virtanen et al. 1994), but in other species (kelp bass and pink salmon) TMA and TMAO are the osmolytes present (Charest et al. 1988).

In salt-stressed spinach (*Spinacia oleracea*) plant leaves increased their betaine content six-fold compared with the normal situation. Betaine content in chloroplasts increased from 26 mM to 300 mM in plants, indicating the way by which the plant adjusts its cells to combat the stress (Robinson and Jones 1986).

Yaas et al. (1994) found in mammalian hybrinoma cell culture that betaine was an effective osmolyte at medium concentrations of 5-30 mM for maintaining growth of cells exposed to osmotic stress caused by NaCl (100mM), KCl (60 mM), or sucrose (175 mM). The results also revealed that, in a comparison of different compounds, the highly methylated agents were the most effective osmoprotectants.

Betaine, acting as an organic osmolyte, has been shown *in vitro* to protect mouse embryos from elevated osmolarity (Dawson and Baltz 1997).

Urine contains high concentrations of urea and NaCl, leading to a hyperosmotic extracellular fluid in renal medullas. In the rat kidney, Sung and Johnstone (1969) demonstrated the existence of sodium-dependent active transport of betaine into kidney cortex cells, which is independent of choline oxidation. Balaban and Knepper (1983) found high concentrations of trimethylamines, including betaine, in rabbit inner medullary cells. In a canine kidney cell culture, high concentrations of betaine, glyserophosphorylcholine, and myo-inositol were found in cells when NaCl was added to make the media hyperosmotic (Nakanishi et al. 1988). Burg et al. (1997) have observed that betaine, taurine, sorbitol, and myo-inositol assist renal medullary cells to compensate for hypertonicity by accumulating these compounds. They also pointed out that there is a genetic control behind the process to relieve the hypertonic stress.
Fig. 2. A simplified scheme on regulation of cell turgor during osmotic stress
In mice, L-carnitine and certain structurally related quaternary amines (trimethylamineoxide, choline, and betaine) have a protective role against acute hyperammonemia (Kloiber et al. 1988, Minana et al. 1996). It has been suggested that the protective effect is a result of their function as osmoprotectants, and that these compounds protect enzymes or other proteins during hyperammonemia.

The osmoregulatory role of betaine in the liver has not yet been studied extensively. However, betaine has been identified to be an important osmolyte at least in liver cell cultures. Hyperosmotic exposure (405 mOsm/l) in cell culture inhibited the function of liver macrophages (Kupffer cells) by 30-40%. This inhibition was prevented completely by the presence of betaine (Warskulat et al. 1996). Betaine has also been found to act as an osmolyte in rat liver Kupffer cells. Betaine transport in Kupffer cells was induced in response to increased osmolarity (Zhang et al. 1996). Together with betaine, taurine and myoinositol functioned as organic osmolytes in sinusoidal endothelial cells. Osmolarity regulates Kupffer cell functions, such as phagocytosis and prostaglandin production, and betaine and taurine interfere with these effects (Wettstein and Haussinger 1997, Weik et al. 1998, Peters-Regehr et al. 1999). Haussinger (1996) proposed that Na⁺-dependent transporters for betaine might exist in hepatocytes. Burnham et al. (1996) confirmed this by their finding of the Na⁺/Cl⁻-dependent betaine/GABA-transporter in the liver.

Liver parenchymal cells oxidize choline to betaine in the mitochondria. From there, betaine escapes to the cytoplasm by simple diffusion. A hyperosmotic media slows betaine efflux from mitochondria, likewise the presence of high concentrations of potassium in the cytoplasm (Ridder and Van Dam 1973, Porter et al. 1993). Betaine is known to be present in liver macrophages (Kupffer cells) and alleviates the function of those macrophages under hyperosmotic exposure in cell culture. As an osmolyte, betaine functions in Kupffer cells and sinusoidal endothelial cells, but not in parenchymal cells (Warskulat et al. 1996, Zhang et al 1996, Wettstein et al. 1998). Betaine is supplied for osmolyte function via cell-to-cell interactions to sinusoidal and Kupffer cells experiencing osmotic stress (Wettstein et al. 1998).
1.4. Betaine as a lipotropic agent

1.4.1. Fatty liver

Free fatty acids are synthesized in the liver to triglyceride (TG) and further to phospholipids or lipoproteins. Phospholipids are primarily used as structural components in biomembranes inside the cell, and lipoproteins are secreted into plasma mainly as very low density lipoproteins (VLDL). Phospholipids are synthesized in the SER or the cytosol. Apoproteins (the most common proteins in VLDLs) are synthesized in the RER, but the final arrangement of lipoproteins takes place in the Golgi apparatus before they are secreted into the plasma as mature micelles (Harper et al. 1979, Mayes 1996).

Fatty liver (hepatic steatosis) due to the accumulation of fat in the form of TGs may result for several different reasons, including nutritional deficiencies (choline, essential amino acids), toxins (aflatoxin), toxic chemicals (carbon tetrachloride, ethanol, white phosphorus), different diseases (diabetes mellitus, pregnancy toxemia of ewes, and ketosis), and intrahepatic metabolic disorders. It may originate from an increased supply or synthesis of fatty acids in liver, decreased fatty acid oxidation, or a block in the excretion of lipoproteins from the liver into the plasma. Intracellular reasons also include a possible decline in protein synthesis in the RER, which may lead to an elevation of TGs in the liver (Lieber et al. 1965, Mookerjea 1965, Lombardi and Oler 1967, Gröhn et al 1983, Dianzani 1991, Halsted 1996, Mayes 1996).

At the organ level, two main types of fatty liver are considered histologically: centrilobular and peripheral. Administration of CCl4 and ethanol induce centrilobular steatosis (Jennings 1955, Edmondson et al. 1967). This also happens in choline deficiency (Cullen and Ruebner 1991). Periportal steatosis manifests, for instance, after ferrosulphate and alloxan treatments (Cullen and Ruebner 1991).

Very little has been published about (induced) fatty liver and betaine in vivo. In this work, the role of choline, CCl4, and ethanol in fatty liver, and their metabolic interrelationships with betaine are reviewed more closely in the next chapters.
1.4.2. Choline and fatty liver


Choline is an essential part of phospholipids in cell membranes (as phosphatidylcholine). Lipid oxidation increases with choline deficiency (Dianzani 1991), which may lead to loss of normal structure and function of biomembranes. Yao and Vance (1989) have shown in cultured hepatocytes that the impaired VLDL secretion from choline-deficient hepatocytes could be corrected by a supplementation of 0.2 mM betaine, indicating the utilization of a methyl group from betaine for phosphatidylcholine formation via methylation of phosphatidylethanolamine. Prolonged fatty liver may lead to subsequent cirrhosis (Teli et al. 1995).

Finkelstein et al. (1982) have shown that both dietary choline and protein levels have a direct effect on the level of betaine in the liver of Sprague-Dawley rats. Increased protein intake decreased the hepatic betaine level, while supplementary choline increased it. Best et al. (1969) observed that the administration of 0.63% betaine to a hypolipotropic diet prevented dietary cirrhosis in rats. Sugiyama et al. (1986) have shown in rats that 1% betaine in a high-cholesterol diet decreased liver cholesterol and total lipid content.

1.4.3. Carbon tetrachloride and fatty liver

Chloroform and carbon tetrachloride (CCL4) have been used as anesthetics since the 1850s. Acute yellow atrophy of the liver was well known by the turn of the century, and the use of these halo-carbonous compounds as general anesthetics has since then ceased (Hardin 1954). Carbon tetrachloride is known to induce hepatic centrilobular steatosis (fatty liver) in rats and has been used to study hepatic steatosis and experimental cirrhosis (Cameron and Karunarathie 1936, Stowell et al. 1951, Recknagel et al. 1960, Krahenbuhl et al. 1991, Sherlock 1995). Several reviews and books on the topic are available.
(Recknagel 1967, Recknagel and Glende 1973, Dianzani 1991). In this context, only a general description of the matter is given.

The chronology of the events in hepatocytes after exposure to CCl₄ are generally known. The first injuries take place in the hepatocytes within a few minutes after the injection of a single dose of CCl₄ (Dianzani 1991, Recknagel et al. 1991). Dissociation of polyribosomes, loss of Ca²⁺-sequestering capacity of the ER, and alleviation of (lipoprotein)protein synthesis are among the first early features in the events leading to hepatic cell damage (Moore et al. 1976, Lowrey et al. 1981, Sweeney 1981). Haloalkylation of cellular lipids and proteins, accumulation of triglycerides in the Golgi apparatus, and declined protein synthesis with declined TG excretion is detected within 10-20 min after CCl₄-induced injury (Schottz and Recknagel 1960, Coleman et al. 1988, Dianzani 1991). Morphological changes in ER and mitochondria are observed within the first hour after the exposure. The peak of liver steatosis occurs 12-24 h after CCl₄ administration, and the reparative phase and the regeneration of hepatocytes occurs 2-6 days after the exposure (Clawson et al. 1991, Dianzani 1991).

Alkyl radicals are considered to play a major role in CCl₄-induced liver damage (Butler 1961, Slater 1966, Tomasi et al. 1980, DeGroot and Haas 1981). The main pathological damage initially caused by alkyl radicals is the inactivation of the microsomal cytochrome P-450 system in the liver (Slater and Sawyer 1969, DeGroot and Haas 1981). The mechanism by which the alkyl radicals are formed in the liver starts with the dehalogenation of CCl₄ to trichloromethyl. This takes place in the SER by a specific cytochrome P-450 isoenzyme, but dehalogenation can also occur in mitochondria (Tomasi et al. 1987, Recknagel et al. 1989). A simplified scheme of the mechanism is presented in Fig. 3.

The consequences of the formation of toxic radicals are many. The trichloromethyl free radical can either produce new organic radicals via hydrogen abstraction or altered structures of organic molecules by reacting covalently with proteins or lipids (haloalkylation). The formed structural changes in lipids and proteins damage different cell organelles (membranes, mitochondria, etc.) and lead to a loss of their normal structure and functioning (Recknagel and Glende 1973, Dianzani 1991, Recknagel et al. 1991). The exposure to CCl₄ causes noticeable histologic changes in different cell organelles in hepatocytes. By using electron microscopy, Oberling and Rouiller (1956) found, within a few hours of CCl₄
injection, a dilated ER, cytoplasmic vacuoles, and liposomes in hepatocytes, and lesions in mitochondria and the Golgi complex. Reynolds (1963) reported rapid (30-120 min post-dosing) alterations in the structure of the ER, plasma membrane, mitochondria, and Golgi complex of the liver parenchymal cell of rats after administration of CCl₄ (orally 0.125 ml/100 g BW). The accumulation of triglycerides in the liver can be seen within 5-6 h of treatment with CCl₄, being independent of mitochondrial changes (Artizzu and Dianzani 1962).

Biochemical changes in the liver parenchyma contribute to a more severe fatty liver (Artizzu and Dianzani 1962). In the presence of oxygen, lipid peroxidation occurs (Fig. 3). Autocatalytic peroxidation of the attacked fatty acids follows, polyunsaturated fatty acids (PUFAs) being the most susceptible (Poli et al. 1987, Dianzani 1991, Recknagel et al. 1991). Lipid peroxidation often produces toxic aldehydes (Recknagel and Glende 1973, Dianzani 1991). Abnormal branched-chain fatty acids occur as a result of end-condensation of trichloromethyl radicals with lipid radicals (Recknagel et al. 1991). Free radicals arising from lipid peroxidation react with proteins, -SH- groups, in particular, are susceptible to covalent binding of CCl₄ cleavage products (Recknagel and Glende 1973).

As phospholipids are an important structural part of cell membranes, altered biomembrane structure can explain several of the toxic effects of CCl₄ in the liver. The fatty acid composition of the ER, and the phospholipid composition of plasma membranes have been shown to change after CCl₄ treatment; especially the phosphatidyl-ethanolamine fraction is attacked by the free radicals, and the amount of unsaturated fatty acids is lowered (Benedetti et al. 1977, Ilyas et al. 1978, Camacho and Rubalcava 1984). James et al. (1986) discovered altered membrane fluidity in hepatocytes of rat liver exposed to CCl₄. By using HPLC-determination to analyze phospholipid peroxidation, Terao et al. (1984) confirmed that phosphatidylcholine and phosphatidylethanolamine were peroxidized in rats after CCl₄ treatment. When radiolabelled choline was given to rats simultaneously with carbon tetrachloride, the synthesis of both phospholipids and triglycerides was inhibited 4-5 h after CCl₄ administration (Shimizu 1969).
\[ \text{CCl}_3 - \text{Cl} = \text{carbon tetrachloride} \]

cytochrome P - 450 in SER

- \text{CCl}_3 = \text{trichloromethyl; free radical}

- \text{H} \quad \text{- C = C - C = C - hydrogen abstraction}

- \text{HCCl}_3 = \text{chboroform}

- \text{H} \quad \text{- C = C - C = C - organic free radical}

- \text{H} \quad \text{O}_2

- \text{- C - C - C - C = C - organic peroxide (unstable)}

- \text{H} \quad \text{- O O}

- \text{H} \quad \text{- C - C - C = C - haloalkylation}

- \text{O O}

- \text{H} \quad \text{reacts covalently with}

- \text{proteins}
- \text{lipids}
- \text{membranes/phospholipids}

- \text{Cell damage, plasma membrane damage}

- \text{Decreased lipoprotein secretion}

- \text{FATTY LIVER}

Fig. 3. A simplified scheme for the initiation of a radical reaction induced by carbon tetrachloride and subsequent peroxide formation, leading to cell damage and fatty liver.
Damage to biomembranes disturbs water and electrolyte balance, and inhibits normal cell function. Rapid collapse of structure and function in the ER takes place after exposure to CCl₄, but mitochondrial elements are affected later (Recknagel and Glende 1973). In mitochondria of rat liver, CCl₄ has been shown to induce swelling (Recknagel and Malamed 1958, Hoffsten et al. 1962). The mitochondrial Ca⁺⁺ pump, which is vital for cell-function, is involved early (10-15 min after exposure) in CCl₄-induced damage (Joseph et al. 1983, Albano et al. 1985). The redistribution of intracellular Ca²⁺ is considered to lead to pathological effects in hepatocytes (Brattin et al. 1984, Albano et al. 1985). The increase in Ca²⁺ in hepatocytes is maximal 1-1.5 h after CCl₄ exposure (Cheville 1994). The loss of Ca²⁺ sequestering activity of the ER results within 5-10 min after haloalkane addition (Sweeney 1981). Moreover, the oxidation of fatty acids has been shown to be closely synchronized with the rise in mitochondrial calcium (and decrease in potassium) in CCl₄-induced poisoning of rat liver mitochondria (Reynolds et al. 1962).

The main pathway for TG discharge from liver cells to plasma is represented by lipoprotein secretion, secreted mainly as VLDL (Dianzani 1991). Accumulation of triglycerides leading to hepatic steatosis after exposure to CCL₄ begins with a haloalkylation-dependent block in the secretion of lipoprotein micelles from the Golgi apparatus. Obviously, the apolipoprotein is attacked and is the reason behind the decreased secretion of lipoproteins (Dianzani 1991). Poli et al. (1985) have demonstrated in vitro and in vivo that secretion of lipoproteins is inhibited by CCl₄ because of the functional impairment of the Golgi apparatus. Fatty liver results due to the blockage of hepatic triglycerides to the plasma as VLDLs, because triglyceride biosynthesis in the ER-membranes remains normal after CCl₄ administration (Recknagel and Glende 1973, Dianzani 1991, Recknagel et al. 1991), and due to the increase in lipid peroxidation and the accumulation of triglycerides and lipoprotein particles in hepatocytes (Benedetti et al. 1977, Poli et al. 1987).

There are publications on different attempts to reduce hepatotoxic effects of CCl₄ by various chemical compounds. Carbon tetrachloride-induced hepatotoxic effects have been beneficially retarded in rats by adenosine (Hernandez-Munoz et al. 1990), Liver Growth Factor (a hepatic mitogen)(Diaz-Gil et al. 1999), alfa-tocopherol (Poli et al. 1987), and in mice, by vitamin E (Liu et al. 1995). Vitamin E (alphatocopherol) donates a hydrogen atom to the lipid radical and hence terminates the chain reaction. The
selenium-containing glutathioneperoxidase is known to convert the formed peroxides to corresponding alcohols (Recknagel et al. 1991). Choline has been shown to reduce hepatic tissue damage induced by CCl4 in dogs (Martin et al. 1984); serum alkaline phosphatase (AFOS) and alanine aminotransferase (ALAT) were reduced by oral administration of 88 mg/kg choline citrate after a single dose of 4 ml/kg CCl4. Gasso et al. (1996) have found that daily injections of SAM (10 mg/kg i.m.) reduced the changes in rat liver leading to CCl4-induced cirrhosis and reduced lipid peroxidation. Demonstrated with rats, some compounds, e.g., ethanol and chlordecone, potentiated the negative effects of a mild exposure to CCl4 and induced the development of significant liver injury (Hall et al. 1991, Kodavanti et al. 1991).

Recent research has revealed that defects in methylation may also have a role in preventing the noxious effects of CCl4. Carbon tetrachloride has been shown to cause DNA hypomethylation in liver, which can be corrected by administration of 10 mg/kg/d of S-adenosylmethionine (Varela-Moreiras et al. 1995). Hypomethylation of DNA is a clearly negative event, contributing to the onset of oncogenesis (Hoffman 1985). Kim et al. (1993) and Murakami et al. (1998) have studied betaine in rats and mice in the context of CCl4 intoxication; both reports indicating that betaine alleviates the toxic effects of CCl4. Kim et al. (1993) administered CCl4 (0.5 mg/kg i.p.) to rats in the presence of additional betaine (100 mg/kg i.p.). They found that betaine reduced the centrilobular necrosis in rat liver and elevated the reduced activities of some cytochrome P-450 enzymes. Murakami et al. (1998) reported that either intraperitoneal (3 mg/kg i.p.) or oral (15 mg/kg) administration of betaine significantly decreased serum ALAT in male mice after acute CCl4 intoxication. Kim et al. (1998) later found in mice that betaine reduced the chloroform-induced hepatotoxic damage in strong correlation with hepatic glutathione levels.

In summary, CCl4 causes toxic changes by free alkyl radicals in the ER, mitochondria, and biomembranes of hepatocytes (e.g. peroxidation of lipids, altered molecular structures, loss of Ca2+ homeostasis and ionic control). The function of the vital cell organelles alters, resulting in decreased protein synthesis, defects in lipoprotein synthesis, etc., leading to decreased lipoprotein secretion via the Golgi complex. Pathological effects are manifested as an accumulation of triglycerides and as the formation of liposomes (lipid droplets) in hepatocytes.
1.4.4. Ethanol and fatty liver

Among the medical problems associated with excessive use of ethanol, hepatic disorders are at the forefront. Prolonged ethanol intake leads to fat accumulation in the liver (alcoholic fatty liver), which is regarded as a risk of progression to more severe liver diseases, such as cirrhosis and fibrosis (Teli et al. 1995). In human liver, 30 g/d ethanol for men and 20 g/d for women is considered safe daily usage. For severe liver damage, ingestion of more than 160 g of ethanol daily for 5 years is normally needed (Sherlock 1995). The increased susceptibility of women to ethanol may be dependent on their less active alcohol dehydrogenase enzyme in the gut mucosa (Mayes 1996).

Enlargement of the liver is among the first signs of hepatic damage caused by ethanol. Lipids account for only about 50% of the increase in dry-weight liver (Lieber et al. 1965); the rest is accounted for by an increase in proteins (Baraona et al. 1975). A single dose (6 g/kg BW) of ethanol increases hepatic triglyceride levels significantly in periportal (centroacinar) zones of the rat liver lobules, which is noticeable 4-16 h after the dose and disappears by 24-36 h (Porta et al. 1970). Histologically, enlargement and coalescence of mitochondria, disarrangement of ER, hyperplasia of the Golgi complex, and depletion of glycogen are observed in hepatocytes after an administration of a single large dose (Hartroft and Porta 1966, Iseri et al. 1966). Cirrhosis after years of chronic ethanol abuse is a multifactorial disease typically associated with protein-energy malnutrition and multiple micronutrient deficiencies (Hartroft and Porta 1966, Halsted 1996, Mayes 1996).

Numerous explanations for fatty liver after ethanol intake have been tendered. Ethanol is oxidized in the hepatic cytosol by alcohol dehydrogenase, leading to increased endogenous fatty acid and triacylglycerol synthesis, retarded fatty acid oxidation, and decreased citric acid cycle activity, all of which contribute to alcoholic fatty liver (Mayes 1996). Alcohol dehydrogenase produces acetaldehyde as the main product in the metabolism of ethanol which appears to have a direct role in collagen formation and may later lead to cirrhosis (Casini et al. 1981, Halsted 1996). Liver damage attributable to chronic ethanol consumption may occur by the covalent binding of acetaldehyde to cellular proteins, producing significant amounts of acetaldehyde-altered proteins (Nomura and Lieber 1981, Sorrell and Tuma 1987). Nanni et al. (1978) proposed that fatty liver induced by ethanol was the result of impaired hepatic
lipoprotein secretion, probably due to altered mechanisms of terminal glycosylation of VLDLs in the Golgi apparatus, similar to the CCl4-induced fatty liver (Dianzani 1991). Ethanol is known to cause depletion of glutathione (an antioxidant) in the liver (Shaw et al. 1983, Hirano et al.1992) which may favor peroxidation of lipids (Wendel et al. 1979). Furthermore, ethanol-induced microsomes increase generation of free organic radicals, resulting in enhanced peroxidation of membrane phospholipids and accumulation of lipids in liver (DiLuzio and Hartman 1967, Cederbaum 1989, Lieber 1997, 1999).

Many authors have related some of the noted changes in the liver after prolonged ethanol intake to an impairment in methylation (Klatskin et al. 1954, Finkelstein and Kyle 1968, Barak and Beckenhauer 1988, Trimble et al. 1993, Barak et al. 1996a, Halsted et al. 1996). Dietary choline and methionine were shown to alleviate the development of ethanol-induced fatty liver (Finkelstein et al. 1974), with the choline requirement in rats increasing with ingestion of ethanol (Klatskin et al. 1954). However, ethanol ingestion may produce fatty livers in man and rats despite maintenance of adequate diets (Lieber et al. 1965). Recently, Trimble et al. (1993) found that chronic ethanol exposure produces disturbances in methylation pathways, with resultant abnormalities in production of phosphatidylcholine, which may play a significant role in liver damage. This is supported by the fact that chronic ethanol intake has been found to lead to an accumulation of N5-methyltetrahydrofolate (N5-MTHF) in the liver (Hillman et al. 1977, Horne et al. 1978), which is a consequence of either a reduction in bile folate flow (Hillman et al. 1977) or the inhibition of methionine synthetase (MS, Fig. 1.) (Barak et al. 1987, Banerjee and Matthews 1990, Halsted et al. 1996). As MS converts homocysteine in the presence of vitamin B12 and N5-MTHF to methionine, the inhibition of this enzyme results in impaired methylation reactions, and may decrease the biosynthesis of membrane phospholipids (Hirata and Axelrod 1980). Simultaneous depletion of glutathione (Shaw et al 1983) may increase the susceptibility of phospholipids in membranes to oxidation (Wendel et al. 1979). Barak et al. (1984b, 1987) noticed that an enhancement of the enzyme BHMT (Fig. 1) was a response to inhibition of MS in the liver by ethanol. In addition, the marked lowering of hepatic betaine levels in the liver after ethanol intake, is regarded as a compensatory reaction for the inhibition of MS in order to remethylate homocysteine to methionine and to maintain normal hepatic SAM levels (Barak et al. 1987, Trimble et al.1993). Furthermore, Trimble et al. (1993) observed that ethanol increased methionine catabolism by a factor of 2.9. Hepatic methionine levels remained, however, unchanged by the increase in turnover of the methyl groups of choline and betaine in
response to ethanol. In baboons (Papio hamadryas) Lieber et al. (1990) recorded that chronic ethanol consumption (50% of energy for 18-36 months) led to a depletion of hepatic SAM and glutathione. Halsted et al. (1996) reported a similar occurrence in micropigs, with a simultaneous reduction in MS activity.

Attempts have been made to decrease the negative effects of ethanol in the liver by promoting methylation. Lieber and De Carli (1966) have shown that dietary choline reduced liver triglycerides in rats when fatty livers were produced by prolonged ethanol intake. The accumulation of folate can be prevented in the rat by 0.5% dietary betaine (Barak et al. 1993), which has been shown to promote generation of SAM in rat liver and to reduce the alcohol-induced fatty infiltration (Barak et al. 1994, 1996b, 1997). Lieber et al. (1990) demonstrated in baboons that the provision of 0.4 mg/kcal S-adenosylmethionine attenuated liver injury caused by chronic ethanol feeding. Mato et al. (1999) established that 1200 mg/d SAM given orally may improve survival of human patients with alcoholic liver cirrhosis.
2. AIMS OF THE RESEARCH

The main objectives of the present work were the following:

1. to study the effect of dietary betaine on the osmoregulatory ability of Atlantic salmon (*Salmo salar*) smolts (I)

2. to study the cryoprotective effects of betaine on the survival of stallion sperm in the freezing process (II)

3. to study the effect of orally administered betaine on methionine metabolism and fatty liver (steatosis) in Sprague-Dawley rats when steatosis was induced by ethanol (III)

4. to study the effects of orally administered betaine on the key enzymes in methionine metabolism and on fatty liver induced by carbon tetrachloride (IV)

5. to study the effects of orally administered betaine on the cell organelles of the perinuclear cytoplasm in the liver after carbon tetrachloride-induced fatty liver (V).
3. MATERIALS AND METHODS

3.1. Animals and experimental design

3.1.1. Fish in the osmoregulation experiment (I)

One-year-old Atlantic salmon, *Salmo salar* L., from the stock of River Neva, which runs into the Baltic Sea, were used in the experiment. The fish were reared on a private fish farm, Hankakoski Ltd., in Hankakoski, Finland and then transferred to the Department of Zoology, University of Helsinki at the size of about 15-20 g MBW. The fish were acclimatized at an experimental temperature of 9.7 ± 0.9 °C for 1 month before the experiment. During this period, they were fed the commercial salmon feed "Smoltti" (Suomen Rehu Oy, Turku, Finland).

The experiment was carried out in four circular fiberglass tanks (diameter 0.9 m, volume ca. 200 l). One hundred fish, individually weighed and measured, were put into each tank. Water was recirculated at a rate of 10 l/min, with the addition of 1 l/min of pure dechlorinated tap water per tank. Two of the tanks were supplied with the control feed "Smoltti", two of the tanks with experimental feed. Except for 1.5% (w/w) commercial betaine/amino acid additive for fish feed ("Finnstim", Finnsugar Bioproducts, Helsinki Finland), the experimental feed was the same as the control feed. Finnstim is a mixture containing mainly (97%) anhydrous betaine, the remainder being a mixture of branched chain amino acids (alanine, isoleucine, leucine, and valine). The feeds were approximately isocaloric and isonitrogenous in their composition. Feeding rates were ca. 1.15%/day. The fish were fed continuously during the entire light period (8 h) with an automatic belt feeder.

After 4 weeks in fresh water, the fish were lightly anesthetized with MS 222 (Sandoz, Switzerland, 0.1 g/l), weighed, measured, and allowed to recover for 4 days. One experimental and one control tank were connected to a salt water reservoir. Salinity was gradually increased in 8 days to 39 ± 1 ppt by adding balanced Instant Ocean sea salt (Aquarium systems, France). The fish were then reared at 39 ppt salinity for an additional 4 weeks. The same salt water pool recirculated in both tanks. Feeding was adjusted based on nitrite levels in the water.
The remaining two tanks served as freshwater controls, and were treated in the same way as during the first 4 weeks of the experiment, except for an adjusted feeding rate (1%/day).

Samples of 7-15 fish (13 fish on average) were taken from tanks for different analyses. Samples were collected at the start of the experiment, after 4 weeks rearing in fresh water, and after the gradual acclimatization to seawater, at 1, 3, 7, 17, and 28 days after the final salinity was achieved.

In order to standardize handling stress just prior to sampling, the fish were enclosed in individual restrainers for 2 days before sampling (Soivio et al. 1977). Restrainers were not used in the sea water challenge test, in which fish were individually netted for sampling.

3.1.2. Stallion sperm in the cryoprotection experiment (II)

Semen from Finnhorse stallions (State Horse Breeding Institute, Ypäjä, Finland) was used in the study. The first three semen-rich fractions of ejaculate were collected by using an open-ended vagina. The semen was mixed with an extender to produce a final concentration of 20-40 million spermatozoa per milliliter of semen. The extended semen was packaged in 1 ml aliquots in polypropylene tubes for freezing, which was carried out by a programmable freezer (Cell Freezer R 204, Planer Products Ltd, UK). Two different cooling rates were used: slow and moderate. At the slow cooling rate, tubes were kept at 32 °C for ten minutes, then cooled at a rate of 1 °C/min to -7 °C at which temperature they were held for ten minutes, then at a rate of 0.3 °C/min from -7 to -30 °C, and finally at a rate of 0.1 °C/min from -30 to -33 °C. After that the tubes were plunged into liquid nitrogen. At the moderate cooling rate, tubes were cooled from 32 °C to -35 °C at a rate of 3 °C/min and then plunged into liquid nitrogen. The time from semen collection to plunging varied from about one hour to three hours for moderate and slow cooling rates, respectively.

3.1.3. Rats in the models for steatosis (III, IV, V)

Male Sprague-Dawley rats, 24 animals/study (Study III, IV), mean body weight (MBW)184.3 ± 6.4 g (III), 225.4 ± 7.8 g (IV), and 85 male Han:Wistar rats (specific pathogen-free animals), MBW 268.9 ±
7.8 g (V) were used in the studies. All rats received humane care in accordance with applicable institutional, local, and national guidelines.

Rats were divided (Study III, IV) into 4 groups (six animals per group) and housed separately in stainless steel cages (21 ± 1 °C, rH 44 - 62%). Rats in groups 1-3 were group-fed the amount of diet consumed by rats in group 4 to ensure isocaloric intake of the rats in all groups.

The liquid diet technique of ethanol administration to achieve alcoholic fatty liver was used in Study III (Lieber and De Carli 1989). Group 1 was fed the semi-liquid control diet. Group 2 was fed the same diet, to which 0.5% (w/v) betaine was added. In our previous studies (unpublished) dietary betaine at concentrations of up to 2% had been found harmless in rats. Group 3 received the semi-liquid ethanol diet (5 g ethanol/dl = 36% ethanol of total energy), in which dextrin-maltose had been isocalorically replaced with ethanol. Group 4 was fed the semi-liquid ethanol diet containing 0.5% (w/v) betaine. Rats were maintained with the dietary regimen for 4 weeks, after which they were sacrificed (light ether anesthesia followed by exsanguination via abdominal aorta) in the non-fasting state. Extracted livers were freeze-clamped at -70 °C and stored at this temperature until analysis.

Study IV rats were fed the semi-liquid control diet (Lieber and De Carli 1989) as a basal regimen, with the animals being acclimated to this diet for three days prior to the start of the study. The diets in groups 2 and 4 contained 2% (w/w) betaine (Betafin BP, Finnsugar Bioproducts, Helsinki, Finland). Groups 1 and 2 received subcutaneous injections of olive oil (1 ml/kg/d), and groups 3 and 4 were given subcutaneous injections of 40% carbon tetrachloride in olive oil (1 ml/kg/d = 0.4 ml CCl4/kg/d). The injections were given for four consecutive days, and the feeding regimen was continued for seven days, after which the animals were killed in a non-fasting state and their livers excised, frozen, and stored at -70 °C until analysis.

In Study V, the rats were arranged into 3 groups and housed separately (inert bedding, 12 h light, 12 h dark, 21 ± 0.5 °C, rH 49 ± 3%). The animals were given commercial rat feed (R36-feed, Lactamin AB, Stockholm, Sweden) and local tap water ad libitum.
The groups were as follows:
1: Control group; olive oil 1 ml/kg BW subcutaneously (s.c.) for 4 consecutive days and mock treatment with water 0.7 ml/d intragastrically (i.g.)
2: Carbon tetrachloride group; CCl4 1 ml/kg BW s.c. for 4 consecutive days and mock treatment with water 0.7 ml/d i.g.
3: Carbon tetrachloride+betaine group; CCl4 1 ml/kg BW s.c. for 4 consecutive days and treatment with betaine (830 mg/kg/d in 0.7 ml concentrated solution i.g.).

In Study V, all animals were acclimatized to the diet and the intragastric administration of betaine-solution/mock treatment with water for 7 days prior to the onset of CCl4 injections. After this acclimation period of 7 days, five rats which had received water intragastrically and five animals receiving betaine-solution were killed (narcotized in CO2 atmosphere followed by immediate decapitation) to obtain day 0 samples in order to study the effect of betaine alone. The daily CCl4/olive oil injections were started on day 0 and they lasted for 4 days. Five rats were killed from each group on the day following every injection (day 1, 2, 3, 4) and 3 days after the fourth CCl4/olive oil injection (day 7). Feed consumption and body weight were recorded throughout the study. The rats were individually sacrificed (see above) and their livers were excised, weighed, and directly processed for chemical and histologic analyses.

3.2. Physiological tests and performed measurements

Seawater challenge tests were carried out at the start of the experiment, after 4 weeks, and after 9 weeks of rearing in fresh water. A total of 15-20 fishes from each group were challenged for 48 h at 39 ppt salinity as described by Soivio and Virtanen (1985).

The length (cm) and weight (g) of fish were measured, and the condition factor was calculated using the formula \( CF = \frac{w(g)}{L^3 \text{ (cm)}} \times 100 \). The distinctness of parr marks was estimated visually using a scale from 0 to 4.
Stallion sperm in the frozen tubes were thawed in a 45 °C water bath for one minute. Progressive sperm motility was evaluated using the laser Doppler technique (Lazymot, BTG Biotechnik GmbH, Germany).

Growth of the rats and liver weights were measured by weighing the animals/livers individually over the course of the studies. Feed consumption was measured daily. Growth rate (IV) was calculated using ln(BWgain)/duration (d).

3.3. Biochemical and chemical analyses

3.3.1. Blood and tissue samples in the osmoregulation of fish (I)

Blood samples were aspirated into heparinized 1-ml tuberculin syringes from the caudal vein of the stunned fish. Filaments of the first gill arch, the posterior kidney, a piece of the lateral white muscle, and the caudal part of the liver were also removed. All samples were frozen in liquid nitrogen and either stored in it (gill and kidney samples) or at -20 °C (plasma, carcass, and liver samples). Blood samples were taken at the start of the experiment, after 4 weeks of rearing in fresh water, and after the gradual acclimatization to seawater, at 1, 3, 7, 17, and 28 days after the final salinity was achieved.

Samples were analyzed for blood hematocrit value, hemoglobin, Na⁺, K⁺, Mg²⁺, and Cl⁻ concentrations in the plasma, red cell and muscle water content, liver glycogen content, total body fat content, activity of Na/K -activated ATPase in gills and kidney, and plasma thyroxine concentration using the methods described by Soivio and Virtanen (1980), and Virtanen and Soivio (1985). The betaine concentration in blood and whole fish was determined from the pooled samples using HPLC (Rajakylä and Palosposki 1983).

3.3.2. Blood and tissue samples in rat studies (III, IV, V)

Chemical and biochemical values were obtained by using the methods described in Table 1.
Table 1. Methods used in chemical and biochemical analyses (Studies III, IV, V)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Study</th>
<th>Method</th>
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<tr>
<td>liver triglyceride</td>
<td>III, IV</td>
<td>lipid extraction</td>
<td>Folch et al. 1957</td>
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<tr>
<td>liver triglyceride</td>
<td>V</td>
<td>colorimetric</td>
<td>Frings and Dunn 1970</td>
</tr>
<tr>
<td>hepatic protein</td>
<td>III</td>
<td>colorimetric</td>
<td>Lowry et al. 1951</td>
</tr>
<tr>
<td>hepatic betaine</td>
<td>III, IV</td>
<td>spectrophotometric</td>
<td>Barak and Tuma 1979</td>
</tr>
<tr>
<td>SAM</td>
<td>III, IV</td>
<td>paper chromatographic</td>
<td>Eloranta et al. 1976</td>
</tr>
<tr>
<td>MS</td>
<td>III, IV</td>
<td>paper chromatographic</td>
<td>Mudd et al. 1970</td>
</tr>
<tr>
<td>BHMT</td>
<td>III, IV</td>
<td>colorimetric</td>
<td>Ericson and Harper 1956</td>
</tr>
<tr>
<td>plasma ALAT</td>
<td>IV</td>
<td>UV-spectrophotometric</td>
<td>Committee on Enzymes 1974</td>
</tr>
<tr>
<td>plasma ASAT</td>
<td>IV</td>
<td>UV-spectrophotometric</td>
<td>Committee on Enzymes 1974</td>
</tr>
</tbody>
</table>

3.4. Histologic studies (III, IV, V)

3.4.1. Light microscopy (III, IV, V)

Liver samples for histologic analyses from left liver lobes were placed into buffered 10% formalin immediately after excision of livers. For histologic lipid staining, Sudan IV-method (for frozen sections) was used. In addition, semi-thin (1-2 µm) liver sections (glutaraldehyde-fixed, epon embedded; prepared for TEM analyses, see 3.4.2.) were stained with toluidin blue in order to detect fat by light microscopy. The samples were further processed for light microscopy, according to conventional techniques (Romeis 1948, Luna 1968, Bancroft et al. 1990). Hepatic lipidosis was estimated from the Sudan IV-stained slides by a 100-point eyepiece graticule (Leica E10A, mesh size 0.5 mm) using a x10 eyepiece and a x10 objective lens. Each graticule square with noticeable lipid was calculated as a "point". In total, 1000 squares/liver with 4-6 replicates were calculated, which means that 4000-6000 points/group were analyzed. All histologic examinations were conducted on a random and blind basis.
3.4.2. Transmission electron microscopy (V)

Two freshly cut liver sections (about 1 mm$^3$ each) from the left lobes of the livers were taken immediately after excision of the livers and immersed in 2% glutaraldehyde, dehydrated in ethanol, and embedded in propylene oxide-epoxy resin. The embedded liver samples were further processed by conventional techniques for TEM analyses.

TEM-samples were examined with a Jeol JEM 100 S -electron microscope (Jeol, Tokyo, Japan). Micrographs were taken (magnification 21700 x) at random and on a blind basis starting from the left upper-corner of the grids. From the day 0 liver samples, 25 micrographs/group were taken and from the day 7 liver samples, 100 micrographs/group. The volume fraction of perinuclear cytoplasmic organelles was determined by using a 100-point lattice (16 cm x 19 cm). The volume densities of cell organelles elaborated by the lattice were recorded as described by Weibel et al. (1966). In total, 2500 points/group were observed from the day 0 samples and 10,000 points/group from the day 7 samples. The following cell structures were recorded: mitochondria, lipid globules, Golgi complex, SER, RER, glycogen, peroxisomes, other microbodies, lysosomes, myelin figures, cytoplasmic vacuoles, and undefined cytoplasm (Chardially 1988). Furthermore, the total number of mitochondria was calculated from each micrograph and the relative size of mitochondria was calculated as follows: mitochondrial points/total number of mitochondria.

3.5. Statistical analyses

Student's t-test and one-way ANOVA were used in Study I. Statistical analysis of the data in Study II was performed using the analysis of least squares (Harvey 1970). Student's t-test was used for statistical analyses in Study III. Biochemical data in Study IV were analyzed by using Student's t-test, and the histologic data by using one-way ANOVA followed by Tukey’s test for pairwise comparison when the group effects were statistically significant (SAS 1988). Student's t-test was used in Study V in comparing feed intake, weight gains, and liver weights and histologic data were analyzed by using ANOVA followed by Tukey’s test. In the cases of unequal variances (analyzed by Bartlett’s test) rank transformed data were used (SAS 1988).
4. RESULTS

The results are summarized in the tables and figures of the original publications.

4.1. Betaine as an osmolyte

4.1.1. Osmoregulation of ATS smolts (I)

When fish were fed with the betaine-supplemented feed in fresh and salt water, the betaine content of the whole fish carcass after 5 weeks of feeding was 7-8 times higher (= 0.070-0.090% of wet weight) in the experimental group than in the control group. The betaine content of the whole body did also tend to increase (0.012 to 0.028% of wet weight) in the control group after 5 weeks in seawater. However, no significant differences were present between the groups in blood-betaine values (0.042-0.043% of wet weight) after 9 weeks in 39 ppt salinity.

After 4 weeks of rearing in fresh water, the betaine-fed group differed significantly from the control group in several physiologic parameters; blood hemoglobin, plasma potassium, and especially muscle water content were lower in the betaine group.

When seawater challenge tests, in which smolts were directly transferred from fresh water to seawater (48 h, 39 ppt salinity), were performed after 4 weeks and 9 weeks of rearing in fresh water, mortality was high in both groups. In the betaine-fed group lower plasma Na\(^+\), Cl\(^-\), K\(^+\), and plasma Mg\(^{2+}\) levels were present than in the control fish. After 9 weeks of feeding on the diet containing betaine/amino acid additive, these fish had higher muscle water content after the seawater challenge test than the control fish.

The measured physiologic parameters in the seawater challenge test after 9 weeks rearing in fresh water revealed significant increases in the betaine group in red blood cell water content, liver glycogen content, gill Na/K-ATPase activity, and plasma thyroxine. Furthermore, there were less parr marks (indicators of desmoltification in ATS) and less total lipids in fish in the betaine group.
When salinity of water was gradually increased to full seawater salinity (39 ppt) in 2 of the 4 tanks used, the gill Na/K-ATPase activity of the betaine-fed fish was significantly elevated and stayed slightly higher than that of the control fish throughout seawater stage (Fig. 4). In contrast, plasma Na\(^+\)- and Mg\(^{2+}\)-concentrations remained lower in the betaine-fed fish than in the control fish during the 4 week period of full seawater salinity. There was also a tendency for lower mortality in seawater for fish receiving betaine/aminoacid additive in their diet.

![Graph](image)

Fig. 4. Gill Na/K-ATPase activity, mean ± SD (nmol PO\(_4\)/mg prot h\(^{-1}\), of Atlantic Salmon in seawater (SW) after transfer from fresh water to full salinity seawater. Experimental group receiving diet containing betaine additive (Study I).

4.1.2. Deep freezing of stallion sperm (II)

The addition of 0 to 3% (w/v) betaine into stallion semen extender revealed improved sperm post-thaw motility at betaine concentrations above 1.5%, the optimum being at 2.5%. When the initial motility after sperm collection was 60%, the post-thaw spermatozoal motility decreased to about 10% and was increased by the addition of betaine to 41 ± 12.8%. The measured post-thaw motility of semen was thus at best 95% of the original motility (Fig. 5).
Fig. 5. Post-thaw motility of sperm (% of fresh semen motility) at different betaine concentrations (% w/v) (Study II).

When two different slow-cooling rates and extenders without or with betaine (2.5% w/v) were used in the freezing of sperm from 5 different stallions and post-thaw motilities were compared between the groups, betaine extender increased significantly (P<0.001) the progressive motility of spermatozoa.

4.2. Betaine as a lipotrope

4.2.1. Ethanol model for steatosis (III)

Administration of ethanol (36% of total energy of the diet) to the semi-liquid diet of rats for 4 weeks resulted in hepatic lipidosis (measured as hepatic triglyceride), which could be prevented by the administration of 0.5% betaine to the diet. Ethanol affected methionine metabolism (Fig. 1) by retarding the activity of methionine synthetase (MS), while simultaneously increasing the activity of betaine
homocysteine methyltransferase (BHMT). Addition of betaine significantly promoted this increase and also enhanced the biosynthesis of S-adenosylmethionine (SAM), the principal methylating agent in the body (Fig. 6). Even though receiving the same energy intake as the other groups, the ethanol-fed group alone lost weight. Rats receiving both betaine and ethanol gained more weight than rats in the respective control groups.

![Graph showing S-adenosyl methionine (SAM) levels of rat livers with and without ethanol treatment, mean ± SD. C = control diet; CB = control diet + 0.5% betaine; E = semi-liquid ethanol diet; EB = semi-liquid ethanol diet + 0.5% betaine (Study III).]

4.2.2. Carbon tetrachloride models for steatosis (IV, V)

Subcutaneous carbon tetrachloride injections at two different dosages, 0.4 ml/kg/d (= 0.07 mg/rat/d) (IV) and 1.0 ml/kg/d (= 0.23 mg/rat/d) (V), for four consecutive days were used to induce hepatic steatosis. Steatosis was apparent both in chemical and histologic analyses. Liver lipid content, based on chemical analyses, increased from about 3% (control group) up to 7.5-10% after CCl₄-injections. Oral administration of betaine reduced steatosis. Significant reduction in liver lipids was also noted in the group receiving betaine but no CCl₄ treatment (IV, Table 1). The lower dosage CCl₄ -injections increased plasma ALAT (IV), and the higher CCl₄-dosage retarded rat growth (V).
Biochemical analyses (IV) showed that administration of betaine significantly increased liver betaine content. CCl4-injections increased also betaine content of the liver slightly. CCl4-injections affected methionine metabolism (Fig. 1) by increasing BHMT and SAM levels in the betaine groups, whereas without the administration of betaine, hepatic BHMT and SAM tended to decrease. The measured SAM values are shown in Fig. 7.

Histologic observations with LM of toluidin blue -stained samples (IV and V) revealed a marked increment in hepatic centrlobular steatosis in the CCl4-injected groups. No necrotic changes were observed at either CCl4-dosage. A systematic analysis of Sudan IV-stained samples showed that the administration of 2% betaine reduced steatosis significantly. The reduction in hepatic steatosis was also evident in morphometric TEM analyses at the higher CCl4-dosage (V, Table 2 and 3). The reduction of steatosis in Studies IV and V due to administration of betaine is illustrated in Fig. 8.

The quantitative morphometric analyses (V, Table 3) from TEM samples revealed a tendency for increased volume density of cytoplasmic vacuoles and a significant increase in number of mitochondrial figures in the perinuclear cytoplasm of hepatocytes in the livers of rats, which had received oral administration of betaine for 7 days before the exposure to CCl4-injections (day 0).

Three days after the CCl4-injections (day 7), there were significant reductions in the number of mitochondrial figures, RER, and Golgi complex in the CCl4-injected rats compared with the control or betaine group. The relative size of mitochondria remained, however, the same in all groups.
Fig. 7. S-adenosyl methionine (SAM) levels (nmol/g liver) of rat livers with and without carbon tetrachloride treatment, mean ± SD. C = control group; CB = control group + 2% betaine; CCl₄ = group exposed to carbon tetrachloride; CCl₄+B = group exposed to carbon tetrachloride + 2% betaine (Study IV).

Fig. 8. Volume density (light microscopy) of lipid content (% present in grid squares) in rat livers after exposure (Day 7) to carbon tetrachloride (CCl₄), with and without administration of 2% betaine (B), mean ± SD (Studies IV and V).
5. DISCUSSION

In biochemical literature betaine is characterized as a methyl donor which participates as an intermediate product in choline oxidation in methionine biosynthesis, and acts as a lipotrope in preventing fatty liver (Mann et al. 1938, Harper et al. 1979, Finkelstein et al. 1983, Finkelstein 1990, Barak et al. 1994). The methyl donor function has traditionally been regarded as the main biochemical function of betaine.


Our aim in this work was:

1) to study osmotic effects of betaine in the transfer of Atlantic salmon smolts from fresh water to seawater (I) and in the deep-freezing of stallion semen (II), and

2) to study the lipotropic function of betaine in rats when fatty livers were induced either by ethanol (III) or by carbon tetrachloride (IV, V).

5.1. Betaine as an osmolyte

5.1.1. Osmoregulation of ATS smolts (I)

Koch and Evans (1959) have demonstrated that the migration of Atlantic salmon smolts from fresh water to seawater is related to their ability to regulate plasma sodium. The ionic and osmotic regulation of young salmon undergoes marked changes during smoltification: the ionic extrusion mechanisms in the gills are activated, and the ion and water transport mechanisms of the gut and kidney alter. As a result, salmon smolts are able to restore their ionic and osmotic balance rapidly after the transfer to seawater (Natochin et al. 1975, Folmar and Dickhoff 1980, Wedemeyer et al. 1980).

A commercial betaine/amino acid additive for fish feed ("Finnstim", Finnsugar Bioproducts, Helsinki, Finland) was tested as the betaine source in Study I. The commercial diet used contained a dietary
supplementation of choline, even though part of the nutritional requirement for choline (750-800 ppm) can be replaced with betaine in salmonids (Rumsey 1991). The intake of betaine via feed was excessive, at approximately 3% of the intake of the dietary protein. The respective intake of amino acids from the protein was approximately 1000x higher than the intake of amino acids from the betaine/amino acid additive. Therefore, the main effects recorded in the study are concluded to represent the effects of betaine alone, and the possible effect of amino acids is considered to be mainly gustatory for fish. This rationale is applied throughout the discussion concerning Study (I).

Plasma ion levels (especially Na⁺ and K⁺) and water content of tissues are the key elements physiologically when the osmoregulation of ATS-smolts is studied. Results from freshwater rearing, gradual adaptation to seawater, and seawater challenge tests revealed significant changes in these parameters. After 4 weeks of rearing in fresh water, plasma K⁺ and muscle water content was lower in the fish fed with feed containing the betaine/amino acid additive, indicating a shift of potassium and water between the extracellular and intracellular compartments. The experimental fish receiving the betaine additive were able to maintain their ion and water balance better than the control fish during adaptation to seawater. The difference was more pronounced in the 48-h seawater challenge tests, indicating that the betaine-fed fish can adapt well to abrupt changes in water salinity. By adding a compound chemically similar to betaine, trimethylamine, to a guppy (Poecilia reticulata) diet, Daikoku (1980) discovered an improved survival time when fish were transferred from fresh water to seawater.

Betaine additive seemed to accelerate active ion extrusion in gills via elevation of Na/K-ATPase activity both after 2 months in fresh water and after gradual acclimatization to seawater. This effect was seen in the reduction of plasma Na⁺ and Mg²⁺ levels. The improved ability to regulate plasma Mg²⁺ might be related to a similar role of betaine in the salmon kidney as observed in the rabbit and the rat (Bagnasco et al. 1986, Lohr and Acara 1990). Clarke et al. (1994) have also found reduced plasma sodium levels in seawater chinook salmon (Oncorhynchus tshawytscha) receiving the same commercial betaine/amino acid additive in dry fish feed. Virtanen et al. (1994) have studied the betaine additive in dry fish feed with rainbow trout (Oncorhynchus mykiss) and found that it decreased the osmotic inhibition of metabolism at seawater transfer. Dietary betaine increased the betaine content also in the muscles of
Atlantic salmon (Salmo salar), but no clear osmoregulatory effect at seawater transfer was evident when fish were fed moist feed (Duston 1993).

The migration from fresh water to seawater is a critical physiological challenge for Atlantic salmon smolts. Attention should be paid to the fact that betaine/ amino acid additive seems to promote the preparation of smolts to this phase, as seen from the above-mentioned parameters and in the a slight tendency towards lower mortality after transfer to seawater in the betaine-receiving group. The mechanisms by which the betaine additive prevents or delay desmoltification remain unknown.

5.1.2. Deep freezing of stallion sperm (II)

The deleterious effects of the freezing process include ice-crystal formation in the extracellular space and a simultaneous increase in salt concentration in the intracellular fluid of spermatozoa. During the process, water is removed from inside spermatozoa to the extracellular space, and spermatozoa become progressively dehydrated. Increase of intracellular ionic strength in the freezing process may cause denaturation of proteins and enzymes, which in the case of spermatozoa may easily reduce post-thaw sperm motility (Amann and Pickett 1987). The survival of spermatozoa is primarily dependent on the amount of unfrozen water in the extracellular environment. In the case when about 15% of the extracellular water remains unfrozen, cell survival stays high (Amann and Pickett 1987).

Smith and Polge (1950) published the fundamental finding that 25% of stallion spermatozoa survived freezing to -79 °C and subsequent thawing when suspended in a buffered extender containing glucose and glycerol. Besides glycerol, certain sugars (e.g. mannose, trehalose, lactose) have been successfully used as cryoprotectants (Higgins et al. 1986, Amann and Pickett 1987). Cryoprotectants are divided into two main categories: those that permeate the plasma membrane and those that are nonpenetrating. The permeating agents reduce the amount of ice and lower the concentration of electrolytes encountered (McGann 1997). The cryoprotective effects of betaine in animal cells have not been studied extensively. Higgins et al. (1986) have shown in vitro using liposomes that betaine and alanine (at concentrations of 3% w/v or less) functioned as cryoprotective agents. Furthermore, in vitro betaine and glycine are known to protect mouse zygotes against the effects of raised osmolarity (Dawson and Baltz 1997).
Betaine (54-106 mM) has been found to improve motility of ram spermatozoa in the deep-freezing process when glycerol and egg yolk were present in the extender (Sanchez-Partida et al. 1992). Recently, Lindeberg et al. (1999) found that high levels of betaine (4-5%) in a lactose-EDTA medium retarded post-thaw motility of stallion semen; however, at lower concentrations (0.25%), betaine tended to increase motility.

In Study II the slow cooling rates used in the freezing of stallion semen (which should have reduced formation of large extracellular ice crystals and allow time for betaine to enter spermatozoa) promoted most successfully post-thaw sperm motility with use of 2.5% betaine concentration in the extender. The post-thaw progressive motility of spermatozoa increased 1.8 to 2.9-fold compared with the respective controls. In the freezing of spermatozoa, the formation of intracellular ice crystals is believed to reduce cell survival due to mechanical and physicochemical reasons. Removal of water as ice and the subsequent altered tonicity leading to a hypertonic media may be fatal for cells in the freezing process (Smith and Polge 1950). An explanation for the positive effect of betaine might be its possible role as an intracellular osmolyte in stallion sperm, a role similar to that exhibited by betaine in other cells (Le Rudulier et al. 1984, Arakawa and Timasheff 1985, Haussinger 1996). It is also possible that betaine in this context prevents the denaturation of structural proteins and/or enzymes caused by the progressive dehydration of cells and the osmotic effects of solutes in the intracellular fluid of spermatozoa (Arakawa and Timasheff 1985, Pegg 1997). However, as the fertilizing capacity of semen was not tested in this study, the actual benefit of the observed increase in post-thaw motility of spermatozoa remains open for discussion. It is also apparent that the benefits of betaine in different deep-freezing processes for semen are dependent on several factors, including the use of an appropriate concentration of betaine and a favorable composition of the extenders (Sanchez-Partida et al. 1992, Lindeberg et al. 1999).

5.2. Betaine as a lipotrope

5.2.1. Ethanol model for steatosis (III)

Altered folate metabolism has long been recognized as one of the major derangements caused by chronic ethanol consumption (Sullivan and Herbert 1964, Hillman et al. 1977, Horne et al. 1978), and many
reports have related ethanol-induced liver changes to an impairment of the methylation process (Klatskin et al. 1954, Finkelstein and Kyle 1968, Barak and Beckenhauer 1988, Halsted et al. 1996). The metabolism of methionine and folate are closely bound together (Fig. 1); Barak et al. (1981) showed that a marked lowering of hepatic betaine levels occurred simultaneously with the accumulation of folates in chronic alcohol consumption. Later, Barak et al. (1985) found that ethanol may impair methionine production through the MTHF-dependent methionine synthase reaction and that enhanced betaine-homocysteine-methyltransferase activity was compensatory for this impairment. Furthermore, alcoholic fatty liver is considered to be a risk factor for cirrhosis and fibrosis (Teli et al. 1995). As betaine has been shown to have a noticeable curative effect against dietary cirrhosis in rats on hypolipotropic diet (Best et al. 1969), and choline, a precursor of betaine, has been shown (Lieber and DeCarli 1966) to alleviate ethanol-induced fatty liver, it made sense to study betaine in the context of ethanol-induced fatty liver.

When the respective blood parameters related to transmethylation were recorded in this study, the results (Study III) demonstrated that the supplementation of dietary betaine increased hepatic levels of betaine in both control and ethanol-fed rats. In addition, betaine supplementation increased BHMT activity in control animals and further increased the already elevated activity of this enzyme in the ethanol-fed animals. As a result of these two effects, hepatic SAM levels increased in the controls and were significantly elevated in the livers of ethanol-fed rats. Increased betaine levels and BHMT activity resulted in increased methionine and subsequent SAM synthesis via the alternate pathway for methionine synthesis, despite decreased MS activity. Both histologic examination and hepatic triglyceride analyses showed that livers were protected against the infiltration of fat that resulted from ethanol ingestion. The mechanism by which SAM is involved in protecting the liver from steatosis was not elucidated. One possibility might be SAM’s role in phospholipid sequential methylation, its aid in the function and repair of membranes (Hirata and Axelrod 1980), and thus in facilitating the transport of fat from the liver. SAM may also participate via cystathionine (Hoffman 1985) in the synthesis of glutathione (Lieber et al. 1990), which may protect the cell from reaction with toxic substances (Corrales et al. 1992), or in nucleic acid and protein methylation reactions (Varela-Moreiras et al. 1995), which are required to maintain the integrity of the cell (Friedel et al. 1989).
The finding in this study (III) that oral administration of betaine elevated hepatic SAM in control and ethanol-fed rats, and drastically protected against ethanol-induced fatty livers had not, to our knowledge, been reported earlier. Since then, however, Barak et al. (1994, 1996b, 1997) have published more on the matter. Our finding was not necessarily an expected result, although Lieber and DeCarli (1966) had demonstrated that choline and methionine partially prevented production of ethanol-induced fatty livers.

5.2.2. Carbon tetrachloride models for steatosis (IV, V)

Carbon tetrachloride (CCL4) has been known for decades to be a strong hepatotoxic agent and its effects on the liver have been studied extensively. Carbon tetrachloride treatment has been used as a model to induce and study hepatic steatosis (fatty infiltration) of the liver (Recknagel 1967, Meeks et al. 1991), and has also been used as a means of producing experimental cirrhosis (Cameron and Karunarathe 1936, Stowell et al. 1951). Some investigators have questioned the role of lipidosis in the development of advanced liver disease (Sherlock 1995). This, however, has been discounted by others who believe that the longer fat remains in the liver, the more damaging it is because of the injurious effects of lipid peroxidation (Kamimura et al. 1995, Teli et al. 1995). In these studies (IV, V), hepatic steatosis was induced by CCL4 and is regarded as a negative feature for the vitality and function of hepatocytes.

Free alkyl radicals are thought to be the main reason for the toxic effects of CCL4 on liver cell membranes (Butler 1961, Slater 1966, Tomasi et al. 1980, DeGroot and Haas 1981, James et al. 1986, Fig. 3). Published literature reveals different approaches for preventing the harmful effects of CCL4 on hepatocytes: vitamin E therapy has been used in the treatment of acute CCL4-induced hepatic injury in rats (Liu et al. 1995), hepatic stimulator substance (from weanling rats) in mice (Mao-Hua et al. 1993), and adenosine in rats (Hernandez-Munoz et al. 1990). As cell membranes are also damaged by CCL4, transmethylation and especially formation of SAM may have a role in the repairing process of existing lesions via the biosynthesis of phospholipids (Hirata and Axelrod 1980).

Very little published information on betaine in the context of CCL4-induced fatty liver is available. The only reports found are those of Kim et al. (1993, 1998) and Murakami et al. (1998). Kim et al. (1993)
found that betaine (100 mg/kg i.p.) reduced CCl₄-induced (0.5 mg/kg i.p.) centrilobular necrosis in rat livers and increased total cytochrome P-450 content. Murakami et al. (1998) reported on a reduction in CCl₄-induced (10 ml/kg) liver necrosis in mice after oral (15 mg/kg) or intraperitoneal (3 mg/kg) administration of betaine.

Both of the present studies (IV, V) showed that the administration of CCl₄ (0.4 ml/kg and 1 ml/kg s.c.) to rats for four consecutive days resulted in extensive centrilobular hepatic steatosis and that dietary (2% ww) or oral (830 mg/kg/d) administration of betaine significantly reduced this lipidosis. The CCl₄-treatments used in both studies were so mild that no necrotic changes could be detected histologically. In Study IV, CCl₄-treatment without dietary betaine caused an increase in plasma ALAT, indicating that the greatest hepatocellular damage took place in the group receiving CCl₄ but not betaine.

Because choline-deficiency produces fatty liver in rats (Forbes and Vaughan 1954, Estes and Lombardi 1969, Kuksis and Mookerjea 1978), it follows that the marked reduction in hepatic lipidosis in the betaine group is partly due to betaine improving choline metabolism and promoting biosynthesis of SAM.

Dietary betaine increased liver betaine but betaine uptake did not appear to be affected by CCl₄. In addition, BHMT activity was enhanced (Fig. 1.) by betaine and resulted in elevated hepatic SAM. It is probable that the elevated SAM prevented steatosis through its methylating role in the function and repair of damaged cell membranes (Hirata and Axelrod 1980, Barak et al. 1994, Varela-Moreiras et al. 1995). The elevated SAM may have had other beneficial effects on hepatocytes as well. Corrales et al. (1992) have shown that CCl₄-treatment reduced S-adenosylmethionine synthetase activity and glutathione levels, which could be corrected by treatment with SAM (3 mg/kg/d i.m.). Both of these compounds are important concomitants of liver fibrosis in vivo.

The increased volume density of RER (Study V) in the betaine group might indicate facilitated production of lipoproteins, and thus, reduction of steatosis by VLDL excretion via the Golgi complex. This is supported by the volume density of the Golgi complex being higher in the betaine group than in
the CCl₄-treated group, thereby allowing for a more efficient excretion of lipoproteins. Thus, the reduction in the volume density of the Golgi complex in the CCl₄-group may have been partly responsible for the increased lipid content of the hepatocytes. Reynolds (1963) has earlier reported that the administration of CCl₄ to rats transformed the Golgi complex into a cluster of dilated vacuoles.

Along with its role in transmethylation reactions, betaine plays a part in the hydration of liver Kupffer cells and sinusoidal endothelial cells, and may thus promote the function of liver cells in general (Zhang et al 1992, Wettstein et al. 1998). However, based on our data, betaine either protects or aids the recovery of hepatocytes in counteracting the effects of such toxic compounds as ethanol and CCl₄ by assisting transmethylation reactions, which may be backed up by the increased activity of macrophages in the liver.
6. CONCLUSIONS

The objective in Study I was to examine whether dietary betaine could alleviate the physiological stress imposed on Atlantic salmon (*S. salar*) smolts in their adaptation from fresh water to seawater conditions.

Atlantic salmon smolts were able to maintain their ion and water balance slightly better during adaptation and after transfer to seawater when the commercial dry feed used was supplemented with a 1.5% betaine/ami no acid additive (Finnstim®). The additive seemed to somewhat accelerate the active ion extrusion in gills via elevation of Na/K-ATPase activity. The exact mechanism by which this occurred requires further study.

The objective in Study II was to test, whether the betaine-supplemented extender used in the freezing of spermatozoa could bring about cryoprotective effects, measured as the post-thaw motility of stallion sperm.

The addition of 2.5% betaine to the extender used in the stallion sperm-freezing processsed increased the post-thaw progressive motility of spermatozoa at slow cooling rates. Being a preliminarty study, without any known previously published data, the mechanism by which betaine acted (i.e. intracellularly, extracellularly, or both) was not clarified and must be studied later. The effects of betaine on the fertility of the post-thawed stallion sperm remains to be studied.

As methionine metabolism is known to participate in the deleterious reactions in the liver caused by chronic ethanol intake, the objective in Study III was to investigate the effects of dietary betaine on the main biochemical intermediates related to methionine metabolism in the liver after hepatic steatosis was induced by dietary ethanol intake.

Oral administration of 0.5% betaine prevented hepatic steatosis induced by chronic ethanol feeding in male Sprague-Dawley rats. Perhaps the most important finding was that dietary betaine promoted the
generation of hepatic S-adenosylmethionine, which is the major methylating agent in the body, possibly participating in biosynthesis of cell membranes and DNA.

The objective in Studies IV and V was to induce hepatic steatosis by CCl₄ and to examine the effects of oral administration of betaine on the main intermediates of methionine metabolism in the liver, as well as to analyze and quantify the histopathologic changes in the liver during and after exposure to CCl₄.

In Study IV, dietary betaine (2%) reduced CCl₄-induced centrilobular steatosis and increased S-adenosylmethionine content in the livers of male Sprague-Dawley rats.

In Study V, quantitative morphometric analyses of the perinuclear cytoplasm of hepatocytes revealed that oral administration of 830 mg/kg/d of betaine prevented CCl₄-induced centrilobular steatosis in male Han-Wistar rats, increased the number of mitochondrial figures in cytoplasm and volume density of RER, and protected the Golgi complex against hepatotoxic effects of CCl₄.
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